[1] Adhesion of Microbial Pathogens to Leukocyte Integrins: Methods to Study Ligand Mimicry

By EVA ROZDZINSKI and ELAINE TUOMANEN

Introduction

Integrin receptors are eukaryotic transmembrane glycoproteins which mediate cell–cell and cell–matrix interactions. The heterodimers are composed of a noncovalently bound α and β chain, both of which participate in the formation of ligand recognition sites (for review, see Refs. 1 and 2). Bacteria have been demonstrated to interact with the leukocyte-restricted β_2 integrins that include Mac-1 ($a_M\beta_2$, CD11b/CD18, CR3), LFA-1 ($a_L\beta_2$, CD11a/CD18), and p150/95 ($a_X\beta_2$, CR4, CD11c/CD18). The β_2 integrins play an important role in the pathogenesis of infections as they have been shown to contribute to the phagocytosis of complement-coated particles[3] and to transendothelial migration of leukocytes from the bloodstream to sites of inflammation.[4] The interaction of bacteria with β_2 integrins leads to uptake of the bacterium into the phagocyte without inducing an oxidative burst, offering access to and survival in the eukaryotic host cell.[3,5] In another volume of this series,[6] we have described methods to determine if a bacterium adheres to a leukocyte integrin in an Arg-Gly-Asp (RGD) dependent fashion and how to identify the specific integrin and bacterial ligands involved. Although these methods are reviewed here in brief, this chapter focuses on bacterial mimicry which allows integrin binding independent of RGD.

To determine if bacteria ligate leukocyte β_2 integrins, a downmodulation experiment can be used. In the assay, leukocytes are plated on surfaces coated with an anti-CD18 antibody, for example, monoclonal antibody

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[4] D. Anderson, F. Schmalsteig, M. Finegold, B. Hughes, R. Rothlein, L. Miller, S. Kohl, M. Tosi, R. Jacobs, T. Waldrop, A. Goldman, W. Shearer, and T. Springer, *J. Infect. Dis.* **152,** 668 (1985).
[5] A. I. M. Hoepelman and E. I. Tuomanen, *Infect. Immun.* **60,** 1729 (1992).
[6] E. Rozdzinski and E. Tuomanen, this series, Vol. 236, p. 333.

(MAb) IB4,[7] 60.3,[8] or TS1/18.[9] Because integrins move within the leukocyte membrane, receptors bearing CD18 are sequestered beneath the leukocytes by the antibody, resulting in a depletion of the apical surface of CD18 receptors.[10,11] If bacteria adhere to the leukocyte integrin, bacterial adhesion to the apical surface of the leukocytes will be reduced. The specific β_2 integrin can be identified by performing the same assay on surfaces coated with monoclonal antibodies directed against the three different α chains of the leukocyte integrins: TS1/22 against a_L (which is CD11a of LFA-1),[12] OKM1 or OKM10 against a_M (CD11b of CR3),[7] or Leu-M5 against a_X (CD11c of p150/95).[13] The bacterial ligand for the integrin can be determined by testing the purified putative adhesin for the ability to downmodulate the leukocyte integrin in the aforementioned assay. Another method to specify the bacterial ligand is to test the putative adhesin for the ability to compete with bacteria for the integrin. To confirm observations made in the downmodulation and competition assays, bacterial mutants which do not express the putative adhesin may be tested for a reduced ability to adhere to leukocytes spread on albumin-coated plastic surfaces in comparison to the wild type.[14]

We have described three different types of bacterial binding to leukocyte integrins, two of which focus on bacteria that bear the RGD sequence as the key motif recognized by the integrin and one of which focuses on bacterial lectins that bind carbohydrates decorating the integrin.[6] Here we focus on a new type of integrin–bacteria interaction that is protein–protein in nature but RGD-independent. This type of binding is characterized by bacterial mimicry of the natural ligands of the leukocyte integrin, for instance, the CD11b/CD18 ligands endotoxin,[15,16] coagulation factor X,[17,18]

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and C3bi.[19] We describe methods to study *in vitro* and *in vivo* the consequences of RGD and non-RGD binding for neutrophil adhesion and transendothelial migration in the host. The interaction of *Bordetella pertussis* with neutrophils, monocytes, and macrophages is used as an example.[20–22] The assays are based on methods published elsewhere in this series.[6,23]

Methods

Competition Assays to Show Bacterial Ligation of Leukocyte Integrins by Mimicking Natural Ligand

Mimicry can be suspected when comparison of the amino acid sequence of the bacterial adhesin reveals similarities with that of a natural ligand of the integrin. Once suspected, mimicry can be demonstrated at the functional level in competition assays. If the adhesin binds to the leukocyte integrin by mimicry it should compete with the natural ligand and therefore inhibit its binding to the integrin. The adhesin filamentous hemagglutinin (FHA) of *Bordetella pertussis* will serve as an example. FHA is made up of several regions with sequence similarities to the regions of the coagulation factor X that bind the integrin CD11b/CD18.[22] Thus, FHA and synthetic peptides representing the factor X-like regions of FHA should inhibit the binding of radiolabeled factor X to the CD11b/CD18 present on neutrophils or monocytes.

Labeling of Factor X with $Na^{125}I$ by Iodogen Method. The following method is based on Ref. 24.

1. To coat 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen, Pierce, Rockford, IL) on a glass tube, dry 33 μg of Iodogen dissolved in 100 μl dichloromethane under nitrogen according to the recommendations of the manufacturer.
2. To the Iodogen-coated tube add 200 μg of human factor X (Sigma, St. Louis, MO, or Hematologic Technologies, San Diego, CA) in phosphate-buffered saline (PBS), pH 7.2, and 2 mCi of carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, IL).
3. Incubate for 15 min on ice (cover the tube with Parafilm).

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[23] S. D. Wright, this series, Vol. 132, p. 204.
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4. Isolate ^{125}I-labeled factor X by chromatography on Sephadex G-25 at room temperature as follows. Equilibrate the Sephadex G-25 column with 25 ml of 1× PBS, pH 7.2. Run radiolabeled factor X in 2.5 ml PBS, pH 7.2, over the column and discard the flow through. Elute with 3.5 ml PBS and collect fractions of 0.5 ml. Measure the protein concentration and gamma radiation of the fractions in a gamma counter and pool the fractions containing radiolabeled factor X. The specific radioactivity should range from 0.3 to 1 μCi/μg protein. Protein recovery is expected to be 95%.

5. Dilute the pooled iodinated factor X with PBS, pH 7.2, to a final concentration of 310 ng/30 μl, or 10.33 μg/ml.

Preparation of Leukocytes. The binding reaction can be performed with freshly isolated monocytes, neutrophils, or cells of the monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD). Techniques for isolating human neutrophils and monocytes have been described previously in this series.[23,25] Although primary cells are the theoretically preferred target cells, both neutrophils and monocytes have technical drawbacks to their use. Neutrophils are easily activated by the isolation procedure and therefore tend to agglutinate. Monocytes represent only 5% of the leukocyte population in human peripheral blood, which means that a large volume of blood must be harvested to get the needed amount of cells. In addition, monocytes are usually contaminated with platelets that have to be removed by incubation with autologous serum containing 5 m*M* EDTA for 30 min at 37°. Therefore THP-1 cells are the preferred cell type in this assay. THP-1 cells are maintained in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) containing 10% (v/v) heat-inactivated fetal calf serum, 20 m*M* HEPES, 100 μg/ml gentamicin, 2 m*M* L-glutamine, and 10 μ*M* 2-mercaptoethanol.

Binding Reaction. Binding of ^{125}I-labeled factor X to THP-1 cells can be measured based on a method described by Altieri *et al.*[18] The inhibition of binding by the bacterial peptide is expected if the bacterium mimics factor X as a ligand for CD11b/CD18.

1. Supplement 200 μl of TPH-1 cells (2×10^7 cells/ml RPMI 1640; M. A. Bioproducts) with 17.5 μl of 50 m*M* $CaCl_2$ (final concentration 2.5 m*M*). Activate the THP-1 cells by adding 3.5 μl of 100 μ*M* NH_2-formyl-Met-Leu-Phe (N-fMLP, Sigma). Add 100 μl of bacterial peptide dissolved in PBS to get a final concentration of 0.5 m*M*. Add 30 μl of ^{125}I-labeled factor X to get a final concentration of 15 n*M*. For control, use a scrambled peptide. Incubate for 20 min at 22°.

[25] D. Roos, this series, Vol. 132, p. 225.

2. Separate neutrophil-bound ^{125}I-labeled factor X from unbound labeled factor X by layering 300-μl aliquots over 50 μl of a mixture of Hi phenyl silicone oil DC 550 and methyl silicone oil DC 200 5:1 (Nye Inc. Specialty Lubricants, New Bedford, MA) and centrifuging at 12,000 *g* for 2 min (room temperature).

3. Count aliquots of the supernatant (cell-free ^{125}I-factor X) and the cell pellet (cell-bound ^{125}I-factor X) collected in the amputated tip of the tube in a gamma counter.

4. Determine the nonspecific binding by measuring the amount of ^{125}I-labeled factor X bound to the cells in the presence of a 100-fold molar excess of unlabeled factor X (~5–10% of counts in the presence of control peptides). Subtract the counts due to nonspecific binding from the total to calculate specific binding.

Assay of Bacterium–Leukocyte Integrin Interaction Affecting Integrin-Dependent Adhesion and Transendothelial Migration of Leukocytes

The CD18 integrins promote leukocyte adhesion to endothelia during inflammation by recognizing endothelial cell receptors.[26–31] Binding of a bacterial ligand to an integrin can interfere with that normal bioactivity, particularly if the bacterium mimics endothelium or an inflammatory component recruited to inflammed endothelium. For example, FHA binds to CD11b/CD18 and the anti-FHA monoclonal antibody 13.6E2[32] binds to cerebral endothelia and blocks leukocyte recruitment into the cerebrospinal fluid (CSF) during experimental meningitis.[33] These findings suggest that FHA may mimic an unknown endothelial ligand for CD11b/CD18 which participates in inflammation-induced leukocyte extravasation. To identify the key sequence of the "endothelial-like" region of FHA, synthetic peptides can be tested for their ability to inhibit leukocyte adhesion to endothelia as described below.

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Adhesion of Neutrophils to Endothelial Cells in Vivo

The following assay is based on a method described by Lo *et al.*[30] The ability of a bacterial peptide to block neutrophil adhesion to endothelial monolayers is an indication of binding to CD18 integrins. The example below focuses on CD11b/CD18.

Preparation of Monolayers of Human Umbilical Vein Endothelial Cells

1. Coat 60-well Terasaki tissue culture plates (Robbins Scientific, Sunnyvale, CA) with fibronectin (50 μg/ml; Sigma) and incubate for at least 15 min at 37°.
2. Wash Terasaki plates once with tissue culture medium M199 (Sigma).
3. Add 9 μl of human umbilical vein endothelial cells (HUVEC; first passage, Clonetics, San Diego, CA) per well, resuspended in complete medium [M199 containing 0.2% sodium bicarbonate (Sigma), 2.5 m*M* HEPES buffer (Sigma), 15% (v/v) heat-inactivated human serum (Sigma), 350 μg/ml L-glutamine (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 2.5 μg/ml amphotericin B (Sigma), 50 μg/ml endothelial growth cell mitogen (Biomedical Technologies, Stoughton, MA), and 10 U/ml heparin (Sigma)]. Let HUVEC settle for 2 to 3 hr at 37° and in the presence of 5% (v/v) CO_2 before refeeding with complete medium. Incubate for at least 24 hr until monolayers are confluent.
4. Wash confluent HUVEC monolayers twice with M199 and activate the cells with tumor necrosis factor α (TNFα) (10 ng/ml complete medium; Boehringer Mannheim, Indianapolis, IN) at 37°, 5% CO_2 for 4 hr.

Isolation, Fluorescent Labeling, and Preincubation of Human Neutrophils

1. Isolate neutrophils from human blood using neutrophil isolation medium as described by the manufacturer (Cardinal Associates, Santa Fe, NM).
2. Label human neutrophils by suspending the cells in 5 ml of 50 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Molecular Probes Inc., Eugene, OR) in Dulbecco's phosphate-buffered saline (DPBS; Bio Whittaker) (the fluorescent probe is prepared from a 5 mg/ml ethanol stock solution centrifuged at 300 *g* for 3 min at room temperature to remove undissolved particles). After incubating cells for 10 min on ice in the dark, wash the neutrophils 3 times with ice-cold DPBS. Resuspend the cells in M199 and adjust the density of cells to 10^6 cells/ml.
3. Preincubate 30 μl of fluorescently labeled neutrophils (10^6 cells/ml M199) with 30 μl of bacterial peptide (1 m*M* in DPBS) for 15 min at 37°. For a negative control, preincubate neutrophils with HAP buffer (PBS

containing 0.5 mg/ml human serum albumin, 3 m*M* glucose, and 0.3 U/ml aprotinin) or a scrambled peptide. For a positive control, preincubate neutrophils with a monoclonal antibody against CD18, such as IB4 (50 μg/ml; Merck, Rahway, NJ), which decreases neutrophil adhesion by up to 40%.

Adhesion of Neutrophils to Human Umbilical Vein Endothelial Cells

1. Remove TNFα from the endothelial monolayers by washing twice with warm M199 medium. After the second wash, turn the plate over and slam it moderately hard on a paper towel to remove fluid from the wells.
2. To each well, add 9 μl of preincubated neutrophils. Allow them to adhere to the monolayer for 15 min at 37°.
3. Remove unbound cells by submersion of the plate in warm M199.
4. To fix the cells, overlay with freshly prepared 2% (v/v) paraformaldehyde (pH 7.4) for 10 min.
5. Count the number of adherent neutrophils in a 40× microscope field using an inverted fluorescence microscope. Express the amount of adherent neutrophils as a percentage of adhesion in control wells with neutrophils treated with HAP buffer alone. Prior to counting, plates can be stored overnight in the refrigerator in the dark.

To determine which epitope of the leukocyte integrin is recognized by the bacterial adhesin, a variation of the assay can be used. Neutrophils can be preincubated with peptides in the presence of monoclonal antibodies that are directed against different epitopes of the integrin. If the peptide/antibody combination does not increase inhibition of neutrophil adhesion over the peptide alone, one can conclude that the peptide recognizes the same epitope as the monoclonal antibody. If the inhibition of neutrophil adhesion by the peptide and the monoclonal antibody is additive, one can assume that each binds to different epitopes, both of which are relevant for leukocyte adhesion.

Transendothelial Migration of Neutrophils

The experiment to study transendothelial migration of neutrophils is based on a method described by Muller and Weigl.[31] The ability of a bacterial peptide to block neutrophil transmigration across endothelial monolayers is an indication of binding to CD18 integrins. The example below focuses on CD11b/CD18.

Subculture and Activation of Human Umbilical Vein Endothelial Cells

1. Coat 96-well flat-bottomed plates (Fisher Scientific, Pittsburgh, PA) with 50 μl of collagen solution consisting of 8 ml of ice-cold bovine type I

purified collagen (Vitrogen 100, Celtrix Laboratories), 1 ml of 10× Medium 199 (Sigma), and 5 ml of 0.1 *N* NaOH (Sigma).

2. Incubate collagen-coated plates overnight at 37°, 5% CO_2.

3. The following day, cover the wells with 100 μl of M199 (Sigma) containing penicillin (100 U/ml) and streptomycin (100 μg/ml).

4. Before seeding the HUVEC, aspirate the fluid and add 25 μl of fibronectin (50 μg/ml in 0.5% NaCl, Sigma) per well and incubate for 15 min at room temperature.

5. Aspirate the fluid and add HUVEC resuspended in complete medium (see above) to each well. Refeed cells every 2–3 days and incubate until the monolayer becomes confluent (several days).

6. Wash confluent monolayers with M199 and activate endothelial cells by adding TNFα (10 ng/ml complete medium) for 4 hr.

7. Remove TNFα by washing three times with warm M199.

Isolation, Fluorescent Labeling, and Preincubation of Human Neutrophils

1. Isolate human neutrophils as described above.

2. Suspend neutrophils in ice-cold Hanks' balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+} (Sigma) and add 3.3 μl/ml 5- and 6-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes). After incubating cells for 30 min on ice, wash the neutrophils twice in cold HBSS with Ca^{2+} and Mg^{2+} and suspend them in cold M199 (Sigma) to a final density of 10^6 cells/ml.

3. Preincubate neutrophils (10^6 cells/ml) with 10 μ*M* of peptides for 5 min on ice. For a positive control, preincubate neutrophils with 25 μg/ml anti-CD18 mAb IB4 (Merck). For a negative control, treat neutrophils with 25 μg/ml mAb W6/32 against HLA class I antigen (Dako, Carpinteria, CA).

Transendothelial Migration of Neutrophils

1. Add 100-μl aliquots of the preincubated neutrophils to the endothelial cells and incubate for 1 hr at 37°, 5% CO_2.

2. To terminate transmigration, aspirate the supernatant fluid and fill the wells with warm 1 m*M* EGTA (Sigma) in HBSS without Ca^{2+} and Mg^{2+}.

3. Cover the plates with a plate sealer (Dynatech, Alexandria, VA) and centrifuge in an inverted position at 250 *g* for 3 min at room temperature.

4. To remove nontransmigrated neutrophils, wash monolayers twice with 200 μl of warm Hanks' solution without Ca^{2+} and Mg^{2+}.

5. Quantitate fluorescence using a fluorescence counter (Millipore, Bedford, MA Cytofluor 2300). The percentage of transmigrated neutrophils is determined by comparing the fluorescence of test wells to wells coated with

collagen and fibronectin but lacking endothelial cells. Fluorescence counts are corrected for contamination of the neutrophil preparation with lymphocytes (as assessed by light microscopy) which do not transmigrate. Percent inhibition of transendothelial migration of neutrophils is calculated by comparing the number of transmigated cells in wells containing neutrophils treated with peptides to those in wells containing neutrophils treated with M199 alone.

Rabbit Model for Meningitis

Because leukocyte transmigration in the model of experimental pneumococcal meningitis described here is strongly CD18-dependent,[34] it is well suited to study bacterial peptides for their ability to interfere with CD18 *in vivo*. Peptides which bind to CD18, either by mimicking a natural ligand or by other means, should be able to inhibit leukocyte extravasation from the bloodstream into the cerebrospinal fluid (CSF). Because leukocyte transmigration is associated with increased blood–brain barrier permeability leading to an influx of serum proteins into the CSF, the same peptides might be able to prevent blood–brain barrier injury and therefore reduce the influx of serum proteins into the CSF. The following rabbit model is a modification of a protocol developed by Dacey *et al.*[35,36]

1. Anesthetize specific pathogen-free, New Zealand White rabbits (2 kg; Hare Marland, Nutley, NJ) with valium (2.5 mg/kg, subcutaneously; Hoffman-LaRoche, Nutley, NJ), ketamine (35 mg/kg, intramuscularly; Aveco, Ford Dodge, IA), and xylazine (5 mg/kg, intramuscularly; Miles Laboratories, Shawnee, KS).
2. Affix a helmet of dental acrylic (Fastray, Harry J. Bosworth, Skokie, IL) to the calvarium by molding around 4 screws implanted in the skull.
3. Twenty-four hours later, anesthetize the rabbits with ethyl carbamate (1.75 g/kg; Aldrich, Milwaukee, WI) and pentobarbital (15 mg/kg; Abbott Laboratories, Abbott Park, IL) and place them in a stereotaxic frame.
4. Introduce a spinal needle (25-gauge, 3.5-inch; Becton Dickinson, Fairview Lakes, NJ) into the cisterna magna and withdraw 300 μl of CSF. Inject intracisternally an inflammatory stimulus, for example, 10^8 heat-killed, unencapsulated pneumococci in 200 μl of saline, or endotoxin (time 0).
5. One hour later, inject animals intravenously with bacterial peptides dissolved in 1 ml of saline into the right marginal ear vein.

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6. Draw 200-μl samples of CSF at hourly intervals and measure the leukocyte density using a counter (Coulter Electronics, Hialeah, FL). Spin CSF samples at 10,000 *g* for 5 min and store at −70°. Assay the supernatant for protein concentration using the bicinchoninic acid method (BCA kit, Pierce) and lactate concentration (lactate dehydrogenase kit, Sigma). A peptide which is effective in decreasing leukocyte recruitment *in vivo* would diminish a normal leukocytosis of over 6000 cells/μl CSF at 6 hr after challenge to a value below 2000 cells/μl. If the decrease in leukocytosis protects against blood–brain barrier injury, values for protein and lactate concentration will remain at normal values (<1.0 and <20 mg/ml, respectively).

[2] Association of Bacteria with Human Phagocytes

By RICHARD F. REST

Introduction

The purpose of this chapter is to present methods and approaches to study and to quantify the adhesion of bacteria to human phagocytic cells, specifically polymorphonuclear neutrophils (PMNs), monocytes, and macrophages. Adherent bacteria are often, but not always, internalized by the phagocytes to which they adhere. If the reader wishes to study and quantify phagocytic killing, or the oxidative response of phagocytic cells to bacterial challenge, other books, manuals, and chapters are available. Volume 132 of this series, "Immunochemical Techniques, Part J: Phagocytosis and Cell-Mediated Cytotoxicity," and Volume 236, "Bacterial Pathogenesis, Part B: Interaction of Pathogenic Bacteria with Host Cells," are excellent companion references, as well as several chapters in this volume. The methods presented here are not comprehensive, but rather are the beginning of an organized approach to study bacterial adhesion to human phagocytes. Neither are the methods absolute. They may or may not be precisely proper for the bacteria under investigation, or the research questions being posed, and it may be necessary to change buffers, alter pH, alter osmolarity, alter ion content, change concentrations and relative ratios of bacteria and phagocyte populations, add serum or other body fluids, or grow bacteria under different conditions.

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Before presenting specific assays, a short introduction to the interacting components and their general characteristics and interactions is required. The assays, which are generally rather simple to set up and perform, consist of incubating bacteria and phagocytes, sometimes with serum or other body fluids, and then quantifying adhesion. Bacteria attach to phagocytes by two broad mechanisms: (1) directly, by using specific molecules expressed on outer cell surfaces such as fimbriae (pili)[1-3] and nonfimbrial ligands, often called adhesins, such as the Opa proteins of *Neisseria gonorrhoeae*[4]; and (2) indirectly, by binding host components, most often immunoglobulins and complement components, which subsequently bind to phagocyte receptors.[5-7] Immunoglobulin- and complement-mediated binding and subsequent internalization are together termed opsonin-dependent phagocytosis, or opsonophagocytosis, whereas direct binding is often referred to as opsonin-independent binding. Opsonin-independent binding and internalization can be mediated by host or bacterial lectins, or both (the process being termed lectinophagocytosis by Ofek and Sharon[1,8]), or by protein–protein interactions.[9] Adhesion mediated by lectins can be further defined by inhibition with mono- or oligosaccharides, or with purified lectins.

Regardless of mechanism, the methods used to quantify adhesion are similar. The ability of bacteria to bind to phagocytes can vary tremendously depending on the bacterial strain or variant, and growth conditions and growth phase, among other variables. In addition, many bacterial surface ligands vary independently of growth conditions or phase; such variations are not rare events.[10] This brief discussion of adhesion mechanisms is meant to impress on the reader the importance of studying bacterial adhesion to phagocytes in the presence and absence of serum or other body fluids of interest and relevance to the particular system in question, to be aware of environmental influences on bacterial adhesin expression, and to define fully the bacterial strains being used.

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[7] M. M. Frank and L. F. Fries, *Immunol. Today* **12,** 322 (1991).
[8] I. Ofek, R. F. Rest, and N. Sharon, *ASM News* **58,** 29 (1992).
[9] D. Relman, E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright, *Cell (Cambridge, Mass.)* **61,** 1375 (1990).
[10] B. D. Robertson and T. F. Meyer, *Trends Genet.* **8,** 422 (1992).

Preparation of Human Phagocytes

*Isolation of Human Neutrophils**

The method presented here is based on the Ficoll–Hypaque method, as described by Ferrante and Thong.[11,12] It is rapid, has a high recovery (≥80%), and yields very pure (≥93%) resting (or at least minimally activated) neutrophils. Contaminating cells are mostly eosinophils (depending on the eosinophil counts of the donors), with an occasional monocyte or lymphocyte. The method is easiest for volumes of blood not exceeding 200 ml (yielding $\leq 5 \times 10^8$ neutrophils), which is often enough for phagocyte association assays performed in 1 day. For isolation of larger numbers of neutrophils, refer to chapters elsewhere in this series (Vols. 162 [44] or 163 [28]).

Some simple rules will allow purification of neutrophils that give consistent results each time they are isolated. Use the highest purity water available; assiduously avoid endotoxin contamination. Use polypropylene plasticware, including pipettes; do not use glassware. Neutrophils stick to glass, leading to neutrophil activation and poor yields. (If glassware is used, treat it with a commercially available siliconizing solution, as directed by the manufacturer.) Expose the neutrophils to as few temperature fluctuations as possible; do not subject the cells to temperature shock, for example, do not repeatedly move tubes of neutrophils from the ice bath to room temperature and back again. Store neutrophil suspensions on ice, in calcium- and magnesium-free buffer until ready to use. Do not vortex, pipette, or mix neutrophil suspensions vigorously. Do not aerate neutrophil suspensions, that is, do not generate bubbles while pipetting or vortexing. The same considerations generally hold true for monocytes and macrophages, the isolation and handling of which are described later in the chapter.

Neutrophils prepared by this method are minimally primed or stimulated. Indeed, they may act or respond more slowly, or to a lesser degree, than neutrophils obtained by other methods. For instance, gonococci do not adhere well to freshly isolated neutrophils. On the other hand, when neutrophils are plated on a plastic surface, or stimulated with reagents that induce degranulation [i.e., induce the fusion of granules (or lysosomes) with the plasma membrane], they bind almost 10-fold more gonococci.[13]

* Also see this series, Vol. 108 [9], [28]–[32]; Vol. 132 [3], [8]; Vol. 162 [44]; Vol. 163 [28]; and Vol. 236 [8], [10]. Refer to Vol. 236 [10] for a more in-depth discussion of isolation of human neutrophils.

[11] A. Ferrante and Y. H. Thong, *J. Immunol. Methods* **36,** 109 (1980).

[12] A. Ferrante and Y. H. Thong, *J. Immunol. Methods* **48,** 81 (1982).

[13] C. Farrell and R. F. Rest, *Infect. Immun.* **58,** 2777 (1990).

This is because neutrophil receptors for gonococci are stored within granule membranes that, during degranulation or upregulation, become part of the plasma membrane. Methods to measure such activities are discussed below.

Materials and Reagents

9% (w/v) Ficoll. Using a powder funnel, add 18 g of Ficoll (molecular weight 400,000, Sigma, St. Louis, MO, No. F 4375) to 160 ml of very rapidly stirring water in a 500-ml Erlenmeyer flask. When the Ficoll is completely dissolved, make up the volume to 200 ml. The 9% Ficoll solution can be stored in the refrigerator for months and can be autoclaved if desired. When working with neutrophils, the Ficoll–Hypaque solution (described below) need not be sterile, as the neutrophils will not be cultured for any length of time

Hypaque-M, 75%, brand of diatrizoate meglumine and diatrizoate sodium injection, available from Winthrop Pharmaceuticals, Division of Sterling Drug, New York, NY

Ficoll–Hypaque solution. Mix 13 parts of 9% Ficoll with 3 parts of Hypaque-M, 75%. The two solutions are very dense; mix thoroughly. The Ficoll–Hypaque solution can be kept in the refrigerator for months. Sterile solutions of Ficoll–Hypaque of various densities for neutrophil, monocyte, and lymphocyte isolation are commercially available: Lympholyte from Accurate Chemical and Scientific (Westbury, NY), Histopaque from Sigma, and neutrophil isolation medium from Cardinal Associates (Santa Fe, NM). Follow the instructions provided with each separation medium. It is much less expensive to make your own Ficoll–Hypaque solution

Blood drawing supplies and reagents, including heparin (for the methods described here, it is easier to use 20- or 50-ml syringes with 18- to 20-gauge needles than it is to use several Vacutainer tubes; for volumes of blood greater than 50 ml, use an 18- to 20-gauge butterfly setup, and change 50-ml syringes)

15- and 50-ml polypropylene, sterile, disposable, conical, screw-cap tubes (or equivalent)

20- to 50-ml syringe with an 18-gauge needle to which has been attached about 3 inches of thin-walled plastic tubing; alternatively, an 18- to 20-gauge butterfly setup can be used, with the needle cut off, leaving about 3 inches of tubing remaining attached to the Luer Hub

Dulbecco's phosphate-buffered saline (DPBS) (without calcium or magnesium) with 1 mg/ml gelatin, pH 7.3 (DPBSG). Heat (do not boil) the buffer to dissolve the gelatin. The buffer contains, per liter, 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4. The buffer can be made up (or purchased) at 10× concentration, without gelatin,

filter sterilized, and stored for months at room temperature (it may crystallize in the cold) in a tightly stoppered bottle. The 10× buffer can be used to make up the 1× buffer quickly with added gelatin

Distilled and/or deionized water, stored in the refrigerator

3.6% (w/v) NaCl, stored in the refrigerator

Methods. Human blood should be handled with caution owing to the possible presence of human immunodeficiency viruses and other pathogens. Acquaint yourself with and use Universal Precautions. (Contact your Institutional Biosafety Officer, the Centers for Disease Control and Prevention, or the National Institutes of Health for information on Universal Precautions.) Wear gloves. Use needles and other "sharps" only when absolutely necessary, discarding needles in proper needle-proof containers, not in the normal waste or trash. Do not recap needles prior to disposal, as this enhances the risk of puncture injury. All materials (beakers, flasks, pipettes and pipette tips, tubes, syringes, gauze, benchtop diapers, etc.) that have been in contact with blood or blood products should be sterilized (preferably by autoclaving, or by soaking or treating with 10% bottled bleach for several hours) before being discarded or reused. If you will be handling human blood on a regular basis you should be immunized against hepatitis B.

Slowly layer 7–8 ml of whole, freshly drawn anticoagulated (10 U/ml heparin, final concentration) blood over 5 ml of room temperature Ficoll–Hypaque in 15-ml-conical screw-cap tubes. For 50 ml of blood, seven tubes are needed. With a little practice, the blood can be layered over the Ficoll–Hypaque directly from the syringe used to draw the blood (with the needle removed). For larger volumes of blood, layer 20–25 ml of blood over 20 ml of Ficoll–Hypaque, in a 50-ml conical screw-cap tube. (*Note:* It is the column height of Ficoll–Hypaque through which the blood cells travel that is important in their separation, not the absolute volume of Ficoll–Hypaque.[14]) Tightly cap the tubes and centrifuge for 30 min at 200 *g* at 20° (not 3°), with the brake set off or low.

Place the centrifuge tubes on ice. The neutrophils will have migrated through the Ficoll–Hypaque as a diffuse (cloudy) band above the erythrocye pellet, and below the Ficoll–Hypaque/plasma interface (where the mononuclear cells are located; see below). Remove the neutrophils from each tube with a syringe and tubing. Place the tip of the tubing in the middle of the neutrophil "cloud" and, while slowly swirling the syringe, withdraw as much of the Ficoll–Hypaque layer as possible, without contaminating the neutrophils with erythrocytes from the pellet or monocytic cells from the Ficoll–Hypaque/plasma interface. At this point, there will be some erythrocytes and platelets contaminating the neutrophil suspension. Before collecting the neutrophils from each subsequent tube, wipe the

[14] J. Auwerx, *Experientia* **47,** 22 (1991).

plastic tubing with a tissue, to minimize contaminating the neutrophils with mononuclear cells. Pool about 15 ml of the neutrophil suspension to as many fresh 50-ml tubes (on ice) as needed. (To avoid bubbles and to avoid activating or damaging the neutrophils, dispense the neutrophils from the syringe slowly, down the sides of the 50-ml tubes.) Add about 30 ml of cold DPBSG to each tube, mix well but gently, and centrifuge for 10 min at 200 *g* at 3°. After centrifugation, the neutrophil pellet(s) should be red because of contaminating erythrocytes. The supernatant(s) may be cloudy owing to the presence of platelets. Any platelets remaining in the neutrophil pellet(s) will be eliminated during erythrocyte lysis (see below). [If you choose not to lyse the erythrocytes, repeat the washing procedure until the supernatant(s) is clear.] Carefully, in one rather slow motion, decant and discard the supernatant(s) from the neutrophil pellet(s). Be careful as the pellet(s) may be loose. Do not drain the tubes well; leave about 0.5–1 ml of liquid.

Contaminating erythrocytes in the neutrophil pellet are removed by hypotonic lysis. Perform the lysis on each 50-ml tube separately. With slow vortex mixing (by hand, not vortex mixer) gently and thoroughly suspend the neutrophil pellet (and contaminating erythrocytes) in the small amount of liquid remaining in the tube. Rapidly add 12 ml of cold water, down the side of the tube, and vortex gently for 10 sec. This will lyse the erythrocytes, yet minimally harm the neutrophils. Immediately add 4 ml of cold 3.6% (w/v) NaCl, down the side of the tube, and vortex gently; this restores isotonicity. Centrifuge the neutrophils and lysed erythrocytes for 8 min at 125 *g* at 3°. The supernatant should be pale red, and the pellet a light tan (with a greenish tinge, if no erythrocytes are present). Carefully decant and discard the supernatant. If there is still a red color to the neutrophil pellet, the hemolysis may be repeated, but only once. Alternatively, erythrocytes may be lysed with ammonium chloride (as described in Vol. 132 [3]).

After the erythrocytes are lysed, pool the neutrophil pellets in a volume of cold DPBSG equal to about one-tenth the volume of blood drawn (e.g., 5 ml of DPBSG if 50 ml of blood was drawn). Suspend the purified neutrophils either by gentle vortexing or by gentle mixing with a disposable plastic transfer pipette, preferably with one having a large bore to avoid damaging or activating the neutrophils. To determine the concentration of neutrophils, dilute the suspension 1:10 (e.g., 10 μl in 90 μl) in 3% (v/v) acetic acid (room temperature). This lyses erythrocytes (if you have chosen not to lyse them), and, with a little practice, allows clear distinction between neutrophils and contaminating monocytic cells on examination with light or phase-contrast microscopy. Immediately charge a hemocytometer according to the manufacturer's directions, wait a minute to allow the neutrophils to settle, and quantify the number of neutrophils. Generally, suspending the purified neutrophils in DPBSG equal to 1/10 the volume of

blood drawn will yield about $1–2 \times 10^7$ cells/ml (i.e., 50 ml blood yields about $5–15 \times 10^7$ neutrophils). Add enough cold DPBSG (without calcium or magnesium) to bring the suspension to $1–2 \times 10^7$ cells/ml.

Determine cell viability by trypan blue "exclusion" (also see this series, Vol. 132 [3]). On a microscope slide, mix 1 part (e.g., 10 μl) of neutrophil suspension with 1 part of a solution of 0.25% trypan blue in 0.85% (w/v) NaCl for 5 min at room temperature, place a coverslip over the cells, and observe under 200× to 400× magnification with a light microscope. A hemocytometer can be used, if desired. Viable neutrophils are clear, whereas dead neutrophils are light blue. Do not allow the neutrophils to sit in the trypan blue solution for any length of time, as the dye is toxic and an unrepresentative number of cells will turn blue.

*Isolation of Human Blood Monocytes**

Materials and Reagents

Isotonic Percoll. Mix 1 part sterile 10× Hanks' balanced salts solution (HBSS) to 9 parts of Percoll (Pharmacia, Piscataway, NJ)

HBSS (1×), sterile, containing, per liter, 8 g NaCl, 0.4 g KCl, 48 mg Na_2HPO_4, 60 mg KH_2PO_4, 60 mg $MgSO_4 \cdot 7H_2O$, 0.14 g $CaCl_2$, 1 g glucose, 0.1 g $MgCl_2$, and 0.35 g $NaHCO_3$, pH 7.2

Material and reagents to collect sterile, autologous human serum

RPMI 1640 cell culture medium, complete, endotoxin-"free," containing 100 units/ml penicillin and 100 μg/ml streptomycin

Disposable, wide-mouth, plastic transfer pipettes (2–5 ml), or 20-ml syringes, sterile

40-ml Oak Ridge (or similar) centrifuge tubes, sterile

50-ml polypropylene, conical, disposable, screw-cap centrifuge tubes, sterile (or equivalent)

Density marker beads (Pharmacia, Cat. No. 17-0459-01)

Methods. To obtain autologous serum, draw 20 ml blood, using no anticoagulant, and transfer to a sterile glass tube. (If the blood is drawn in Vacutainer tubes, it need not be transferred to a new tube.) Let the blood clot at 37° for 1 hr, gently stirring clotted blood for a few seconds with a sterile wooden stick or transfer pipette at 30 and 60 min. Place the tube(s) of blood on ice for 2 hr, then centrifuge the clotted blood for 10 min, at 1000 *g*, at 3°. Carefully remove the serum, leaving all erythrocytes and the clot behind, and transfer to a fresh sterile tube. Properly sterilize and dispose of the clot. Keep the serum on ice at all times. Fresh serum can

* Also see this series, Vols. 132 [7] and 236 [11].

be frozen for months at −70° in small samples. Do not store serum in the refrigerator overnight; use freshly prepared serum.

One can isolate about 20% as many monocytes as neutrophils from human blood (about 4–8 × 10^7 monocytes from 100 ml blood). The method described here yields mononuclear cells that are 90–95% monocytes. Prepare blood (≥100 ml) exactly as above for neutrophil isolation, using sterile reagents and aseptic technique. Using a pipette, carefully transfer the mononuclear cell layers from the Ficoll–Hypaque/plasma interfaces to 50-ml plastic centrifuge tubes, being careful to aspirate as little of the Ficoll–Hypaque solution (which contains neutrophils) as possible. Wash the mononuclear cells twice (10 min, 200 *g*, 3°), each time with about 40 ml of cold HBSS, and suspend to 2–5 × 10^7 mononuclear cells/ml in cold HBSS. (Ficoll–Hypaque is toxic to monocytes and should be removed as quickly as possible.)

To each of two 40-ml Oak Ridge-type centrifuge tubes, dispense 22 ml of isotonic Percoll, 1 ml of autologous serum, and 14.7 ml of HBSS, and mix well. To tube 1, add 10 μl each of the various beads from the density marker kit (Pharmacia). Add an equal volume of HBSS to tube 2. Tube 1 is used as a reference to mark the position of the monocytes to be isolated in tube 2. To form a Percoll gradient, centrifuge both tubes, without cells, in a fixed-angle rotor, for 15 min, at 30,000 *g*, at 3°, with the brake off. When the gradients are ready to load, gently but thoroughly suspend the washed mononuclear cells prepared above and layer them (in a volume of about 5 ml) onto the preformed gradient in tube 2. Add an equivalent volume of HBSS to tube 1, and centrifuge both tubes in a swinging-bucket rotor for 20 min, at 1200 *g*, at 3°. After centrifugation, the monocytes band at a density of 1.076 (marker 5, blue beads), just above the lymphocytes. Using a pipette, or a 20-ml syringe with attached plastic tubing, carefully transfer the monocytes to a sterile, 15-ml polypropylene, conical, screw-cap centrifuge tube, and dilute with at least 5 volumes of cold HBSS. Wash the monocytes twice for 10 min, at 200 *g*, at 3°. After the second centrifugation, suspend the monocytes in 5 ml of RPMI 1640 plus penicillin and streptomycin, and determine the cell density, viability, and purity (by Wright stain). Purity can also be determined by cytochemical staining (see this series, Vol. 132 [8]). Adjust the suspension to 1 × 10^7 monocytes/ml.

Preparation of Human Monocyte-Derived Macrophages

Materials and Reagents

Materials for isolating and autologous serum, as described above
Sterile (autoclavable) Teflon beakers or screw-cap Erlenmeyer flasks, 20–60 ml (e.g., Teflon FEP flasks or Teflon PTFE beakers, Cole Parmer Scientific, Niles, IL)

Methods. Isolate monocytes and autologous serum, as described above. Supplement the RPMI 1640 culture medium with 15% (v/v) autologous serum and dispense the monocytes to the carefully washed sterile Teflon beakers or flasks. The number of monocytes per beaker or flask can be varied significantly. As a guide, dispense $1–2 \times 10^6$ monocytes in 2–4 ml of culture medium in 20-ml flasks, or 5×10^6 monocytes in 10 ml of culture medium in 50- to 60-ml flasks. Incubate for 4–7 days at 37°, with 5% (v/v) CO_2, and "feed" with an additional 5% autologous serum every 3 days; the medium need not be changed. Monocytes can be removed after cooling the flasks or beakers on ice for 15 min and vigorously (with no air bubbles) pipetting the monocytes with a plastic pipette. Observe for morphological changes by light microscopy and by cytochemical staining (see this series, Vol. 132 [8]) and for viability by trypan blue exclusion (see above). For purification of mouse mononuclear cells and neutrophils, refer to this series, Vol. 236 [10].

Human Neutrophil-, Monocyte-, and Macrophage-like Cell Lines

It is often useful to use a continuous cell line for some studies, such as those attempting to identify and purify specific receptors, to clone a gene encoding a receptor or a gene responsible for receptor regulation, to study the regulation and differentiation of receptors, or to compare receptors on myeloid versus monocytic cell lines. By using cell lines, one can get a consistent and essentially unlimited source of membranes, DNA, and mRNA. For neutrophils, which are fully differentiated cells containing relatively little mRNA and a relative abundance of nucleases, this becomes particularly useful. Quite interestingly, and of extreme utility, HL-60 and U-937 cells, for example, can quite easily be induced to differentiate into monocytic or myelocytic cells (albeit immature), depending on the stimulus used. Differentiated cell lines can be very valuable for studying adhesion, but only a relatively few cell lines have been used to study the interaction of bacteria with phagocytes. Some of these are listed in Table I, along with a few references, a brief description of the cell lines, and their ATCC (American Type Culture Collection, Rockville, MD) catalog numbers; all of the cell lines can be purchased from the ATCC. HL-60[15,16] (also see this series, Vol. 190, p. 118) and U-937,[17,18] the most commonly used myelomonocytic cell lines, have been rather well-characterized vis-à-vis bacteria–

[15] G. D. Birnie, *Br. J. Cancer* **9,** 41 (1988).
[16] M. C. Leglise, G. A. Dent, L. H. Ayscue, and D. W. Ross, *Blood Cells* **13,** 319 (1988).
[17] H. Hosaya and T. Marunouchi, *Cell. Struct. Func.* **17,** 263 (1992).
[18] M. T. Chateau and R. Caravano, *FEMS Immunol. Med. Microbiol.* **7,** 111 (1993).

TABLE I
HUMAN NEUTROPHIL-, MONOCYTE-, AND MACROPHAGE-LIKE CELL LINES

Cell line	Derived from	Description	ATCC number	Ref.
HL-60	Promyelocytic leukemia	Undifferentiated, nonadherent; differentiates to myelocytic or monocytic lineage in presence of various stimuli; very commonly used; well-characterized	CCL 240	*a*
K-562	Chronic myelogenous leukemia; erythroleukemia line	Undifferentiated, nonadherent; differentiates to myelocytic or monocytic lineage in presence of various stimuli; not commonly used or well-characterized for phagocytosis studies	CCL 243	*b*
KG-1	Acute myelogenous leukemia	Undifferentiated, nonadherent; differentiates to myelocytic or monocytic lineage in presence of various stimuli; not commonly used or well-characterized for phagocytosis studies	CCL 246	*c*
THP-1	Acute monocytic leukemia	Monocyte- and macrophage-like characteristics	TIB 202	*d, e*
U-937	Histiocytic lymphoma	Undifferentiated, nonadherent; differentiates to myelocytic or monocytic lineage in presence of various stimuli; commonly used; well-characterized	CRL 1594	*f*

[a] S. J. Collins, R. C. Gallo, and R. E. Gallagher, *Nature (London)* **270,** 347 (1977).
[b] C. B. Lozzio and B. B. Lozzio, *Blood* **45,** 321 (1975).
[c] H. P. Koeffler and D. W. Golde, *Science* **200,** 1153 (1978).
[d] S. Tsuchiya, M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Kono, and K. Tada, *Int. J. Cancer* **26,** 171 (1980).
[e] S. Tsuchiya, Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, and K. Tada, *Cancer Res.* **42,** 1530 (1982).
[f] C. Sundstrom and K. Nilsson, *Int. J. Cancer* **17,** 565 (1977).

phagocyte interactions and cellular differentiation.[19] KG-1[20] and THP-1[14] are less well characterized, and they have not been as commonly used.

Measurement of Bacteria–Phagocyte Interaction

Quantitation of Phagocyte-Associated (Adherent and Ingested) Bacteria

Be aware that bacteria may interact with phagocytes quite differently depending on bacterial growth conditions and growth phase. Anaerobically

[19] R. Hass, *Eur. J. Cell Biol.* **58,** 1 (1992).
[20] A. J. Furley, B. R. Reeves, S. Mizutani, L. J. Altass, S. M. Watt, M. C. Jacob, P. van den Elsen, C. Terhorst, and M. F. Greaves, *Blood* **68,** 1101 (1986).

and aerobically grown bacteria, cells from exponential- and stationary-phase cultures, agar- versus broth-grown bacteria, and bacteria grown in the presence of absence of specific nutrients (e.g., iron) might adhere to phagocytes quite differently. As mentioned above, the degree of neutrophil priming or activation and of monocyte or macrophage differentiation can dramatically affect bacterial adhesion.

Materials and Reagents

Phagocytes at $1-2 \times 10^7$ cells/ml in DPBSG or HBSS (prepared as above)

Bacteria at $1-2 \times 10^8$ cells/ml in DPBSG or HBSS (prepared as above)

DPBSG (see above) with 1 m*M* Ca^{2+} and 1 m*M* Mg^{2+} or HBSS, sterile (see above)

1.5-ml conical, microcentrifuge tubes, or 3- to 4-ml snap-cap, round-bottomed polypropylene tubes, sterile

2.5 mg/ml cytochalasin B or D in dimethyl sulfoxide (DMSO); the stock should be kept in small aliquots, frozen at −20°, and thawed and used only once

Cytocentrifuge (Cytospin, Shandon Southern Instruments, Sewickley, PA)

Wright stain, glass slides, light microscope, immersion oil

Methods. This is a very simple assay consisting of bacteria, phagocytes, buffer, and other components under investigation, such as serum, other body fluids, or inhibitors, all tumbling in a tube. Each adhesion assay utilizes a 1.5-ml snap-cap centrifuge tube containing 2×10^7 bacteria, 5×10^5 phagocytes (ratio of 40 : 1, bacteria to phagocytes), and buffer to 500 μl. Cap the tubes tightly and tumble them end-over-end, at about 15 rpm, in a 37° incubator. If a rotator is not available, the tubes may be rocked end-to-end. At 30–120 min (at least for initial assays), deposit 150 μl of assay mixture on a glass slide using a cytocentrifuge. Subject the "dot" to Wright stain and quantify the number of associated bacteria per 100 contiguous phagocytes by oil immersion (1000× magnification) light microscopy. To acquire data objectively, cover all identifying marks on the slides with opaque tape, number the slides randomly, and then quantify results. Break the code, that is, remove the pieces of tape, only after all data have been recorded. It is also helpful to have a second member of the laboratory quantify a few slides, to determine if, using a specific set of criteria, similar results are obtained. These criteria should include, for instance, whether bacteria that are adjacent to but not squarely over a phagocyte are counted as adherent or whether a bacterium "trapped" between two phagocytes is counted as adherent.

If, on observation of the stained preparations, the phagocytes are too closely packed to assess bacterial adhesion accurately, decrease the phago-

cyte density in the assay. If the phagocytes are too clumped to assess bacterial adhesion, decrease the bacterial or phagocyte density, or both, or decrease the assay time. It is possible, in this situation, that the bacteria activate the phagocytes to such a degree that clumping naturally occurs, and, regardless of density, phagocytes will irreversibly clump. In these cases, quantitation can be difficult; it may be better to perform adhesion assays with adherent phagocytes than with phagocytes in suspension (see below). Be conscious of the pH of the medium during the adhesion assay. The DPBSG contains no bicarbonate or glucose, whereas HBSS contains both. The two buffers have quite different buffering capacities in air than in a CO_2 atmosphere. For peace of mind, measure the pH of a few tubes after the assay is over; it should remain between pH 7.2 and pH 7.4.

Cytochalasins inhibit microfilament polymerization and phagocytosis. Although adhesion assays can (and should initially) be done without cytochalasin, it is often easier to quantify associated bacteria when cytochalasin is used. This may be due to the degradation of ingested bacteria when cytochalasin is not used, or to the fact that cytochalasin changes the morphology of the phagocytes, making the stained bacteria easier to visualize. More importantly, cytochalasin assures that bacteria are not internalized by the phagocytes. Thus, when results are quantified, there is no concern that there are both extracellular and intracellular bacteria. Cytochalasins are used at a final concentration of 5 μg/ml (i.e., 1 μl of the 2.5 mg/ml stock in the 500-μl assay).

Cytochalasin activity is reversible; thus, the reagent must be present in the assay buffer at all times, and it should be added to the phagocytes about 10 min prior to the addition of bacteria. In our hands, results are the same with or without cytochalasin, at assay times below 30 min. Remember that when viewing stained preparations from phagocytosis assays, one cannot determine whether bacteria are adherent versus ingested, regardless of what the observer might think they look like under the microscope. A technique is described below to quantify adherent versus ingested bacteria (in the absence of cytochalasins). Why would one not use cytochalasin? Cytochalasin dramatically disrupts microfilament and cell function. Because microfilaments are associated with many phagocyte membrane receptors, it is reasonable to think that disrupting microfilament structure and function might also affect receptor structure or function. This is at least one reason why preliminary assays should be done with and without cytochalasins.

Assays can be made proportionally larger (and 3- to 4-ml snap-cap tubes used) for long assays from which several samples are to be drawn, or for assays in which multiple samples will be drawn, for instance, when wishing to prepare samples for fluorescence or electron microscopy. The shape of the tube makes no difference, as long as the phagocytes tumble

freely. The size of the tube should be no larger than 5 times the volume of the assay mixture.

The phagocyte adhesion assay system should be tailored or optimized for each individual species of bacteria and for the phagocyte under investigation. This assay is simple enough that optimization can easily be done within 1 or 2 weeks. For instance, better adhesion may be achieved with a particular buffer (HBSS, DPBSG, or a more complete cell culture medium). The density of phagocytes and the ratio of bacteria to phagocytes should be varied. Especially for monocytes and macrophages, the time of the assay can be extended. Neutrophils, however, do not remain viable for long periods, and results from assays over 3 hr should be viewed with caution. For this reason, and because some bacteria are toxic to phagocytes, it is mandatory to determine phagocyte viability at the end of all adhesion assays, at least the first few times a new bacterial strain or variant is used.

Although the assay is more tedious to quantify, there are several reasons to prefer this assay to radioactive determinations, where bacteria are radiolabeled, mixed with phagocytes, incubated, separated from phagocytes by centrifugation, and counted. First, depending on the bacteria and the phagocytes (especially neutrophils, which are denser than monocytes or macrophages), it may not be possible to separate bacteria from phagocytes quantitatively by centrifugation. This is certainly true for gonococci (Rest, unpublished observations). Second, important observations can be made by light microscopy that cannot be made by radioactive determinations concerning the association of bacteria with phagocytes. These include the state of the phagocytes (e.g., whether they are clumped, activated, or lysed) and the absolute numbers of bacteria per phagocyte (e.g., whether there are even or uneven distribution of bacteria per phagocyte).

The results obtained from the tumbling tube adhesion assay may indicate that there is little adhesion of bacteria to phagocytes, especially to neutrophils. As mentioned above, however, the preliminary data must be interpreted with caution and further experiments performed, as follows. Neutrophils obtained by the method described above are minimally activated. This means that there is a paucity of some receptors on their surface. This is certainly true for the human neutrophil receptors for *N. gonorrhoeae*[13] and for microbes that use complement receptor 3 (CR3), which is upregulated in stimulated neutrophils. There are at least two ways to determine if such a situation is true.

The first is to stimulate the neutrophils, before they are used in the adhesion assay, with one of three commonly used stimuli: phorbol myristate acetate (PMA), the chemotactic peptide formylmethionylleucylphenylalanine (FMLP), or the calcium ionophore A23187. A stock solution of 100 μg/ml PMA in DMSO is stored at $-20°$ in a frost-free freezer, in 50-μl

aliquots. Each aliquot is used only once, then discarded. Similarly, the FMLP stock solution is 1 m*M* in DMSO, and the A23187 stock solution is 10 m*M* in DMSO. Pretreat neutrophils for 10 min with a final concentration of 100 ng/ml PMA, 0.2 μM FMLP, or 10 μM A23187. Cytochalasin B, at 5 μg/ml, is used in conjunction with FMLP. If, on stimulation with one of the reagents, phagocytes still do not bind bacteria, it can be safely assumed that the bacteria do not adhere to phagocytes.

The second method to determine if phagocyte receptors for the bacteria under study need to be upregulated, or brought to the surface of the phagocyte, is to perform the adhesion assays in a monolayer system. Adherent phagocytes are upregulated or "stimulated," similar to being treated with some mild stimuli. The monolayer system is essentially identical for each of the three types of phagocytes, which are prepared exactly as described above. Assays are most conveniently performed in 24-well tissue culture plates, or in petri dishes, on individual round (generally 12–13 mm) glass or tissue culture plastic coverslips (obtained through any scientific distributor or company dealing in cell and tissue culture products). Suspend phagocytes in DPBSG or medium with calcium and magnesium to 1×10^6 cells/ml, deposit about 2×10^5 phagocytes per coverslip, and incubate at 37° in a humidified incubator for 1 hr. Rinse the coverslips twice gently with warm DPBSG or medium containing gelatin, albumin, or heat-inactivated serum, to remove unbound phagocytes, and immediately and gently deposit 100–200 μl of bacteria (at $1–10 \times 10^7$ cells/ml) onto the adherent phagocytes. Let the bacteria adhere for up to 2 hr and gently rinse the monolayers with warm DPBSG or medium containing 10% heat-inactivated serum, to remove unbound bacteria. (Gelatin, serum, or albumin added to the rinse buffer or medium preserves phagocyte morphology for staining.) If desired, adherent phagocytes can be further stimulated with PMA, A23187, or FMLP, as discussed above. Stain and analyze the monolayers as above for the tumbling tube assay.

Differentiation of Adherent versus Ingested Bacteria

In the following procedure, first described by Hed for yeast,[21] bacteria are prelabeled with fluorescein and incubated with phagocytes, aliquots of the phagocytosis mixture are mixed with trypan blue, and the mixture is viewed with fluorescence and phase-contrast microscopy. Intracellular bacteria fluoresce, whereas the fluorescence of adherent (but not ingested) bacteria is quenched by the trypan blue. With the technique, adherent and intracellular bacteria can be quantified, as can the percent ingested bacteria.

[21] J. Hed, *FEMS Lett.* **1**, 357 (1977).

The assay originally described the use of crystal violet as a quenching agent, but more recently Hed reported that crystal violet, being lysosomotropic, can enter phagolysosomes and quench the fluorescence of intracellular bacteria as well (see this series, Vol. 132 [6]).

There are drawbacks to the assay. Owing to the physicochemical makeup of the cell surface, the bacterial strain(s) might be resistant to significant fluorescein isothiocyanate (FITC) conjugation, or FITC conjugation may decrease or destroy the binding function or specific surface molecules. Some of these problems may be insurmountable, making this technique less useful.

Materials and Reagents

Same materials and reagents as described above for the adhesion assay
Fluorescein isothiocyanate (FITC), 0.1 mg/ml in 0.1 *M* carbonate buffer, pH 9.5, freshly prepared
Trypan blue, 2 mg/ml in 0.15 *M* NaCl in 20 m*M* citrate or acetate buffer, pH 4.4
Fluorescence microscope, microscope slides

Methods. To label bacteria with fluorescein, incubate 1×10^9 bacteria in 1 ml of 0.1 *M* carbonate buffer, pH 9.5, containing 0.1 mg/ml FITC for 30 min at 37°. Wash the fluorescein-conjugated bacteria with DPBSG three times by centrifugation, and suspend to 5×10^8 cells/ml in DPBSG.

To determine total adherent and intracellular bacteria, incubate bacteria with phagocytes as indicated in the adhesion assays described above. At time points determined empirically by initial adhesion assays, deposit 5 μl of assay mixture on a glass slide, add 5 μl of trypan blue solution, mix gently, cover with a coverslip, and immediately observe with phase-contrast and fluorescence microscopy. It is important to observe intracellular and extracellular bacteria immediately after their removal from the phagocytosis assay, because phagocytosis may continue on the glass slide. Intracellular bacteria will fluoresce, whereas adherent (extracellular) bacteria will not. Under phase-contrast microscopy, intracellular bacteria will be bright, whereas adherent bacteria will be dark. By working with the numbers of blue (adherent) and fluorescent (intracellular) bacteria, "total associated" and "percent ingested" bacteria can be easily determined. Care should be taken in interpreting data from time points taken more than 1 hr after phagocytosis, because the fluorescein might be degraded, released from the surface of the bacteria, or otherwise quenched.

The assay can be readily modified for adherent phagocytes. Variations of the method have been published (e.g., see Ref. 22). For measurement of adhesion of bacteria to phagocytes by flow cytometry, see this series, Vol. 132, pp. 183–192.

[22] D. A. Drevets and P. A. Campbell, *J. Immunol. Methods* **142,** 31 (1991).

[3] Interaction of Bacteria with Mast Cells

By RAVI MALAVIYA and SOMAN N. ABRAHAM

Introduction

When pathogenic bacteria penetrate or circumvent the integumental barrier of the host, they adhere to and activate a variety of inflammatory cells. These cells include neutrophils, lymphocytes, macrophages, and mast cells. Activated inflammatory cells may undergo (i) exocytic reactions, such as degranulation and secretion of inflammatory mediators, and (ii) endocytic reactions such as phagocytosis of adherent bacteria. Together, these events determine both the intensity of the inflammatory response mounted by the host and the rate of bacterial clearance.

Much is known about the interaction between bacteria and neutrophils,[1,2] macrophages,[3,4] or lymphocytes.[5,6] However, only a limited amount of information is available on bacterial interactions with mast cells. Because mast cells are replete with a panoply of potent mediators of inflammation,[7–10] as well as being one of the first inflammatory cells to be encountered by the invading pathogen, their interaction with bacteria is likely to be a critical aspect in the pathogenic process.[11–13] It is our aim to present some of the methods which we have utilized to study the interaction of type 1

[1] K. T. Miyasaki, *J. Periodontol.* **62,** 761 (1991).
[2] W. Konig, *Clinical and Experimental Allergy* **21**(Suppl 1), 58 (1991).
[3] J. Verhoef, *Clin. Ther.* **13,** 172 (1991).
[4] O. D. Rotstein, *Clin. Infect. Dis.* **16**(Suppl. 4), S190 (1993).
[5] H. Band, G. Panchamoorthy, J. Mclean, C. T. Morita, S. Ishikawa, R. Modlin, and M. B. Brenner, *Res. Immunol.* **141,** 645 (1990).
[6] J. W. Simecka, S. E. Ross, G. H. Cassell, and J. K. Davis, *Clin. Infect. Dis.* **17**(Suppl 1), S176 (1993).
[7] L. J. Roberts, R. A. Lewis, J. A. Oates, and K. F. Austen, *Biochim. Biophys. Acta* **575,** 185 (1979).
[8] E. Razin, J. M. Mencia-Huerta, R. A. Lewis, E. J. Corey, and K. F. Austen, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 4665 (1982).
[9] J. M. Mencia-Huerta, E. Razin, E. W. Ringel, E. J. Corey, D. Hoover, K. F. Austen, and R. A. Lewis, *J. Immunol.* **130,** 1885 (1983).
[10] Y. Wei, K. Heghinian, R. L. Bell, and B. A. Jakschik, *J. Immunol.* **137,** 1993 (1986).
[11] M. K. Church, S. Norn, G. J.-K. Pao, and S. T. Holgate, *Clin. Allergy* **17,** 341 (1987).
[12] R. Malaviya, E. A. Ross, J. I. MacGregor, T. Ikeda, J. R. Little, B. A. Jakschik, and S. N. Abraham, *J. Immunol.* **152,** 1907 (1994).
[13] R. Malaviya, E. Ross, B. A. Jakschik, and S. N. Abraham, *J. Clin. Invest.* **93,** 1645 (1994).

fimbriated *Escherichia coli* and mast cells. These techniques could serve as a paradigm for studies relating to the interaction of bacteria with other inflammatory cells.

Preparation of Mast Cells

Based on different phenotypical, biochemical, and ontogenic properties, two distinct populations of mast cells have been defined in rodents. These populations are referred to as connective tissue and mucosal mast cells. Analogous differences appear to be present in human mast cells.[14-16] The connective tissue mast cells (CTMC) are found in the skin, peritoneal cavity, serosa, as well as in the muscularis propria of the intestine. Their granules contain heparin and large amounts of histamine. Mucosal mast cells (MMC) are primarily found in the intestinal mucosa and intraepithelium, within the crypts and bases of villi. Their major proteoglycan is chondroitin sulfate, and their histamine content is approximately 1/10 of the amount in CTMC. In spite of their heterogeneity, both populations of mast cells share several biological functions.

Relatively large numbers of mast cells can be obtained from culturing hematopoietic precursors obtained from the mouse bone marrow. These bone marrow-derived mast cells (BMMC) resemble MMC and are used for most of our studies. CTMC, which cannot be obtained in large amounts, are employed primarily for assays of mast cell degranulation and mediator release. Mast cell lines such as RBL-2H3, CFTL-12, CFTL-15, and P8-18 are also available and have primarily been utilized to study intracellular signal transduction and mediator release.[17-23] However, certain notable

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[17] M. A. Brown, J. H. Pierce, C. J. Watson, J. Falco, J. N. Ihle, and W. E. Paul, *Cell (Cambridge, Mass.)* **50,** 809 (1987).
[18] M. Plaut, J. H. Pierce, C. J. Watson, J. Hanley-Hyde, R. P. Nordan, and W. E. Paul, *Nature (London)* **339,** 64 (1989).
[19] A. Wong, S. M. Hwang, M. N. Cook, G. K. Hogaboom, and S. T. Crooke, *Biochemistry* **27,** 6763 (1988).
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[21] A. Wong, M. N. Cook, S. M. Hwang, H. M. Sarau, J. J. Foley, and S. T. Crooke, *Biochemistry* **31,** 4046 (1992).
[22] G. Henkel, D. L. Weiss, R. McCoy, T. Deloughery, D. Tara, and M. A. Brown, *J. Immunol.* **149,** 3239 (1992).
[23] S. Offermanns, S. V. P. Jones, E. Bombien, and G. Schultz, *J. Immunol.* **152,** 250 (1994).

features associated with typical mast cells, such as evoking an oxidative burst and bactericidal activity on interaction with bacteria, have not been observed in these cell lines.

Culture of Bone Marrow-Derived Mast Cells

Principle. Bone marrow cells obtained from mice proliferate and differentiate into mature mast cells when cultured for over 3 weeks in the presence of growth factors [interleukin 3 (IL-3) and IL-4].[10,24,25] A convenient source of mast cell growth factors is conditioned medium obtained from the culture of myelomonocytic leukemia WEHI-3 cells.[10,26]

Materials

500-ml spinner flask (Bellco Glass, Vineland, NJ); prior to use, the flask has to be siliconized by treatment with 1% (v/v) Procil-28 (PCR Incorporated, Gainesville, FL)
50-ml sterile tubes
Scissors
Forceps
5-ml petri dishes
5-ml syringe
26½-gauge needle
Ethanol (70%, v/v)
Conditioned medium: medium obtained from culture of WEHI-3 cell line (American Type Culture Collection, Rockville, MD)
Enriched medium: add the following to RPMI 1640 (GIBCO/BRL, Grand Island, NY): nonessential amino acids (0.1 m*M*), L-glutamine (2 m*M*), sodium pyruvate (1 m*M*), 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), heat-inactivated fetal bovine serum (5%, v/v) (Hyclone Laboratories, Logan, UT), heat-inactivated bovine calf serum (5%, v/v) (Hyclone), HEPES (15 m*M*) (GIBCO/BRL)
Five 6- to 8-week-old, male BALB/c mice (Jackson Laboratories, Bar Harbor, ME)
Crystal violet (0.2%, w/v) or Alcian blue stain (0.2%, w/v) (Sigma, St. Louis, MO)
Low profile magnetic stirrer (Bellco Glass)

[24] E. Razin, C. Cordon-Cardo, and R. A. Good, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2559 (1981).
[25] R. Malaviya, R. Malaviya, and B. A. Jakschik, *J. Biol. Chem.* **268,** 4939 (1993).
[26] Y.-P. Yung and M. A. S. Moore, *Contemp. Top. Mol. Immunol.* **10,** 147 (1985).

Procedure

1. Mice are anesthetized, sacrificed by decapitation, and drained of blood.
2. The femurs are removed and cleaned with ethanol.
3. Employing a syringe equipped with a $26\frac{1}{2}$-guage needle, the bone marrow is flushed into petri dishes with enriched medium.
4. Approximately $1-2 \times 10^8$ bone marrow cells are harvested and then seeded in a siliconized spinner flask to a density of 1.0×10^5 cells/ml in a mixture of conditioned (25%) and enriched medium (75%).
5. The flask is placed on a stirrer and incubated in a humidified atmosphere containing 5% (v/v) CO_2 at 37°. The speed of the magnetic stirrer in the flask should be carefully controlled so as not to induce spontaneous mast cell degranulation.
6. The culture medium is replaced every seventh day with the mixture of conditioned and enriched medium.
7. After 21 days, the purity of the BMMC culture is ascertained by staining the cells for 5 min with crystal violet or alcian blue. Typically, at this time 98% or more of the cells are stained with the mast cell-specific dyes.

Isolation of Connective Tissue Mast Cells

Principle. A convenient source of fully differentiated connective tissue mast cells (CTMC) is the mouse peritoneum, where approximately 5% of total peritoneal cells are mast cells. Sterk and Ishizaka[27] have described a useful method for isolating CTMC involving the isopycnic gradient centrifugation of peritoneal cells. Because of its unique ability to generate dense solutions of low viscosity and osmolality, metrizamide [2-(3-acetamido-5-*N*-methylacetamido)-2-deoxy-D-glucose] is used as the gradient material.[28]

Materials

Five to ten CBA/J or BALB/c mice (Jackson Laboratory)
50 and 15-ml polystyrene tubes
$26\frac{1}{2}$-gauge needle
5-ml syringe
Forceps
Scissors
Trypan blue stain (0.4%)
Crystal violet stain (0.2%)

[27] A. R. Sterk and T. Ishizaka, *J. Immunol.* **128,** 838 (1982).
[28] A. C. Munthe-Kaas and P. O. Seglen, *FEBS Lett.* **43,** 252 (1974).

10× stock Tyrode's buffer:

Sodium chloride	40.0 g
Potassium chloride	1.0 g
Sodium dihydrogen phosphate monohydrate	0.28 g
MES (Sigma)	4.9 g
HEPES	6.3 g

Bring up to 500 ml with deionized water (final pH 7.0)

Tyrode's gel (TG) buffer: dissolve 0.2 g sodium bicarbonate and 0.2 g D-glucose in 20 ml of 10× Tyrode's buffer, bring up to 100 ml with deionized water, and add an equal volume of 0.2% (w/v) gelatin prepared in deionized water

Metrizamide solution: dissolve 0.9 g metrizamide (Sigma) in 4 ml of TG buffer (22.5%, w/v) and keep at room temperature; the solution should be prepared fresh daily

Procedure

1. CBA/J mice are anesthetized, sacrificed by decapitation, and drained of blood.
2. Five milliliters of TG buffer (25°) is injected into the peritoneal cavity of each mouse.
3. The abdomen is gently massaged and the peritoneal lavage from each mouse is collected and pooled in a 50-ml tube.
4. The pool of peritoneal cells is centrifuged at 450 *g* for 10 min at 25° and suspended in 2 ml TG buffer.
5. The suspension is gently layered over 2 ml of the metrizamide solution in a 15-ml tube.
6. The tube is centrifuged at 450 *g* for 20 min (25°).
7. The peritoneal cells, which collect at the interface, are aspirated carefully and discarded. The supernatant is also aspirated, and a cotton swab is employed to remove all residual cells attached to the walls of the tube.
8. Greater than 95% of the cells that have collected at the bottom of the tube are mast cells as assessed by staining with crystal violet.
9. The viability of the cells is tested and should be greater than 98% as assessed by the trypan blue dye exclusion test.

Growth and Preparation of Bacteria

Ten-milliliter tubes of Luria broth or brain–heart infusion are inoculated with 100 μl of *Escherichia coli* or bacteria of choice and incubated for 1–2 days at 37°. The culture is then centrifuged and the pellet of bacteria is washed once and suspended to the desired density in sterile phosphate-

buffered saline (PBS, GIBCO/BRL) or Hanks' balanced salt solution (HBSS, GIBCO/BRL).

Quantitation of Bacterial Adhesion to Mast Cells

Principle. Although several techniques could be employed to study the adhesion of bacteria to mast cells, the method employing microscopy is particularly useful, because it reveals the characteristic patterns of bacterial adhesion on the mast cell surface as well as the heterogeneity in receptivity among mast cells.

Materials

Light microscope
BMMC suspended to a density of 1.4×10^6 cells/ml in HBSS
Escherichia coli suspended to a density of 1.4×10^8 bacteria/ml in HBSS
1.5-ml sterile polypropylene microcentrifuge tube
Glass slides
Methanol
Methylene blue stain (0.2%, w/v)
HBSS

Procedure

1. One hundred microliters of the mast cell suspension is mixed with an equal volume of the bacterial suspension (mast cell–bacteria ratio, 1 : 100) in a microcentrifuge polypropylene tube.
2. The mixture is incubated at 37° for 1 hr with gentle rocking. Vigorous rocking can degranulate mast cells.
3. Mast cells with adherent bacteria are sedimented by centrifugation (450 *g* for 10 min) and nonadherent bacteria are carefully removed.
4. Any residual nonadherent bacteria are removed by washing the mast cells with HBSS, twice.
5. The final pellet of mast cells with adherent bacteria is suspended in 150 μl of HBSS.
6. Around 50 μl of the suspension is smeared on each of three glass slides.
7. The slides are air-dried, fixed with methanol for 1 min, and stained with methylene blue for 2 min.
8. Approximately 100 mast cells/smear are examined for adherent bacteria by light microscopy ($\times$100 objective lens).
9. The results are presented either as: (a) the number of adherent bacteria/mast cell, which is determined by dividing the total number of

adherent bacteria by the total number of mast cells examined, or (b) the percentage of mast cells with at least one adherent bacterium which is determined by multiplying the ratio of the total number of mast cells with at least one adherent bacterium divided by the total number of mast cells examined by 100.

10. The experiment is performed at least on three separate occasions, and the results are expressed as the mean ± standard error.

Expected Results. Typically with type 1 fimbriated *E. coli,* the mean number of adherent bacteria is 26 per mast cell, and the percentage of mast cells with at least one adherent bacterium is 98%.

Quantitation of Mast Cell Chemiluminescence Response to Bacteria

Principle. Traditional phagocytes, stimulated either by phagocytosis or by contact with a variety of surface-active agents, will emit a prompt burst of light (chemiluminescence). This event is correlated in time with an increase in the formation of singlet oxygen (O_2^-) and other oxygen metabolites that function as potent microbiocidal agents. The singlet oxygen is produced when one of the unpaired electrons of oxygen is lifted to a higher orbit with an inversion of spin. Light is emitted when the electron reverts to the ground state and is termed chemiluminescence. The emitted light is measured by an appropriate fluorometric detection system.[29] The sensitivity level of the assay is increased by using luminol, a cyclic hydrazide, which acts as a substrate for O_2^-. It is possible to obtain an estimate of the proportion of certain oxygen species released by the mast cells by comparing the chemiluminescence reactions in both the presence and absence of specific chelators of superoxide anions and hydrogen peroxides. Superoxide dismutase quenches the chemiluminescence response associated with the release of superoxide anion. Catalase, however, specifically quenches chemiluminescence associated with release of hydrogen peroxide.

Materials

β-Liquid scintillation counter set in the off-coincidence mode (DuPont, Wilmington, DE)
5-ml polypropylene scintillation vials
Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Aldrich, Milwaukee, WI), stock solution of 10 mg/ml dissolved in dimethyl sulfoxide (Sigma), prepared and stored in the dark
HBSS without phenol red (GIBCO/BRL)

[29] J. A. Metcalf, J. I. Gallin, W. M. Nauseet, and R. K. Root (eds.), "Laboratory Manual of Neutrophil Function," p. 103. Raven, New York, 1986.

Zymosan (Sigma), stock solution of 10 mg/ml prepared in HBSS
BMMC suspended in enriched media to a density of 1.0×10^6 cells/ml
Escherichia coli suspended in HBSS to a density of 1.0×10^9 bacteria/ml
Superoxide dismutase (Sigma)
Catalase (Sigma)

Procedure

1. One milliliter of the mast cell suspension is sedimented by centrifugation (600 *g* for 10 min), and the pellet is suspended in 900 μl of luminol (final concentration, 10 μM).
2. The mixture is transferred to a scintillation vial to which 100 μl of the bacterial suspension is added.
3. The vial is swirled for 30 sec, placed into the β-liquid scintillation counter, and counted for 1-min periods continuously for 30 min.
4. Each test is performed in triplicate, and a plot of mean counts per minute (cpm) versus time is generated.
5. To study the effect of sequestering agents, 40 μl/ml of superoxide dismutase or 5000 units/ml catalase is added to the vials containing mast cells before the addition of bacteria, and the vials are counted as described above.
6. The following negative and positive controls should also be included: (i) 100 μl of HBSS mixed with 900 μl of luminol, (ii) 100 μl of HBSS mixed with 900 μl of mast cells suspended in luminol, and (iii) 100 μl of zymosan mixed with 900 μl of mast cells suspended in luminol.
7. The specific mast cell chemiluminescence induced by bacteria can be expressed as the net increase in counts after subtracting background and unstimulated values.

Expected Results. The background counts (empty vials) usually range between 2000 and 5000 cpm, and the unstimulated controls range between 3000 and 9000 cpm. When type 1 fimbriated *E. coli* is employed as the stimulant, chemiluminescence levels usually peak around 10 min and are often 8 to 10 times higher than those of unstimulated controls. Superoxide anion is the predominant oxygen species evoked by type 1 fimbriated *E. coli.* This is because over 70% of the mast cell chemiluminescence elicited is inhibited by superoxide dismutase, whereas less than 5% is inhibited by catalase.

Quantitation of Mast Cell Bactericidal Activity

Principle. The method of quantitating mast cell bactericidal activity is similar to standard killing assays employed for traditional phagocytes using

bacterial colony counts. An inherent problem with killing experiments is bacterial multiplication during the course of the assay, especially of organisms that are not in contact with the phagocyte. This phenomenon tends to mask the killing effect of the phagocytes and renders precise kinetic studies of their bactericidal activity difficult. To reduce the problem, nonadherent bacteria are removed from the incubation mixture early in the experiment. Thus, bacteria are allowed to bind to mast cells for 15 min, after which nonadherent bacteria are removed and replaced with fresh medium, before the incubation is initiated at 37°. Bacterial growth during the course of the experiment is also monitored by incubating bacteria with cultured mouse 3T3 fibroblasts (innocuous cells, with no known bactericidal activity) in a parallel experiment. By comparing bacterial viability in the presence of the two cell types, an accurate assessment of mast cell bactericidal activity can be made.

Materials

Antibiotic-free enriched medium, prepared as enriched medium but the antibiotics are omitted

MacConkey agar or appropriate agar medium (Difco Laboratories, Detroit, MI)

16 hr culture of *E. coli* are suspended to a density of 1.5×10^7 viable bacteria/ml in enriched medium

BMMC suspended to a density of 0.3×10^6 cells/ml in enriched medium

3T3 mouse fibroblasts (ATCC): cells are harvested from plastic flasks by treating the fibroblast monolayer with trypsin/EDTA (0.05%, v/v / 0.02%, w/v); cells are suspended in enriched medium to a density of 1.5×10^5 cells/ml

Triton X-100 (Sigma)

48-well sterile tissue culture plate (Costar, Cambridge, MA)

Procedure

1. Five hundred microliters of the mast cell suspension and an equal volume of the fibroblast suspension are dispensed into several wells of a 48-well tissue culture plate.

2. The plate is incubated at 37° overnight in a 5% (v/v) CO_2 incubator, at which time a monolayer of each cell type is formed within the wells.

3. The monolayers are gently rinsed with enriched medium to remove nonattached cells.

4. Three hundred microliters of the bacterial suspension is dispensed into each well.

5. The plate is incubated for 15 min at 25° to permit bacteria to adhere to the monolayer.

6. Nonadherent bacteria are removed by repeated very gentle rinsing (5–6 times) of the monolayer before 300 μl of enriched medium is added to each well.

7. The plate is placed in a 37° incubator.

8. At time 0 (i.e., immediately) and at 15-min intervals thereafter, for a total of 1 hr, 300 μl of 0.2% Triton X-100 (which lyses the host cells) is added to triplicate wells of each cell type.

9. One hundred microliters of the cell lysate from each well is serially diluted in 0.1% Triton X-100.

10. Ten microliters of each dilution is spread on a MacConkey agar plate.

11. The plates are incubated overnight at 37°.

12. The number of colonies on the plates are counted.

13. The mean number of viable bacteria per milliliter (from triplicate experiments) is determined.

14. The percentage of bacterial viability at a given time point (x) with either cell type is represented as (the colony count at time x/the colony count at time 0) $\times$ 100.

15. The experiment is performed on at least three separate occasions, and the results are expressed as the mean percentage viability $\pm$ standard error at each time point.

Expected Results. When type 1 fimbriated *E. coli* is exposed to BMMC for 1 hr, bacterial viability is reduced by up to 60%. This finding becomes even more remarkable if we consider that, during the same period, a 90% increase in bacterial viability is seen in the presence of fibroblasts.

Assessment of Mast Cell Degranulation

Principle. Morphological studies of mast cell degranulation can be employed to monitor bacteria-triggered mast cell activation and mediator release. Avidin is a glycoprotein with a specific binding affinity for heparin, a constituent of CTMC granules. Avidin tagged with fluorescein isothiocyanate (FITC) is a highly effective probe to monitor the granule content of individual mast cells.[30,31] With the help of an image analyzer which automatically displays the fluorescence intensity of mast cells labeled with avidin–FITC, as digital and quantifiable images, the granule content of mast cells can be accurately measured before and after exposure to bacte-

[30] P. R. Bergstresser, R. E. Tigelaar, and M. D. Tharp, *J. Invest. Dermatol.* **83,** 214 (1984).

[31] M. D. Tharp, L. L. Seelig, Jr., R. E. Tigelaar, and P. R. Bergstresser, *J. Histochem. Cytochem.* **33,** 27 (1985).

ria.[13,32,33] The digitization involves an analog-to-digital conversion of the video signal. When the image acquisition is complete, the software compares, pixel by pixel, the digital image with a predefined scale for color shading correction (bright pink to blue), and, finally, the digitized image is shown on the color screen. In essence, the final digitized image seen on the screen is a reflection of the fluorescent intensity and surface area of the individual mast cell. A disadvantage of the method is that totally degranulated mast cells may not be detected; thus, the degranulation data obtained could be an underestimated value.

Materials

Image analysis system: Epson equity computer III (Epson America, Korea), image analysis software (Microcomp, Southern Micro Instruments, Atlanta, GA), graphic digitizer 39C2 (Numonics, Montgomeryville, PA), Polaroid freezeframe video recorder, low-level intensity SIT 68 camera (Dage MTI, MI), Sony TV monitor (Sony, Ichinomiya, Japan), and epifluorescence microscope (Nikon, Tokyo, Japan), equipped with a mercury lamp (for excitation, use a filter with transmission maximum at 470 nm, and for emission, use a dichroic mirror with 50% reflection and transmission at 495 nm, in combination with a barrier filter with the wedge at 530 nm)

Cytospin 3 centrifuge (Shandon, Pittsburgh, PA)

RPMI–HEPES buffer: 15 m*M* HEPES in RPMI 1640 medium

RPMI–HEPES buffer containing 0.75% (w/v) bovine serum albumin (BSA)

CTMC suspended in RPMI–HEPES buffer containing 0.75% BSA at a density of 8.0×10^5 cells/ml

Escherichia coli suspended in RPMI–HEPES buffer containing 0.75% BSA at a density of 4.0×10^7 cells/ml

Carnoy's fixative:

Ethanol (99%)	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

Mounting medium:

Glycerol	9 ml
PBS	1 ml
DABCO (1,4-diazobicyclo[2,2,2]octane, Sigma)	30 m*M*

Adjusted to pH 8.6 by the addition of 0.1 *M* NaOH

Avidin–FITC (Zymed, San Francisco, CA), 6.25 μg/ml in PBS

[32] Y. Zhang, B. F. Ramos, and B. A. Jakschik, *J. Clin. Invest.* **88,** 841 (1991).

[33] S. Bianchi and L. Mugnai, *Eur. J. Basic Appl. Histochem.* **35,** 161 (1991).

Procedure

1. One hundred microliters of CTMC is incubated for 60 min at 37° with an equal volume of bacteria (mast cell/bacteria ratio of 1:50).
2. As a control (spontaneous degranulation), 100 μl of CTMC is incubated with an equal volume of RPMI–HEPES.
3. One hundred microliters of each mixture is centrifuged onto glass slides in the cytospin (250 *g* for 5 min at 25°).
4. The slides are placed in coplin jars containing Carnoy's fixative for 1 min and then rinsed in PBS (three times).
5. Each of the smears is immersed in avidin–FITC for 2 hr in a moist and dark chamber.
6. The slides are rinsed thoroughly in PBS (in the dark) and placed in mounting medium.
7. Microscopic examination of the slides is performed within 2 hr of staining. A significant drop in fluorescence is evident if the slides are not examined quickly.
8. The fluorescence intensities of approximately 200 mast cells after exposure to bacteria (or to PBS) are determined employing the image analysis system. The mean fluorescence intensity per mast cell is determined and expressed as arbitrary units.
9. The experiment is performed on at least three separate occasions, and the data are expressed as the mean fluorescence intensity ± standard error. Because of the wide variation in the fluorescence intensity of mast cells seen between each experiment, the results from different days are normalized relative to the controls for each experiment before the mean is determined.

Expected Results. The mean fluorescence intensity of CTMC exposed for 1 hr to type 1 fimbriated *E. coli* is typically 50% less than the fluorescence intensity of CTMC exposed to buffer (unstimulated).

Secretion of Inflammatory Mediators by Mast Cells

The interaction of bacteria with mast cells can result in the release of several mast cell mediators, some of which are presynthesized, whereas others are synthesized only on stimulation. The mediators, either functioning alone or in concert, could significantly affect the intensity and duration of the inflammatory response in the host. A quantitative assessment of inflammatory mediators released into the extracellular medium by mast cells after bacterial stimulation can be a useful indicator of mast cell activation. Because the biological activity of the mediators is also known, this information may also be a predictor of the inflammatory potential of the bacteria *in vivo*. We provide assays for the quantitative determination of

TABLE I
MAJOR MAST CELL MEDIATORS

Mediator[a]	Method of estimation based on reference
Preformed	
Hexosaminidase	10, 34
Serotonin	23, 35, 36
TNFα	37, 38
Newly synthesized	
LTB_4	10, 39, 40
PGD_2	41
TXB_2	42, 43

[a] TNFα, Tumor necrosis factor α; LTB_4, leukotriene B_4; PGD_2, prostaglandin D_2; TXB_2, thromboxane B_2.

the mediators histamine (presynthesized) and leukotriene (LTC_4) (*de novo* synthesized) released by mast cells. A list of other mast cell mediators and references describing a method for their estimation is presented in Table I.[34–43]

Quantitation of Histamine Released by Mast Cells

Principle. Histamine [2-(4-imidazolyl)ethylamine], a critical mediator of vasodilation and vascular permeability, is secreted by activated mast cells.[44] The procedure for its quantitation involves extraction of histamine from the reaction supernatants to an aqueous solution followed by condensation with *o*-phthalaldehyde (OPT) to yield a product with strong and stable fluorescence which is measured in a spectrofluorometer.

[34] D. H. Leaback and P. G. Walker, *Biochem. J.* **78,** 151 (1961).
[35] M. Baniyash, I. Alkalay, and Z. Eshhar, *J. Immunol.* **138,** 2999 (1987).
[36] S. K. Kops, T. C. Theoharides, C. T. Cronin, M. G. Kashgarian, and P. W. Askenase, *Cell Tissue Res.* **262,** 415 (1990).
[37] S. I. Abrams and J. H. Russell, *J. Immunol.* **146,** 405 (1991).
[38] Y. Zhang, B. F. Ramos, and B. A. Jakschik, *Science* **258,** 1957 (1992).
[39] B. F. Ramos, Y. Zhang, R. Qureshi, and B. A. Jakschik, *J. Immunol.* **147,** 1636 (1991).
[40] P. Pradelles, J. Grassi, and J. Maclouf, *Anal. Chem.* **57,** 1170 (1985).
[41] L. Xu, R. Malaviya, K. M. Olsen, and B. A. Jakschik, *Prostaglandins* **45,** 385 (1993).
[42] J. Maclouf, M. Pradel, P. Pradelles, and F. Dray, *Biochim. Biophys. Acta* **431,** 139 (1976).
[43] D. F. Reingold, K. Waters, S. Holmberg, and P. Needleman, *J. Pharmacol. Exp. Ther.* **216,** 510 (1981).
[44] P. A. Shore, A. Berkhalter, and V. H. Cohn, Jr., *J. Pharmacol. Exp. Ther.* **127,** 182 (1959).

Materials

Spectrofluorometer (Farrand Optical, New York, NY)
OPT (Sigma) solution (0.05%, w/v, in methanol); the reagent should always be protected from light
Sodium hydroxide (4.5 *N*)
Hydrochloric acid (0.01 *N*)
Sulfuric acid (1 *N*)
Perchloric acid (70%)
Glass tubes (10 × 75 mm)
1.5-ml polypropylene microcentrifuge tubes
RPMI–HEPES buffer
Histamine (Calbiochem, La Jolla, CA): predetermined amounts of histamine prepared from a stock of 20 μg/ml for generation of standard curve
CTMC suspended to a density of 8.0×10^5 cells/ml in RPMI–HEPES buffer
Escherichia coli suspended to a density of 8.0×10^7 bacteria/ml in RPMI–HEPES buffer

Procedure

1. Two hundred fifty microliters of the CTMC suspension is mixed with an equal volume of bacterial suspension in a microcentrifuge tube.
2. For a control (unstimulated), the CTMC suspension is mixed with 250 μl RPMI–HEPES.
3. The mixtures are incubated at 37° for 1 hr and then centrifuged at 450 *g* for 10 min at 4°.
4. The supernatants are collected and centrifuged at 1000 *g* for 5 min.
5. The final supernatants are collected into 10 × 75 mm glass tubes and saved on ice.
6. Five hundred microliters of this material is mixed with 50 μl perchloric acid and vigorously vortexed.
7. This sample is centrifuged at 800 *g* for 10 min, and 300 μl of the supernatant is transferred to a new tube. (At this point, samples can be stored at −20° or −70°).
8. One hundred microliters sodium hydroxide and 400 μl OPT are added to the sample or to 300 μl of known amounts of histamine (standard), and the resulting mixture is vortexed and kept on ice for 40 min.
9. Four hundred microliters of sulfuric acid is added to the tube to stop the reaction.
10. The sample is incubated at room temperature for 20 min, and then 100 μl is transferred to a cuvette.

11. Fluorescence at 450 nm resulting from activation at 360 nm is measured in a spectrofluorometer (wavelengths uncalibrated).

12. The blank is prepared by mixing 270 μl RPMI–HEPES buffer and 30 μl perchloric acid, after which steps 8–10 are followed.

13. The amount of histamine in each sample is determined by comparing the spectrophotometric reading against a standard curve generated with known amounts of histamine.

14. The net histamine released in each experiment is determined by taking the amount of histamine from stimulated mast cells (nanograms) and subtracting the amount of histamine from unstimulated cells (nanograms).

15. The experiment is performed on at least three separate occasions, and the results are expressed as the mean ± standard error.

Expected Results. The histamine released by CTMC after exposure for 1 hr to type 1 fimbriated *E. coli* is typically 2–3 times more than the amounts released in the control (unstimulated).

Assay for Leukotriene C_4

Principle. LTC_4, a modulator of arteriole constriction and plasma exudation, is released into the extracellular medium by mast cells after they interact with bacteria. The mediator can be quantitated employing an enzyme-linked immunosorbent assay (ELISA), which is based on the ability of LTC_4 in the sample to compete with LTC_4-acetylcholinesterase (tracer) for binding sites on immobilized LTC_4-specific antibodies.[10,40,45]

Materials

Multiskan ELISA reader (Bio-Rad, Melville, NY)

96-well microtiter plate (Nunc, Naperville, IL)

BMMC suspended in RPMI–HEPES buffer to a density of 2×10^6 cells/ml

Escherichia coli suspended in RPMI–HEPES buffer to a density of 1×10^7 bacteria/ml

LTC_4-specific rabbit antibody (primary antibody) (Riker Laboratories, St. Paul, MN), diluted 1:10,000 in ELISA buffer

LTC_4-acetylcholinesterase (tracer) Cayman Chemical, Ann Arbor, MI), diluted per manufacturer's direction

LTC_4: predetermined amounts of LTC_4 prepared from a stock of 1.186 μg/ml for generation of standard curve

Rabbit IgG-specific mouse monoclonal antibody (secondary antibody) (Cayman Chemical): 5 mg (1 vial) is dissolved in 500 ml of 50 m*M* potassium phosphate buffer (pH 7.4)

[45] B. A. Jakschik, L. F. Harrington, and R. Malaviya, *Eicosanoids* **5**, 39 (1992).

ELISA buffer:

1 *M* potassium phosphate buffer (pH 7.4)	100.0 ml
Sodium azide	0.10 g
Sodium chloride	23.40 g
Tetrasodium EDTA	0.37 g
Bovine serum albumin	1.00 g

Bring to 1 liter with deionized water

Substrate (Ellman's reagent), 50× stock solution:

Acetylcholine iodide (Sigma)	0.725 g
Dithiobisnitrobenzoic acid (Sigma)	1.487 g
Sodium chloride	4.375 g
1 M potassium phosphate buffer	37.5 ml
Deionized water	62.5 ml

Wash buffer: 0.05% (v/v) Tween 20 made up in 10 m*M* potassium phosphate buffer, pH 7.4

Saturation buffer: ELISA buffer containing sodium azide (0.02%, w/v) and BSA (0.2%, w/v)

Procedure. Before beginning the assay for leukotriene C_4, the 96-well microtiter plate must be coated with secondary antibody. (a) Two hundred microliters of the secondary antibody is dispensed into each well of the microtiter plate. (b) The plate is covered and incubated at 25° for 18 hr. (c) The antibody is removed and replaced with 100 μl of saturation buffer and 200 μl of 50 m*M* potassium phosphate buffer. (d) The plate is incubated at 4° for 18 hr. (e) The microtiter plate is washed repeatedly (three times) with wash buffer.

1. One hundred fifty microliters of BMMC suspension is incubated with an equal volume of bacteria for 1 hr at 37°, in a 5% (v/v) CO_2 incubator.
2. The mixture is centrifuged at 450 *g* for 10 min at 4°, and the supernatant is aspirated and transferred to another tube.
3. The suspension is centrifuged at 1000 *g* for 5 min to remove any bacteria and the final supernatant is collected.
4. To assay for LTC_4, 50 μl of test sample or a predetermined amount of LTC_4 (for the standard) is added to duplicate wells.
5. Fifty microliters of the diluted LTC_4-specific rabbit antibody is added to the wells.
6. Fifty microliters of the primary antibody and an equal volume of the tracer is added to each well.
7. The plate is incubated overnight at 4° and washed three times with wash buffer.
8. Two hundred microliters of the substrate is added to each well, and the plate is placed on a shaker and incubated at 25°.

9. When a color develops (3–5 hr), the optical density at 420 nm of the wells is read with the ELISA reader.

10. The results are expressed as the quantity [absorbance in the presence of competitor (sample or LTC_4 standard)/absorbance in the absence of competitor] × 100.

11. The quantitation of LTC_4 is performed employing a linear log–logit transformation curve.[40,46,47]

Expected Results. BMMC exposed to type 1 fimbriated *E. coli* for 1 hr typically release 5–10 ng of LTC_4 per 10^6 cells.

Acknowledgments

We thank Mrs. Vourdonna Knoeppel for secretarial assistance and Ms. Elizabeth Abraham for editorial assistance. This work was supported in part by the National Institutes of Health (AI 13550), Monsanto-Searle/Washington University Biomedical Program, and Searle (Arthritis and Prostaglandin Research Challenge).

[46] D. W. Rodbard, W. Bridson, and P. Rayford, *J. Lab. Clin. Med.* **74,** 770 (1969).
[47] R. P. Ekins and G. B. Newman, *Acta Endocrinol.* **64,** 11 (1970).

[4] Erythrocytes as Target Cells for Testing Bacterial Adhesins

By JANINA GOLDHAR

Introduction

The capacity of certain bacteria to cause agglutination of red blood cells has been known for almost 100 years. During the last two decades the bacterial hemagglutinins have been intensively studied. Certain methodological aspects of the numerous studies on the adhesins have been reviewed.[1,2]

Red blood cells (RBCs) of various animal species provide a model target for evaluating the specificity of bacterial lectins because of the large number and natural variability of glycoproteins and glycolipids on their surfaces. The recognition of receptors on the surfaces of various types of RBCs is related to the capacity of the bacteria carrying the adhesin to

[1] I. Ofek and R. J. Doyle, *in* "Bacterial Adhesion to Cells and Tissues," p. 16. Chapman & Hall, New York, London, 1994.
[2] J. Goldhar, this series, Vol. 236, p. 211.

9. When a color develops (3–5 hr), the optical density at 420 nm of the wells is read with the ELISA reader.

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[46] D. W. Rodbard, W. Bridson, and P. Rayford, *J. Lab. Clin. Med.* **74,** 770 (1969).
[47] R. P. Ekins and G. B. Newman, *Acta Endocrinol.* **64,** 11 (1970).

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[2] J. Goldhar, this series, Vol. 236, p. 211.

cause hemagglutination (HA). For this reason the HA test represents a convenient experimental model for studying bacterial adhesins. The specific interactions between bacterial adhesins and RBCs can be also studied using other procedures. This chapter focuses on experimental approaches to determine the specificities of the *Escherichia coli* adhesins, using RBCs as target cells. Many of the described procedures were used or can be adapted for studies on other bacterial adhesins and for other target cells.

Basic Experimental Procedures

Hemagglutination of Red Blood Cells by Whole Bacteria or Isolated Adhesins

There are a number of ways to determine the specificity of HA. Duguid and Old[3] were the first to use the agglutination of RBCs of different animal species as a basis for classification of *E. coli* adhesins. They found that the effects of experimental conditions on the results of HA depends on the type of hemagglutinin. The HA activity of group I strains of *E. coli* (later defined as carrying type 1 fimbriae) is best developed in a stationary phase of bacterial culture grown in liquid medium at 37°. The HA activity may be expressed, albeit less strongly, in a culture grown at 20° or on solid medium. In these early studies, the authors also observed differences in the specificities of the hemagglutinins and divided them into two categories: (1) those inhibited by D-mannose and its derivatives, designated as mannose-sensitive (MS), and (2) those not inhibited by D-mannose, designated mannose-resistant (MR). In contrast to MS adhesins, MR adhesins are best expressed on solid medium at temperatures above 18°–20°.

Many *E. coli* clinical isolates can express two or more types of adhesins according to growth conditions, a possibility that must be considered when interpreting results.[1] It is recommended that an *E. coli* isolate tested for expression of type 1 fimbriae or of MR adhesins be subcultured a number of times under appropriate conditions (liquid or solid medium.[4] Evans *et al.*[5] used HA of RBCs of various animal species to detect and type clinical isolates of enterotoxigenic *Escherichia coli* (ETEC). The authors proposed a system involving two criteria of specificity: (1) the type of target cells, namely, agglutination of human group A, bovine, chicken, and guinea pig

[3] J. P. Duguid and D. C. Old, *in* "Bacterial Adhesins" (Receptors and Recognition, Ser. B) (E. H. Beachey, ed.), p. 185. Chapman & Hall, London, 1980.
[4] I. Ofek, J. Goldhar, Y. Eshdat, and N. Sharon, *Scand. J. Infect. Dis. Suppl.* **33,** 61 (1982).
[5] D. J. Evans, Jr., D. G. Evans, and H. L. Du Pont, *Infect. Immun.* **23,** 336 (1979).

RBCs, and (2) inhibition by D-mannose. The procedure, based on a slide agglutination technique (described in detail previously[2]), is suitable for screening purposes. The interindividual differences in RBCs in the same species should also be taken into account.

Titration of HA capacity with good test standardization is performed with freshly prepared bacterial suspensions of known density, 2-fold serially diluted, and mixed [25–50 μl (v/v)] with RBC suspensions in phosphate-buffered saline (PBS, 1–2%). The HA titer is determined as a minimal density of bacteria causing HA (minimal HA unit, MHU). The titration of HA capacity can be performed by slide agglutination or in U-bottom microtiter plates.[6] The latter method is the most convenient for titration and HA inhibition assays. The optimal conditions for the HA must be established for each type of adhesin, including pH of the buffer, temperature of the incubation, type of microtiter plate, and agitation. Hemagglutination is a secondary effect of adhesion and requires a critical density of adhesin molecules on the bacterial surface for the binding of adhesin-carrying bacteria or cell-free adhesin molecules to many erythrocytes.[7] *Escherichia coli* bacteria carrying type 1 fimbriae, P fimbriae, and colonization factor antigen (CFA) fimbriae cause MR HA, whereas the isolated adhesins effect HA only under special conditions.[8–11]

The hemagglutinins (adhesins) of *E. coli* are usually associated with fimbrial structures protruding from bacterial surfaces. Duguid and co-workers were the first to observe by electron microscopy (EM) that not all *E. coli* strains causing HA are fimbriated.[12] Subsequently, *E. coli* strains carrying nonfimbrial adhesins originating from both enteric and extraintestinal infections were isolated and characterized. In contrast to fimbrial hemagglutinins, the isolated nonfimbrial adhesins retained polyvalency and expressed HA activity.[6,13] Jann and Hoschützky[8] reported that isolated and purified fimbriae types 1, S, and P retained the adhesive capacity (optimal at pH 7.2), but HA was observed only at pH 5 and in the presence of divalent ions and not at pH 7–9.

[6] J. Goldhar, R. Perry, J. R. Golecky, H. Hoschützky, B. Jann, and K. Jann, *Infect. Immun.* **55,** 1837 (1987).
[7] G. W. Jones and R. E. Isaacson, *Crit. Rev. Microbiol.* **10,** 229 (1983).
[8] K. Jann and H. Hoschützky, *Curr. Top. Microbiol. Immunol.* **151,** 55 (1991).
[9] D. G. Evans, D. J. Evans, Jr., S. Clegg, and J. A. Panley, *Infect. Immun.* **25,** 738 (1979).
[10] I. E. Salit and E. C. Gotschlich, *J. Exp. Med.* **146,** 1169 (1977).
[11] T. K. Korhonen, V. Vaisanen, H. Saxen, H. Hultberg, and S. B. Svenson, *Infect. Immun.* **37,** 286 (1982).
[12] J. P. Duguid, S. Clegg, and M. I. Wilson, *J. Med. Microbiol.* **12,** 213 (1979).
[13] I. Orskov, A. Birch-Andersen, J. P. Duguid, J. Stenderup, and F. Orskov, *Infect. Immun.* **47,** 191 (1985).

Estimation of Binding of Purified Adhesins to Red Blood Cells or to Erythrocyte Membranes

Binding of Red Blood Cells to Purified Adhesins (Hemadhesion). The binding capacity of RBCs to adhesins isolated from bacteria causing HA can be tested using the hemadhesion test described by Hoschützky *et al.*[14] Briefly, an RBC suspension (1% in PBS) is distributed into the wells of a microtiter plate (round bottom) in which the test adhesin has been immobilized. Prior to adding the RBC suspension, the wells are washed once with PBS, treated with bovine serum albumin (1% (w/v) in PBS) at 4° for 4 hr, and washed twice with PBS. After incubation at 20° for 30 min, the nonadhering RBCs are gently washed out and the remaining (attached) erythrocytes are lysed with water (50 μl/well). The amount of released hemoglobin is measured by an enzyme-linked immunosorbent assay (ELISA) reader, providing quantitation of the binding reaction. The test can be used for both hemagglutinating and nonhemagglutinating isolated adhesins.

Binding of Radiolabeled Fimbriae to Red Blood Cells. Other authors[15] used purified fimbriae radiolabeled with $Na^{125}I$ for binding quantitation. The rate of binding was measured as counts per minute (cpm) per 10^6 cells. For description of technical details, the reader is referred to the original publications.

Binding of Whole Bacteria or of Isolated Adhesins to Erythrocyte Membranes or Membrane Components. The specificity and sensitivity of the binding assay can be improved when isolated immobilized RBC membrane components are used as a target. The details of the procedures are described elsewhere in this volume (see [8] and [12]).

Determination of Specificity of Adhesins

The above-described basic procedures can be used in the following studies for characterization of the adhesins.

Comparing Hemagglutination Capacity of Wild Strain (Clinical Isolate) with Variants, Mutants, or Transformants that Differ in Expression of Hemagglutinin. The biogenesis of P and type 1 fimbriae can be studied via HA by inactivating each gene and examining the effect of the mutation on hemagglutination activity.[16]

Providing Evidence for Expression of Adhesin on Surface of Bacteria.

[14] H. Hoschützky, F. Lottspeich, and K. Jann, *Infect. Immun.* **57,** 76 (1989).
[15] T. K. Korhonen, S. Eden, and C. Svanborg-Eden, *FEMS Microbiol. Lett.* **7,** 237 (1980).
[16] S. J. Hultgren, S. Normark, and S. N. Abraham, *Annu. Rev. Microbiol.* **45,** 383 (1991).

Certain MR adhesins of *E. coli* (both fimbrial and nonfimbrial), for example, are released from the bacteria at temperatures above 45° (eluting adhesins, MRE), resulting in a decrease in the HA activity of the bacterial suspension.[17] Lack of hemagglutination by whole organisms, in contrast to the activity of bacterial extracts, may suggest that the adhesin is intracellular or is "covered" by other cell surfaces,[18] and therefore its role in interaction of the bacterial and host cells should be clarified.

Determining Inhibition of Binding of Bacteria by Adhesin-Specific Antibodies. To perform HA inhibition tests, the polyclonal antibodies, monoclonal antibodies, or Fab fragments serially diluted in PBS are mixed with the bacterial suspension or with the isolated adhesin (4–6 MHU, v/v) in microtiter plate wells. The minimal HA unit should be determined according to a titration performed under the experimental conditions used for inhibition assays. After incubation of the mixture at 37° for 30 min (or at 4° for 2–4 hr), the RBC suspension is distributed to the wells. Following incubation at the optimal conditions for each HA system, the inhibition titer is recorded either as a titer of inhibiting antibodies[19] or as a minimal inhibitory concentration (μg/ml) of the antibody.[20] Appropriate controls should be included. Similar tests can be performed using the hemadhesion procedure.[21]

Specific antibodies provide a useful tool for immunochemical characterization of structural and functional elements of fimbriae. In a study conducted to characterize fimbriae P–receptor interaction, Fab fragments of the antifimbrial antibodies were used for inhibition of HA and bacterial adhesion to human uroepithelial cells.[20] Moch *et al.*[22] used monoclonal antibodies (MAbs) for inhibition of HA caused by S fimbriae of *E. coli* and fimbrial components. The authors demonstrated that HA is caused by whole fimbriae and by the isolated adhesin component, but not by fimbriae lacking the adhesin. The MAbs against the functional adhesin protein, but not those against structural fimbrial component, inhibited the agglutination.

The capacity of adhesin-specific MAbs to inhibit HA caused by the

[17] S. M. Ip, P. B. Crichton, D. C. Old, and J. P. Duguid, *J. Med. Microbiol.* **14,** 223 (1981).

[18] D. Mirelman and I. Ofek, *in* "Microbial Lectins and Agglutinins" (D. Mirelman, ed.), p. 1. Wiley, 1986.

[19] J. Goldhar, R. Perry, and I. Ofek, *Curr. Microbiol.* **11,** 49 (1984).

[20] M. Rhen, P. Klemm, E. Wahlstrom, S. B. Svenson, G. Kallenius, and T. K. Korhonen, *FEMS Microbiol. Lett.* **18,** 233 (1983).

[21] H. Hoschutzky, W. Nimmich, F. Lottspeich, and K. Jann, *Microb. Pathog.* **6,** 351 (1989).

[22] T. Moch, H. Hoschützky, J. Hacker, K.-D. Kronke, and K. Jann, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3462 (1987).

purified adhesin suggests its specificity for a functional epitope of the adhesin. The HA inhibition capacity (in addition to ELISA reactivity) has been used as a method of selection of the MAbs against a nonfimbrial NFA-3 adhesin.[23,24]

Determining Receptor Specificity of Adhesins: Inhibition of Hemagglutination by Putative Receptor Components. As already established with plant lectins, HA is the most convenient test for the receptor specificity of bacterial adhesins. One of the ways of studying of the adhesin (lectin) receptors is inhibition of HA by putative receptor components such as mono-, oligo-, or polysaccharides. The procedure for the HA inhibition test is as described above. A panel of monosaccharides, oligosaccharides, polysaccharides, or glycoconjugates is prepared in solution in PBS (pH 7.1–7.4), generally starting with a concentration of 100 m*M*. An appropriate panel of oligosaccharides possessing various linkages, or differing in a secondary or tertiary structure (linear, branched, or aromatic) allows deduction of the binding site of the lectin that corresponds to the structure causing the greatest inhibition. In general, di- and trisaccharides are more active than monosaccharides; glycolipids and glycoproteins are most active, but the results are difficult to interpret.

Several studies have been successfully conducted using HA inhibition, namely, determination of the configuration of the binding site of the MS lectin of *E. coli,* compared with the MS lectin of *Salmonella* and concanavalin A (all recognize D-mannose), determination of the receptor of S fimbriae, recognizing sialyl(α-2,3)galactosides, sialic acid, as a receptor component of CS2 and K99 adhesins, and determination of glycophorin A^{MM} of human erythrocytes recognized by the nonfimbrial NFA-4 adhesin and glycophorin A^{NN} recognized by the nonfimbrial NFA-3 adhesin.[2,23]

Despite the relative simplicity of the HA inhibition test, it can be problematic. Difficulties include the influence of some complex carbohydrates on pH and on the agglutinability of RBCs (then the high limit concentration of the putative inhibitor must be lowered), the quantitation of results within the range of 2-fold dilution, and, most critically, the choice of an appropriate set of potential inhibitors.

Binding to Red Blood Cells Expressing Known Blood Group Antigens. A number of *E. coli* adhesins recognizing human blood group antigens have been described. Probably, the best known is the Pap (P fimbriae) adhesin recognizing the glycolipid P blood group antigen.[11] The N, M, and

[23] J. Grünberg, R. Perry, H. Hoschützky, B. Jann, K. Jann, and J. Goldhar, *FEMS Microbiol. Lett.* **56,** 241 (1988).

[24] H. Kahana, J. Grunberg, Y. Bartov, R. Perry, N. Smorodinski, I. Boldur, and J. Goldhar, *Immunol. Infect. Dis.* **4,** 161 (1994).

Dr antigen-specific adhesins of *E. coli* were also determined.[21,25–27] One of the procedures employed to determine the blood group antigen specificity of bacterial hemagglutinins is screening a panel of several types of erthrocytes expressing or nonexpressing particular blood group antigens. The panels are commercially available for use by blood bank laboratories. Use of the RBC panel (Data-Cyte Plus, Antigenic Construction Matrix) enabled a presumptive determination of NFA-3 binding specificity of N blood group-recognizing adhesin.[2,23] NFA-3 agglutinated only RBCs that contained N antigen, similar to a plant lectin from *Vicia graminea* (Vg, known to recognize the blood group NN antigen), whereas neither NFA-4 nor anti-M monoclonal antibody agglutinated RBCs lacking M antigen. Nowicki *et al.*[26] employed a set of erythrocytes of known phenotypes expressing or lacking antigens of the IFC complex to demonstrate the receptor similarity of Dr and AFA-I and AFA-III *E. coli* hemagglutinins.

Panels containing RBCs treated with enzymes that selectively destroy some antigens are also available for confirmation of the results. For example, ficin-treated RBC panels are used to confirm the identification of N/M blood group specificity.[28] Such reduction of binding after enzymatic or chemical modification of RBCs aids in determination of the receptor. In addition to the use of RBCs as target cells, similar chemical and enzymatic modifications can be made with isolated putative receptors, receptor components, glycoproteins, polysaccharides, and oligosaccharides used for inhibition of HA. For technical details of the above procedures, the reader is referred to the original articles.[21,23,29,30]

Comments and Conclusions

We have described the methods of studying the hemagglutinins of *E. coli* bacteria. Similar methods have been used for studying hemagglutinins of other bacteria. It has to be mentioned, however, that when RBCs are used as target cells for studying bacterial adhesins, one must realize the

[25] I. Ofek and R. J. Doyle, *in* "Bacterial Adhesion to Cells and Tissues," p. 92. Chapman & Hall, New York, 1994.

[26] B. Nowicki, A. Labigne, S. Moseley, R. Hull, S. Hull, and J. Moulds, *Infect. Immun.* **58,** 279 (1990).

[27] M. Jokinen, C. Ehnholm, V. Vaisanen-Rhen, T. K. Korhonen, R. Pipkorn, N. Kalkkinen, and G. Gahmberg, *Eur. J. Biochem.* **147,** 47 (1985).

[28] P. D. Issit and C. H. Issit, *in* "Applied Blood Group Serology," 2nd ed., p. 183. Cooper Biomedical, Malvern, Pennsylvania, 1980.

[29] P. O. Sjoberg, M. Lindhal, J. Porathand, and T. Wadström, *Biochem. J.* **255,** 105 (1988).

[30] I. Ofek, H. Lis, and N. Sharon, *in* "Bacterial Adhesion" (D. C. Savage and M. Fletcher, eds.), p. 71. Plenum, New York, 1985.

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[31] N. Nakasone and M. Iwanage, *FEMS Microbiol. Lett.* **113,** 67 (1993).
[32] I. W. T. Wibawan, C. Lammler, R. S. Seleim, and F. H. Pasaribu, *J. Gen. Microbiol.* **139,** 2173 (1993).

[5] Adhesin-Dependent Isolation and Characterization of Bacteria from Their Natural Environment

By KAREN A. KROGFELT

Introduction

Bacterial growth *in vivo*, that is, in their natural environment, versus growth in conventional laboratory media has been discussed by many scientists. The bacterial physiology that has been described to date has been mainly restricted to that observed in conventional laboratory media. Bacterial growth in nature most likely takes place utilizing totally different nutrients. Such conditions could result in, for example, vastly different surface characteristics of the bacterium. Furthermore, bacterial physiological studies are performed in pure cultures, whereas the natural environments are,

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for the most part, complex ecosystems in which several microorganisms coexist. Competition for nutrients as well as the presence of bacteriocides and predators might be important in the overall growth physiology and surface characteristics of a bacterium.

Understanding the physiology of bacteria grown *in vivo* in various hosts has been a difficult task. By using the adhesive properties of the microorganism, it has been possible to isolate certain bacteria from their natural environment for further investigations. Pure cultures so obtained do not require growth on an artificial laboratory medium.

Principle of Method

In this context, experiments were performed by using a streptomycin-treated mouse as a host for a human *Escherichia coli* strain, producing type 1 fimbriae *in vivo*.[1] Type 1 fimbriae bind specifically to D-mannose moieties.[2] Sepharose beads coupled with D-mannose were used for isolating bacterial cells from a fecal suspension, and/or from cecal mucus and cecal contents.[3] Then, the isolated intact bacterial cells can be characterized by techniques such as electron microscopy, flow cytometry,[4] and *in situ* hybridization.[5–7] By electron microscopy, cell shape and surface components can be observed. A flow cytometer can be used for determining rapidly and with high precision the cell size of individual bacterial cells as well as the cell size distribution in the isolated population. In addition, DNA content can be measured and the number of genomes per cell calculated.[4] By *in situ* hybridization with labeled ribosomal RNA probes, the rRNA can be measured, which will reflect the metabolic activity of the bacterial cell in the host.[6] Furthermore, the expression of specific genes and gene products can be monitored *in vivo*, if the necessary probes are available. Labeling of the probes can be done with radioisotopes, fluorescent molecules, or biotin. Fractionation of the extracted cells into the different compartments (such as membranes, cytoplasm, periplasm) and further protein purification and characterization by conventional methods may reveal new important antigens produced *in vivo* only.[8]

[1] E. A. Wadolkowski, D. C. Laux, and P. S. Cohen, *Infect. Immun.* **56,** 1036 (1988).
[2] J. P. Duguid and D. C. Old, *in* "Bacterial Adherence" (E. H. Beachey, ed.), Vol. 6, p. 185. Chapman & Hall, London and New York, 1980.
[3] K. A. Krogfelt, B. A. McCormick, R. L. Burgoff, D. C. Laux, and P. S. Cohen, *Infect. Immun.* **59,** 1567 (1991).
[4] E. Boye and A. Løbner-Olesen, *Res. Microbiol.* **142,** 131 (1991).
[5] R. I. Amman, L. Krumholz, and D. A. Stahl, *J. Bacteriol.* **172,** 762 (1990).
[6] L. K. Poulsen, G. Ballard, and D. A. Stahl, *Appl. Environ. Microbiol.* **59,** 1354 (1993).
[7] K. A. Krogfelt, L. K. Poulsen, and S. Molin, *Infect. Immun.* **61,** 5029 (1993).
[8] P. Panigrahi, G. Lovonsky, L.T. DeTolla, and J. G. Morris, Jr., *Infect. Immun.* **60,** 4938 (1992).

Procedure

Covalent Binding of D-*Mannose to Sepharose Beads*

1. Rehydrate 3.5 g of epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) in 100 ml of distilled water for 15 min.
2. Wash the gel material thoroughly on a sintered glass funnel (pore size G 3) with a total of 350 ml of distilled water.
3. Dissolve 200 mg of D-mannose in 10 ml sodium hydroxide solution, pH 13, in a 100-ml flask. Add the washed gel material with a spoon, and be sure to check that the pH is still pH 13. Incubate in a shaker (low speed) in a water bath overnight at 37°. Do not use a magnetic stirrer.
4. Wash out excess mannose from the gel on a sintered glass funnel with 100 ml of distilled water; then with 100 ml of 0.1 *M* bicarbonate buffer, pH 8; and finally with 100 ml of 0.1 *M* acetate buffer, pH 4.
5. Block reactive epoxy groups on the gel by suspending the gel material in 30 ml of 1 *M* ethanolamine in a 100-ml flask. Incubate with gentle shaking overnight at 45°.
6. Wash the gel thoroughly with distilled water (~500 ml) on a sintered glass funnel. Resuspend in 50–100 ml of 0.1 *M* Tris buffer, pH 7.5, and store at 4°.
7. Add 0.02% (w/v) sodium azide for prolonged storage (at least 2 years).

Specific Isolation of Escherichia coli Cells from Fecal Suspension

1. Induce colonization of streptomycin-treated mice by oral challenge[9] with an *E. coli* strain producing type 1 fimbriae.
2. Collect fecal samples of 1 g from each mouse individually. Dilute the samples 1 : 100 in cold phosphate-buffered saline (PBS), pH 7.4, vortex, and allow fecal debris to settle for 10 min at 4°.
3. Remove 2 ml from the top of the fecal suspension and mix with 200 μl of a thick suspension of Sepharose–mannose beads. Incubate at 4° for 15 min on a bottle roller. Type 1 fimbriated bacteria will bind to mannose molecules on the surface of the beads.
4. Let the beads settle to the bottom of the tube and remove the supernatant. Keep cold.
5. Wash away bacteria that are not bound to the beads by adding 2 ml of cold PBS to the beads, mixing gently, and letting the beads settle. Repeat the wash twice.

[9] M. L. Myhal, D. C. Laux, and P. S. Cohen, *Eur. J. Clin. Microbiol.* **1,** 186 (1982).

6. Add 1 ml of 0.5% sodium dodecyl sulfate (SDS) to the beads and incubate for 15 min at 4°, under gentle agitation to release specifically bound bacteria.

7. Pellet the Sepharose–mannose beads by low-speed centrifugation (2000 rpm for 1 min at 4°). The supernatant contains a pure culture of type 1 fimbriated bacteria (if necessary, step 6 can be repeated).

8. Check the purity of the supernatant for *E. coli* by plating on relevant media (nonselective and selective media).

9. Prepare and/or store the isolated bacteria appropriately, depending on further manipulations. For example, fix the bacteria immediately in paraformaldehyde if they are to be used for flow cytometry or *in situ* hybridizations.

Conclusion

The above-described method is not restricted to isolation of type 1 fimbriated *E. coli*, and other specific receptors for bacterial adhesins could be bound covalently to gel matrices and used similarly. Numerous bacterial adhesins and their respective receptors have been described in detail.[10–12]

[10] K. A. Krogfelt, *Rev. Infect. Dis.* **13,** 721 (1991).
[11] N. Sharon and H. Lis, *Science* **246,** 227 (1989).
[12] K. Jann and H. Hoschütsky, *in* "Current Topics in Microbiology and Immunology" (K. Jann and B. Jann, eds.), Vol. 151, p. 55. Springer-Verlag, Berlin and Heidelberg, 1990.

[6] Analysis of Ocular Microbial Adhesion

By LINDA D. HAZLETT

Introduction

The purpose of this chapter is to review currently employed methods used to examine ocular microbial adhesion. We focus on *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen whose interaction with the ocular surface has been most thoroughly examined. The basis for this is that the organism is one of the most common pathogens associated with bacterial corneal ulcers, particularly in contact lens wearers.[1] Because of the rapidity of corneal degradation and the potential for perforation that

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occurs within 24–48 hr after onset of infection, the keratitis produced by the bacterium is one of the most dangerous of corneal infections. The description of techniques included in this chapter details growth and characterization of the bacteria and *in vitro* assessment of bacterial binding to corneal epithelium. We focus primarily on the interaction of *P. aeruginosa* with corneal epithelial proteins, but we also describe methods for immunofluorescent and immunoelectron microscopy (IEM) localization of ganglio-tetraosylceramide (asialo-G_{M1}), representative of a class of neutral glycolipid receptors for bacterial binding to scarified cornea.[2]

Bacterial Growth and Characterization

Stock cultures of *P. aeruginosa* (ATCC strain 19660, Rockville, MD, unless otherwise indicated) are stored at 25° on Tryptose agar slants (Difco Laboratories, Detroit, MI) and used for inoculation of 60 ml of broth medium containing 5% peptone and 0.25% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD). Cultures are grown on a rotary shaker at 37° for 18 hr, centrifuged at 7000 *g* for 20 min at 4°, and washed with 0.9% pyrogen-free saline to a density of 2.0×10^{10} colony-forming units (cfu)/ml, by using a standard curve relating viable counts to optical density at 440 nm. The routine inoculum for *in vitro* binding experiments, using adult (1- to 2-month-old) or infant, 5 postnatal day (P) mouse eyes, is 5 μl (5.0×10^7 cfu) topically applied.

To assess bacterial surface characteristics, such as piliation or the presence of a flagellum in ATCC 19660 and other strains, bacterial suspensions are placed on Formvar-coated copper grids and negatively stained[3] for 1 min with saturated aqueous uranyl acetate. Grids are air-dried for 30 min and are observed and photographed using a JEM-100 CX transmission electron microscope (TEM) operating at 60 kV. About 50 bacteria are routinely observed to provide sufficient morphological information.

Supernatants from overnight cultures also are quickly analyzed for *in vitro* protease production following determination of the protein concentration (BCA protein assay, Pierce, Rockford, IL). If several strains are to be compared, one needs to adjust the total protein concentration similarly for each strain tested. Protease activity is quickly determined using a Bio-Rad Laboratories (Richmond, CA) protease detection kit. In this system, diffusible proteases cause a clear zone in the agar gel whose diameter is

[2] L. D. Hazlett, S. Masinick, R. Barrett, and K. Rosol, *Infect. Immun.* **61,** 5164 (1993).

[3] R. H. Haschemeyer and R. J. Myers, *in* "Principles and Techniques of Electron Microscopy, Biological Applications" (M. A. Hayat, ed.), Vol. 2, p. 101. Van Nostrand-Reinhold, Princeton, New Jersey, 1972.

related to the protease concentration.[4] This assay has also been used to determine protease contamination in reagents used for binding inhibition and other assays.[5] More selective procedures can then be employed which specifically examine for select types of proteolytic activity.

Pili Purification

Pili are purified essentially according to an earlier method.[6] Briefly, bacteria are grown on solid medium in stainless steel pans, harvested, suspended in 15% sucrose in standard saline–citrate (SSC) buffer, pH 7.0, and stirred overnight at 4°. The cell suspension is blended for 2 min at 2000 rpm in a Sorvall Omnimixer (RMC, Tuscon AZ). The cells are removed by centrifugation at 10,000 *g* for 15 min at 4°, and the supernatant solution is dialyzed for 72 hr against tap water at 4°. Ammonium sulfate [$(NH_4)_2SO_4$] is added to the dialyzate to 50% saturation and the solution stirred overnight at 4°. Both flagella and pili are precipitated by this process and are removed by centrifugation at 27,000 *g* for 1 hr at 4°. The pellet is suspended in 20% (w/v) $(NH_4)_2SO_4$ in SSC buffer, stirred for 4 hr at 4°, and centrifuged at 27,000 *g* for 1 hr at 4° to remove the flagella. This aggregation step is most effective when repeated two to three times. The pellet is resuspended in SSC buffer and centrifuged at 3000 *g* for 15 min. The final pellet, enriched for pili, is redissolved in SSC buffer, dialyzed against distilled water overnight, and then negatively stained[3] with 2% uranyl acetate for TEM examination. In addition, samples are electrophoresed on 13% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS)[7] to assess sample purity further. Flagella samples can be similarly treated, following their removal after centrifugation at 27,000 *g*.

Quantitation of Endotoxin in Pili or Flagella Samples and Its Reduction

Endotoxin (lipopolysaccharide, LPS) is quantitated using a quantitative chromogenic *Limulus* amebocyte lysate (LAL) kit (QCL-1000, Whittaker Bioproducts, Walkersville, MD). In the assay, 100 μl of the sample or standard is mixed with 100 μl of LAL in an LPS-free tube and incubated at 37° for 6 min. The reaction is stopped with 10% SDS. The absorbance of each sample is determined spectrophotometrically at 410 nm. Detoxi-Gel AffinityPak columns (Pierce) which are prepacked with 1 ml of the gel support, cross-linked 6% beaded agarose, are used to reduce the amount

[4] O. J. Bjerrum, J. Ramlau, I. Clemmensen, A. Inghild, and T. C. Bog-Hansen, *Scand. J. Immunol.* **4**(Suppl. 2), 81 (1975).
[5] L. D. Hazlett, S. Masinick, R. S. Berk, and Z. Zheng, *Exp. Eye Res.* **55,** 579 (1992).
[6] L. S. Frost and W. Paranchych, *J. Bacteriol.* **131,** 259 (1977).
[7] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).

of LPS in the pili and flagella samples. The columns are equilibrated with 0.1 ml ammonium bicarbonate buffer (pH 7.8) and the flow initiated by the force of gravity. After the protein sample to be purified has completely entered the gel, ammonium bicarbonate buffer is used for elution. After about two chromatographic passes, the amount of LPS in the samples is acceptably reduced to no more than 1.0 endotoxin unit (EU)/mg which represents an LPS concentration of 0.1 ng/ml. Such samples are used for monoclonal antibody (MAb) production as well as binding inhibition experiments.

Iodination of Pilus Proteins

The LPS-reduced pili are labeled with iodine-125 using the solid-phase reagent tetrachlorodiphenylglycoluril[8] (Iodogen, Pierce), according to the method of Hansson *et al.*[9] with some modifications. Briefly, 13 × 100 mm glass tubes are coated with a fine film of Iodogen (250 μg/tube) by adding 0.5 ml of reagent (0.5 mg Iodogen/ml chloroform) and evaporating under a slow stream of nitrogen at room temperature. Tubes are washed with cold phosphate-buffered saline (PBS) three times just before beginning the iodination procedure. About 400–500 μg purified pilus proteins in 400 μl of PBS is added, and the labeling is initiated by the addition of 0.5 mCi of Na^{125} I (carrier free, specific activity 15 Ci/mg; Amersham, Arlington Heights, IL) in 20 μl PBS. The reaction proceeds for 10 min at 4° followed by a further 10 min of incubation at room temperature. Iodination is terminated by removing the sample from the tube. Free from bound radioactivity is separated as described by Lipford *et al.*[10] using an ultrafree MC filter (Millipore, Bedford, MA). The total labeling usually obtained is in the range of 10^7–10^8 counts per minute (cpm). Labeled pili have been used in a solid-phase binding assay[9] in which 10 μg/well of corneal epithelial protein is used to coat experimental test wells. It has been found that ^{125}I-labeled pili bind to corneal epithelial proteins significantly above control values in both adult (Fig. 1) and 5 P epithelium.[11] The binding sites are saturable and are competed for by addition of unlabeled pili.[11] ^{125}I-Labeled pili also significantly bind to asialo-G_{M1}, compared with other glycolipid standards,[12] using solid-phase[9,13] and thin-layer chromatography (TLC)[9] overlay assays.

[8] P. J. Fraker and R. C. Speck, Jr., *Biochem. Biophys. Res. Commun.* **80,** 849 (1978).
[9] G. C. Hansson, K. A. Karlsson, G. Larson, N. Stromberg, and J. Thurin, *Anal. Biochem.* **146,** 158 (1985).
[10] G. B. Lipford, Q. Feng, and G. L. Wright, Jr., *Anal. Biochem.* **87,** 133 (1990).
[11] L. D. Hazlett, X. Rudner, S. Masinick, M. Ireland, and S. Gupta, *Invest. Ophthalmol. Vis. Sci.*, in press (1995).
[12] S. K. Gupta, R. S. Berk, S. Masinick, and L. D. Hazlett, *Infect. Immun.* **62,** 4572 (1994).
[13] H. C. Krivan, V. Ginsburg, and D. D. Roberts, *Arch. Biochem. Biophys.* **260,** 493 (1988).

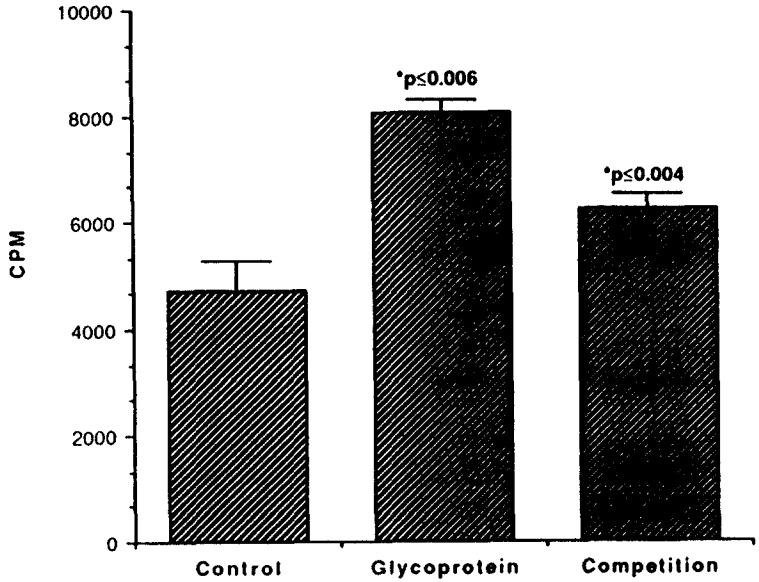

FIG. 1. Binding of ^{125}I-labeled *Pseudomonas* pili protein to adult (37 days postnatal) mouse corneal epithelial glycoproteins using a solid-phase binding assay.[9] Corneal epithelial glycoprotein-coated plates (10 μg/well) were incubated with 35 μl ^{125}I-labeled pili or with a 10-fold excess of unlabeled to labeled pili, whereas control wells (coated with 3% BSA) were incubated with 35 μl labeled pili, all overnight and at room temperature. Pili binding, expressed as counts per minute, was significantly above control values in glycoprotein-coated wells and was significantly decreased by competition with unlabeled pili.

Preparation of Ocular Tissue

Organ Culture

For *in vitro* binding studies an organ culture model[14] has been developed which uses whole mouse eyes, thus maintaining the normal multistratified nature of the corneal epithelium, as well as its relationship to underlying basal lamina and subjacent stroma. The model is extremely useful, providing data that complements ongoing *in vivo* studies.

Adult (1- to 2-month-old) mice are sacrificed and the ocular surface scarified using a 25$\frac{5}{8}$-gauge needle to create three 1-mm incisions in the center of the right and left cornea (under a magnification of 40× using a stereoscopic microscope). The deepest wounds penetrate the epithelial cell basal lamina into the superficial corneal stroma. The depth of the wound

[14] A. Singh, L. D. Hazlett, and R. S. Berk, *Infect. Immun.* **58,** 1301 (1990).

is determined by processing randomly selected eyes for light (1-μm plastic sections) or scanning electron microscopic (SEM) observation.

Eyes of immature, 5 P (5 days postnatal) mice are similarly prepared for organ culture, but in this model it has been determined that, unlike the adult animal, no wounding of the corneal surface is needed to initiate bacterial binding and invasion. The model is of importance, as clinical cases of infection in premature infants have been reported in which not only ocular disease but also death ensued.[15] This also occurs in the *in vivo* 5 P animal model.[16] Eyes are enucleated with a sterile pair of scissors and placed into sterilized prepared culture wells containing 4.5 ml of minimal essential medium with Earle's salts, L-glutamine, and nonessential amino acids without sodium bicarbonate (GIBCO Laboratories, Grand Island, NY), pH 7.2–7.4. The corneal surface, oriented superiorly in the wells, is covered by a thin layer of minimal essential medium.

Culture Wells

Cell culture dishes (GIBCO), 35 × 10 mm in size, and rubber tubing (Norton Plastics, Akron, OH) with diameters of 0.48 and 0.32 cm are used. The larger tubing is inserted with the smaller tubing to a depth of a 0.25 cm mark. Two centered diagonal and vertical cuts are then made on the larger tubing down to the 0.25 cm mark, and horizontal cuts along this plane are made in order to remove four cut sections of tubing. This allows resting of the posterior part of the eye inside the smaller tubing and provides stability and orientation of the corneal surface superiorly. The entire tubing apparatus is glued to the bottom of the cell culture dish with cyanoacrylate adhesive and placed under UV light for sterilization overnight before each use.[14]

Adhesion Assay

A 5-μl bacterial suspension containing 5.0×10^7 cfu of *P. aeruginosa* is delivered to the surface of the eye in culture using a calibrated micropipette with a sterile disposable tip. Eyes are immediatley placed in a water-jacketed CO_2 incubator at 37° and 5% (v/v) CO_2 and cultured for various times (we routinely culture for intervals of 15–60 min). Eyes are then rinsed vigorously in PBS, fixed, dehydrated, and critical point dried for SEM observation.

Prior to initiating these studies, it was necessary to establish the integrity of the organ culture model. This was done using whole eyes in culture as

[15] R. P. Burns and D. H. Rhodes, Jr., *Arch. Ophthalmol.* **65,** 517 (1960).
[16] L. D. Hazlett, D. D. Rosen, and R. S. Berk, *Infect. Immun.* **20,** 25 (1978).

described above, but for 24 hr and without antimycotics or antibiotics. Eyes were free of exogenous bacterial growth for 8 hr. By 12 hr a few bacterial organisms were seen, and by 24 hr the eyes were heavily contaminated. Therefore, the experimental incubation period of 60 min, which is sufficient for binding studies, is well within the 8-hr integrity limits of the model.[14]

Quantitation of Adhesion

Adherent bacteria are quantitated at 15, 30, and 60 min after ocular application by SEM.[14] The number of organisms in a microscopic field of a fixed size is observed. The field size is fixed by using a consistent working distance of 25 mm, fixing the angle from which the sample is viewed, and using a uniform magnification of 3000×. For each test, five randomly selected fields are photographed on each of three eyes and the negatives enlarged to a total magnification of 6000×. Counts are done with a square cut to measure 80 mm^2 at a magnification of 6000×, and the data are expressed as the mean numbers of bacteria per square micrometer of corneal surface. This method yields results which agree well with those obtained from parallel experiments using microbiological culture of infected eyes. For the latter, enucleated eyes are rinsed in PBS, pH 7.45, and individually homogenized in a sterile 2.0-ml tissue homogenizer with 0.1 ml of PBS, pH 7.45, and a 0.1-ml volume of 10-fold dilutions is plated in triplicate on Trypticase soy agar plates. Plates are incubated for 24 hr at 37° and the number of bacteria isolated expressed as cfu/cornea. Three eyes are adequate for each treatment category.[17]

Specimen Preparation for Scanning Electron Microscopy

Following incubation of the cultured eyes with the bacteria, eyes are vigorously rinsed with PBS (pH 7.5) to dislodge nonadherent organisms and immediately fixed at 4° in a fixative containing 2.0% osmium tetroxide, 2.5% glutaraldehyde, and 0.1 *M* phosphate buffer, pH 7.5 (1 : 1 : 1, v/v) for 3 hr with a change to fresh fixative at 1.5 hr. The eyes are dehydrated through a graded series of ethanol to 100% ethanol and dried to the critical point in an AutoSamdri critical point dryer (Tousimis Research Laboratories, Rockville, MD) with liquid CO_2 as the transition fluid. Eyes, with corneas superiorly oriented, are mounted on brass specimen stubs with colloidal silver paint suspended in methanol. A thin layer of gold (15–30 nm) is evaporated onto the surface of each specimen using a Pelco SC-5 (Ted Pella, Redding, CA) sputter coater. Eyes are viewed as described

[17] L. D. Hazlett, M. M. Moon, M. Strejc, and R. S. Berk, *Invest. Ophthalmol. Visual Sci.* **28,** 1978 (1987).

above with a JEOL JSM-840A SEM. The data generated are analyzed for statistical significance using an unpaired two-group Students *t* test.

Preparation of Mouse Corneal Epithelia

Adult (1- to 2-month-old) or infant (5 P) mice are used to prepare corneal epithelial sheets for subsequent use in *in vitro* overlay binding assays. Following sacrifice, the eyes are enucleated (in 5 P mice, the eyelids are fused and must be cut away to enucleate the eye) and placed in PBS containing 20 m*M* ethylenediaminetetraacetic acid (EDTA), pH 7.2–7.4, and incubated for 3 hr at 37°.[18] After incubation, the corneal epithelial sheet is teased from the underlying stroma with a pair of forceps and all conjunctival epithelium removed. About 15–30 sheets are routinely pooled and homogenized using a 1-ml glass tissue homogenizer and 200 μl of Laemmli sample buffer (62.5 m*M* Tris base, 2.0% SDS, and 10% glycerol without 2-mercaptoethanol, pH 6.8), placed into a boiling water bath for 30 sec, and stored at −70° until used.

Polyacrylamide Gel Electrophoresis

Corneal epithelial protein samples (6.65 μg/lane loaded) are electrophoresed on discontinuous SDS–polyacrylamide gels[7] using 10% acrylamide in the separating gel and 3.5% acrylamide in the stacking gel. Before electrophoresis, 2-mercaptoethanol (5%, final concentration) is added to the protein samples. The gel is electrophoresed using 20 mA per gel under constant current using a Bio-Rad Mini Protean II Dual Slab Cell. The electrophoretically separated material is stained with Coomassie Brilliant Blue G-250 (Bio-Rad) for visualization of the proteins, or it is transferred to 0.2-μ pore diameter nitrocellulose paper[19] at 100 V under constant voltage for 1 hr on ice for immunoblot analysis. For staining, gels are fixed in 7% glacial acetic acid, 40% (v/v) methanol overnight, stained with Coomassie Brilliant Blue G-250 (0.25% v/v Coomassie Brilliant Blue G-250, 45.4% methanol, 9.2% glacial acetic acid) for 3 hr, and destained with destain solution (7.5% glacial acetic acid, 5% v/v methanol).

Immunoblotting

After electrophoretic transfer of corneal epithelial proteins, using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad), nitrocellulose blots are blocked for nonspecific binding activity with 3% bovine serum albumin (BSA, essentially fatty acid- and globulin-free; Sigma, St. Louis, MO),

[18] L. Juhlin and W. B. Shelley, *Acta Derm. Venereol.* **57,** 289 (1977).
[19] H. Towbin, T. Staehelin, and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350 (1979).

0.05% Nonidet P-40, 5 m*M* EDTA and 150 m*M* sodium chloride (Sigma), and 0.25% gelatin in 50 m*M* Tris buffer, pH 7.5, at 37° with shaking at 100 rpm overnight. The blots are washed twice with 0.1% Tween 20 in 50 m*M* Tris-buffered saline (TTBS), pH 7.5, at 37° with shaking at 100 rpm overnight.[20] For overlay assays,[21] blots are incubated with LPS-reduced bacterial pili (100 μg/ml) or PBS (controls) at 37° at 100 rpm. After 2 hr the blots are washed three times with TTBS (10 min per wash). A pili-specific MAb, XLR-3 [immunoglobulin (Ig) G2b isotype, or MAb PK3B of the same isotype and specific for the PAK pilin subunit,[22] 1:1000 dilution], in 1% normal rabbit serum in TTBS is incubated with the blot at 37° for 1 hr at 100 rpm. The blots are washed three times with TTBS. A goat anti-mouse IgG(heavy and light chain-specific)–alkaline phosphatase (AP) conjugate (Cappel, Organon Teknika, Durham, NC) at a 1:3000 dilution in TTBS with 1% normal goat serum is added and incubated at 37° for 1 hr at 100 rpm. The blots are washed three times with TTBS and once with TBS. The color development solution used is 0.3 mg of *p*-nitro blue tetrazolium chloride per milliliter, 0.15 mg of 5-bromo-4 chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad) per milliliter in 0.1 *M* sodium bicarbonate, 1.0 m*M* magnesium chloride, pH 9.8. Color development is stopped by rinsing the blots in distilled water.[20]

Lectin Blotting

To determine if host corneal proteins which bind pili in overlay assays are glycosylated, a GlycoTrack carbohydrate detection kit from Oxford GlycoSystems (Rosedale, NY) is used. Specific lectins may also be used for more detailed analysis of glycosylation patterns of host proteins.[11,20] Corneal epithelial blotted proteins are washed with PBS for 10 min and incubated in 10 m*M* sodium periodate in sodium acetate–EDTA buffer for 20 min in the dark at room temperature. The blots are washed three times with PBS (10 min per wash) and incubated with hydrazide solution (2 μl hydrazide to 10 ml sodium acetate–EDTA) for 60 min. Blots are washed three times with Tris-buffered saline (TBS) and blocked with 0.5% nonfat dry milk for 30 min. After washing three times with TBS, blots are incubated with streptavidin–AP conjugate (S-AP, 5 μl S-AP to 10 ml TBS) for 60 min. Blots are washed with TBS and developed with AP color developing solution as described above.

[20] X. L. Rudner, Z. Zheng, R. S. Berk, R. T. Irvin, and L. D. Hazlett, *Invest. Ophthalmol. Visual Sci.* **33,** 2185 (1992).

[21] P. Doig, W. Paranchych, P. A. Sastry, and R. T. Irvin, *Can. J. Microbiol.* **35,** 1141 (1989).

[22] P. Doig, P. A. Sastry, R. S. Hodges, K. K. Lee, W. Paranchych, and R. T. Irvin, *Infect. Immun.* **58,** 124 (1990).

The data provide information on glycosylation of proteins migrating at similar molecular weight as those which bind pili. Electroelution[23] of specific molecular weight proteins that are identified as binding pili in overlay assay also is done by cutting them from one- or two-dimensional polyacrylamide gels run in parallel. Proteins are then examined by dot-blot[24] analysis of the eluted proteins, employing pili in an overlay assay or analysis by lectin staining, allowing direct comparison of the proteins for their pilus binding and glycosylation patterns. This approach complements comparisons that rely on the similar migration patterns of proteins run in parallel gels.

Blots are also used to determine the effects of carbohydrates on pilus binding. Sialic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, methyl-α-D-mannopyranoside, L-fucose, and diacetylchitobiose (Sigma) (0.25, 1, 10, and 40 mg/ml) are preincubated individually with pili (100 μg/ml) at 37° for 1 hr before incubation with the corneal epithelial blots.[20] All of the sugars except sialic acid when combined with PBS are in a neutral pH range (pH 6.35–7.37); sialic acid solutions must be adjusted to neutrality with 2 *N* NaOH. As an additional control because of this adjustment, pili are incubated with PBS which first has been acidified with HCl (pH 2.0) and then adjusted to neutrality with NaOH. This is done to ensure that the inhibition of binding effect observed with sialic acid is not merely due to adjusting the sugar from acid to neutral pH. Immunoblots are prepared and color development done as described above.

Periodate Oxidation of Corneal Epithelial Protein Blots

Carbohydrates are removed by mild periodate oxidation[25] of corneal epithelial blotted proteins as another way to examine the relative importance of sugar residues in bacterial adhesion.[11,20] The BSA-blocked blots are rinsed with 50 m*M* sodium acetate buffer (pH 4.5); then control blots are incubated in the same buffer for 1 hr. Experimental blots are incubated with various concentrations of periodate (0–30 m*M*, Sigma) in 50 m*M* sodium acetate buffer, pH 4.5, for 1 hr in the dark at room temperature. Control and experimental blots are rinsed with sodium acetate buffer and incubated with 50 m*M* sodium borohydride (Sigma) in PBS for 30 min in the dark at room temperature. The blots are rinsed six times with PBS and assessed for pilus binding as described above.

[23] E. Jacobs and A. Clad, *Anal. Biochem.* **154,** 583 (1986).

[24] N. Panjwani, T. S. Zaidi, J. E. Gigstad, F. B. Jungalwala, M. Barza, and J. Baum, *Infect. Immun.* **58,** 114 (1990).

[25] M. P. Woodward, W. W. Young, Jr., and R. A. Bloodgood, *J. Immunol. Methods* **78,** 143 (1985).

Another alternative experiment to determine the role of glycosylation in bacterial binding consists of incubation of corneal epithelial blots with sialidase [we use type V, purified from *Clostridium perfringens* (2.2. U/ml activity), but other sialidases[26] may also be employed to decrease sialic acid residues selectively.] Blots treated in this way can be analyzed by immunoblot overlay procedures, described above, as well as probed with specific sialic acid labeling lectins, such as *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) lectin, to determine which proteins are deglycosylated by the enzyme treatment.[11] For the latter assay, corneal epithelial blots are incubated first with 3% BSA for 2 hr. After two washes with TBS, blots are incubated with either digoxigenin-labeled MAA (5 μg/ml, specific for sialic acid linked $\alpha 2 \rightarrow 3$ to galactose, Boehringer Mannheim, Indianapolis, IN) or digoxigenin-labeled SNA (1 μg/ml, specific for sialic acid linked $\alpha 2 \rightarrow 6$ to galactose) for 1 hr, each according to the dilution recommended by the manufacturer.[11] Blots are washed with TBS three times and then incubated with antidigoxigenin–AP (0.75 U/ml, Boehringer) for 1 hr. The AP color development is carried out as described above. These lectins are also used in overlay binding inhibition experiments.[11] For these, blots of corneal epithelial proteins are blocked with 3% BSA overnight at 37° and with shaking at 100 rpm. Blots are washed twice with TTBS and once with lectin buffer (0.15 *M* sodium chloride, 10 m*M* manganous chloride, 50 m*M* Tris-HCl, 10 m*M* calcium chloride, and 0.05% Tween 20, pH 7.0). Blots are incubated with lectin buffer (controls) or SNA or MAA lectin (20–500 μg/ml in lectin buffer) at 37° for 1 hr with shaking at 100 rpm. The blots are washed twice with lectin buffer, once with PBS, and are then assessed for the ability to bind pili by the overlay technique described above.

Cryosections

Following organ culture of eyes (wounded or not) with various treatments, they can be subjected to cryosectioning and eventual microscopic analysis.[2] For light microscopic immunofluorescence, enucleated unfixed eyes are immediately immersed in Tissue Tek (OCT) (Miles, Elkhart, IN) and frozen in liquid nitrogen, with care taken to orient eyes so that cross sections through the cornea are obtained. Sections (10–12 μm thick) are cut on a cryostat (Hacker-Bright microtome, Bright Instruments, Huntington, England) and collected onto freshly coated polylysine glass slides. Slides are stored at −20° until used. The nature of the wound site and the morphological integrity of the frozen corneal tissue are verified by random slide selection and toluidine blue staining.

[26] R. Schauer, *Adv. Carbohydr. Chem. Biochem.* **40,** 131 (1982).

For immunostaining, slides are brought to room temperature and dried overnight at 37°. For immunostaining, all slides are preincubated at room temperature with 0.1 *M* PBS containing 1% BSA to block nonspecific activity by placing a 20-μl drop on each section and placing the slides in a moist chamber for 30 min. Slides are rinsed with 0.1 *M* PBS three times and drained dry for 1–2 min. A 20-μl drop of antibody (we examined the localization of asialo-G_{M1} glycolipid using a mouse antiasialo-G_{M1} MAb, a generous gift of H. Krivan) at the determined appropriate dilution (in the range of 1:5 or 1:10) is placed on each section and incubated for 1 hr. The specificity of the MAb is determined separately using a solid-phase assay.[2,27] Slides are rinsed three times with PBS and drained dry. Each section is treated with a 20-μl drop of 50 m*M* Tris-HCl containing 0.1% Tween 20 for 5 min in a moist chamber. Slides are rinsed with 50 m*M* Tris-HCl buffer three times and dried. The second antibody consists of 20 μl of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC, Sigma), used at a 1:50 dilution per section. Slides are placed in a moist chamber for 30 min under reduced lighting conditions which helps prevent exhaustion of the fluorochrome. Slides are rinsed three times with Tris-HCl buffer and then with distilled water to remove excess unbound antibody and buffer, all under reduced lighting. Coverslips are applied with a modified mountant (10 mg *p*-phenylenediamine, 1 ml of 10 m*M* PBS, pH 7.4, and 9 ml glycerol[28] which retards fluorescence exhaustion) and stored in the dark in slide boxes at 4° prior to observation and photography.

Control sections are similarly treated with the exception that the primary antibody is either omitted or substituted with an irrelevant, similarly prepared one. We used the MAb anti-HLA-DR5 (ATCC clone SFR3-DR5), which recognizes human class II antigen present principally on B cells, antigen-presenting cells, and thymic epithelial cells. All tissue sections are photographed using a microscope equipped with a fluorescent filter; the wavelength (450–480 nm) facilitates visualization of the fluorochrome conjugate. Equivalent times of photographic exposure are used for experimental and control sections. Similarly, all darkroom techniques, such as negative exposure and development times, are standardized.

Immunoelectron Microscopy

For IEM localization of asialo-G_{M1} glycolipid,[2] eyes from adult mice whose corneas are scarified are placed into freshly prepared fixative (10 m*M* sodium periodate, 50 m*M* lysine, and 2.0% formaldehyde in 0.1 *M*

[27] H. C. Krivan, D. D. Roberts, and V. Ginsburg, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6157 (1988).
[28] G. D. Johnson and G. M. Araujo, *J. Immunol. Methods* **43,** 349 (1981).

phosphate buffer)[29] for 30 min at room temperature. Eyes are rinsed three times for 5 min each at room temperature with filtered 100 m*M* glycine in 50 m*M* phosphate buffer, washed overnight in cold phosphate buffer, rinsed with 0.1 *M* phosphate buffer, and dehydrated in a graded series of ethanol to 100%. Specimens are infiltrated with Lowicryl K4M polymerizing solution (Polysciences, Warrington, PA) with gentle agitation. Eyes are transferred to Beem capsules containing freshly prepared K4M polymerizing solution and UV-irradiated under a General Electric 15-W Black lite bulb, 10 cm from the source, for 45 min at 4°.

For postembedding IEM staining, thin secions (90 nm) of the cornea, oriented to obtain cross sections, are cut on a Reichert-Jung Ultracut E and placed on nickel grids, and the grids are preincubated section side down in a 20-μl drop of PBS containing 0.1% BSA, 0.05% Tween 20, and 5% fetal calf serum (all Sigma) to block adventitious binding sites. Grids are transferred to another 20-μl drop of specific or nonspecific (control) antibody (dilution must be determined for each) and incubated at room temperature for 1 hr. Grids are washed sequentially in a multiwell culture plate in PBS–BSA–Tween 20–fetal calf serum to remove unbound antibody. Grids are transferred to another drop of goat anti-mouse IgG conjugated to 10-nm gold particles (Sigma) at a 1 : 5 dilution for 30 min, rewashed in PBS–BSA–Tween 20–fetal calf serum, and stained with uranyl acetate and lead citrate. Control sections are similarly prepared with either omission of the primary antibody or its substitution with an irrelevant one. Grids are observed and photographed with a JEOL 100 CX transmission electron microscope operating at 60 kV. Some loss of the glycolipid will occur with this procedure, owing to the use of a graded series of alcohol, but labeling parallels immunofluorescence data in which no fixative is used before antibody labeling.[2]

Iodination of Surface Membrane Proteins

Radioiodination of surface membrane-associated corneal epithelial proteins is employed to determine which among the proteins that bind pili in overlay immunoblots are membrane associated and which are intracellular. Enzymobeads (Enzymobead radioiodination reagent, Bio-Rad) are rehydrated with 0.5 ml distilled water at least 1 hr before use. Then 135 μl of the iodination mixture [50 μl Enzymobeads, 25 μl of 1% β-D-glucose (Sigma), 10 μl of 1.0 mCi $Na^{125}I$ (ICN Biomedicals, Irvine, CA), and 50 μl of 0.2 *M* phosphate buffer, pH 7.2) is added to freshly enucleated eyes (5 eyes from 5 P mice is sufficient) in a microcentrifuge tube.[11] Iodination is allowed to

[29] I. W. McLean and P. K. Nakane, *J. Histochem. Cytochem.* **22,** 1077 (1974).

proceed at room temperature for 30 min with agitation every 5 min. Eyes are then washed three times with PBS, pH 7.5, and placed in EDTA buffer to incubate for 3 hr to allow removal of the corneal epithelium from each eye (as detailed above), and the proteins are electrophoresed on 10% SDS–polyacrylamide gels, as described above. Parallel control eyes are similarly treated but are incubated in radioiodination mixture lacking Enzymobeads. The resulting gels are fixed and stained overnight with 0.25% Coomassie Brilliant Blue G-250 (Bio-Rad), 9.2% glacial acetic acid, and 5% methanol. The gel is equilibrated in 2% glycerol for 3 hr with a change to fresh glycerol every 30 min and dried with a Bio-Rad Model 483 slab dryer for 2 hr. The dried gel is autoradiographed using Kodak (Rochester, NY) Diagnosis Film X-Omat RP at −70° overnight.[11] Gels also are electrophoretically transferred onto nitrocellulose and the transferred proteins similarly autoradiographed. The same transferred proteins are used for overlay assays with pili and for binding inhibition assays with lectins as described herein. The latter procedure allowed us to identify a corneal epithelial surface-localized protein of 57 kDa molecular weight which is α-2,6-sialylated, binds pili in the overlay assay and whose interaction with pili is the initial pathogenic event in the unwounded eye model.[11]

Acknowledgments

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[7] Isolation and Structural Characterization of Adhesin Polysaccharide Receptors

By FREDERICK J. CASSELS and HERMAN VAN HALBEEK

Introduction

Bacterial polysaccharides have been shown to serve as receptor molecules for the carbohydrate-binding adhesins of genetically distinct bacteria in the human oral cavity.[1-4] It is believed that the bacteria involved in these coaggregation interactions possess a selective advantage, utilizing these mechanisms to form human dental plaque by sequential accretion.[2] The binding is carbohydrate-specific, as demonstrated by saccharide inhibition studies of two interacting bacterial partner cells, inhibition with purified polysaccharide or oligosaccharide, and lack of polysaccharide on coaggregation-defective mutants.[1,5,6] The binding of bacteria to the tooth surface, via acquired pellicle, is an important first step in the formation of dental plaque[2]; the characterization of some pellicle glycoprotein receptors is discussed elsewhere.[7,8] The early colonizers in dental plaque, primarily members of the genera *Streptococcus* and *Actinomyces,* are involved not only in intrageneric cell-to-cell interactions (streptococci), but also in interactions between members of the two genera and between these genera and many additional genera.[1,2] The later colonizers are presented with a different substrate for attachment once the primary colonizers establish themselves. In the cell-to-cell interactions characterized as adhesin-to-carbohydrate, six polysaccharide receptors or putative receptors have been purified and structurally determined.[1,9,10] All polysaccharides are from *Streptococcus*

[1] P. E. Kolenbrander, N. Ganeshkumar, F. J. Cassels, and C. V. Hughes, *FASEB J.* **7,** 406 (1993).
[2] P. E. Kolenbrander and J. London, *J. Bacteriol.* **175,** 3247 (1993).
[3] P. E. Kolenbrander, this volume [31].
[4] J. London, this volume [32].
[5] F. J. Cassels and J. London, *J. Bacteriol.* **171,** 4019 (1989).
[6] J. O. Cisar, M. J. Brennan, and A. L. Sandberg, *in* "Molecular Basis of Oral Microbial Adhesion" (S. E. Mergenhagen and B. Rosan, eds.), p. 159. American Society for Microbiology, Washington, D.C., 1985.
[7] A. Prakobphol, H. Leffler, and S. J. Fisher, this volume [12].
[8] B. L. Gillece-Castro, A. Prakobphol, A. L. Burlingame, H. Leffler, and S. J. Fisher, *J. Biol. Chem.* **266,** 17358 (1991).
[9] G. P. Reddy, C. C. Chang, and C. A. Bush, *Anal. Chem.* **65,** 913 (1993).
[10] C. Abeygunawardana and C. A. Bush, *Adv. Biophys. Chem.* **3,** 199 (1993).

oralis or *S. sanguis* and serve as receptor molecules for adhesins borne on *Actinomyces* spp. and *Capnocytophaga* spp.

Methods for the structural characterization of the polysaccharides include various chemical, chromatographic, and spectroscopic techniques. Among them, nuclear magnetic resonance (NMR) spectroscopy provides a powerful nondestructive means to characterize carbohydrates structurally, and it has become an integral part of the current methodology of oligosaccharide sequencing.[10-18] This chapter illustrates the role of combined ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy as a method for obtaining the characterization of the primary structure of adhesin polysaccharide receptors. For carbohydrate sequencing, NMR spectroscopy is most useful when combined with chromatographic and other spectroscopic methods (mass spectrometry in particular). This chapter describes the purification and analysis of the phosphorylated hexasaccharide repeating unit of the adhesin polysaccharide receptor obtained from *Streptococcus oralis* ATCC 55229 (American Type Culture Collection, Rockville, MD; previously characterized as *Streptococcus sanguis* H1)[19,20] to exemplify the integrated approach to polysaccharide structural determination.

Experimental Procedures

Isolation of Crude Cell Wall Material

Streptococcus oralis ATCC 55229 (*S. sanguis* H1) cells are grown in a complex medium containing tryptone (5.0 g/liter) (Difco, Detroit, MI), yeast extract (5.0 g/liter) (Difco), Tween 80 (0.5 ml/liter) (Difco), and $K_2HPO_4 \cdot 3H_2O$ (5.0 g/liter) (Aldrich, Milwaukee, WI) with 0.3% glucose [added as a 50% (w/v) solution after filter sterilization to autoclaved and cooled medium of 1 liter or greater] under anaerobic conditions (Becton-Dickinson, Hunt Valley, MD, Gas Pak 150). A 10-ml tube of prereduced

[11] A. S. Serianni, *in* "Glycoconjugates: Composition, Structure, and Function" (H. J. Allen and E. C. Kisailus, eds.), p. 71. Dekker, New York, 1992.
[12] H. van Halbeek, *in* "Frontiers of NMR in Molecular Biology" (D. Live, I. M. Armitage, and D. Patel, eds.), p. 195. Alan R. Liss, New York, 1990.
[13] C. A. Bush, *Bull. Magn. Reson.* **10,** 73 (1988).
[14] H. J. Jennings and I. C. P. Smith, this series, Vol. 50, p. 39.
[15] J. Dabrowski, this series, Vol. 179, p. 122.
[16] T. A. W. Koerner, J. H. Prestegard, and R. K. Yu, this series, Vol. 138, p. 38.
[17] H. van Halbeek, this series, Vol. 230, p. 132.
[18] R. A. Byrd, W. Egan, M. F. Summers, and A. Bax, *Carbohydr. Res.* **166,** 47 (1987).
[19] F. J. Cassels, H. M. Fales, J. London, R. W. Carlson, and H. van Halbeek, *J. Biol. Chem.* **265,** 14127 (1990).
[20] J. Glushka, F. J. Cassels, R. W. Carlson, and H. van Halbeek, *Biochemistry* **31,** 10741 (1992).

and prewarmed (37°) medium is inoculated with 1 ml of a frozen stock culture and incubated for 16–24 hr. The entire 10 ml is then used to inoculate 100 ml of prereduced, prewarmed medium, and the culture is grown for 16–24 hr. The approximately 10-fold inoculum is continued to the l-liter level, and the 1-liter volume is added to a 20-liter carboy (16 liters of medium). The medium is not prereduced, and the incubation period is extended to 60–72 hr. Approximately 32 g wet weight of bacteria per carboy is harvested by centrifugation.

The covalently attached polysaccharide is removed from *S. oralis* ATCC 55229 cell walls by treatment with mutanolysin after deproteinization.[5,6] Deproteinization of *S. oralis* ATCC 55229 cells involves sequential treatment with 0.1% Triton X-100 (v/v, Boehringer Mannheim, Indianapolis, IN), 0.1% Pronase (w/v, Calbiochem, San Diego, CA), and 6 *M* guanidine hydrochloride (Sigma, St. Louis, MO), with extensive washing after each treatment. The resulting crude cell wall preparation, at a concentration of 26 mg/ml (corresponding to 4 mg/g initial wet weight *S. oralis* ATCC 55229 cells), is then digested by incubation with mutanolysin (Sigma) for 16 hr at 37°. After clarification, the supernatant is adjusted to a concentration of 5% trichloroacetic acid (w/v, Sigma) with stirring (30 min, on ice), and acid-precipitable material is removed by centrifugation. The supernatant is neutralized by adding solid Tris (Bio-Rad, Richmond, CA), dialyzed extensively against deionized water, and freeze-dried. Approximately 0.9 g crude cell wall material (dry weight) per carboy is obtained.

At this point in the procedure, either the polysaccharide or its repeating hexasaccharide unit may be obtained directly from the crude cell wall material. Alternatively, the hexasaccharide may be obtained from the purified polysaccharide after initial isolation of the polysaccharide (with lower yield of purified hexasaccharide).[5]

Purification of Streptococcus oralis ATCC 55229 Adhesin Polysaccharide Receptor

Rehydrated mutanolysin extract from *S. oralis* ATCC 55229 yields purified polysaccharide by anion-exchange chromatography on a Mono Q HR 10/10 column (Pharmacia, Piscataway, NJ). Polysaccharide is eluted at a flow rate of 2.0 ml/min with a 0.1 to 0.5 *M* NaCl gradient prepared in 2 m*M* Tris-HCl, pH 8.0. All fractions are monitored for the presence of neutral carbohydrate by the phenol–sulfuric acid assay.[21] Neutral carbohydrate is detected in the initial column wash and later as a sharp peak that elutes at approximately 200–225 m*M* NaCl. Immunoelectrophoresis

[21] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.* **28,** 350 (1956).

of column fractions has proved useful in purity determination.[5] The mutanolysin extract, as well as the salt-eluted peak, inhibit coaggregation between *S. oralis* ATCC 55229 and *Capnocytophaga ochracea* ATCC 33596 whole cells in a semiquantitative microdilution plate assay (used primarily for screening purposes).[5] Organic phosphate is detected in the polysaccharide.[22] Up to 200 mg of purified polysaccharide is obtained from the crude cell wall material derived from one carboy.

Purification of Streptococcus oralis ATCC 55229 Hexasaccharide Repeating Unit

Freeze-dried material is treated with 48% hydrofluoric acid (HF) (w/w, Baker Analyzed, J. T. Baker, Phillipsburg, NJ) for 4 days at 4°. HF is first removed under a stream of nitrogen gas; the residue is then rehydrated with distilled, deionized water, followed by evaporation (SpeedVac, Savant Instruments, Farmingdale, NY). Rehydration and evaporation are repeated two additional times. Evaporated material is suspended in high-performance liquid chromatography (HPLC) grade water (Burdick and Jackson) and passed through an ultrafiltration membrane (Centricon 3, Amicon, Beverly, MA). The filtrate is injected onto a C_{18} reversed-phase HPLC column (4.6 mm × 25 cm, Zorbax, DuPont, Wilmington, DE) mounted on a Hewlett-Packard 1090L chromatograph and run isocratically in HPLC grade water (0.5 ml/min). Fractions are evaporated to dryness (SpeedVac, Savant); fractions containing visible residue are spotted onto high-performance thin-layer chromatography (HPTLC) plates.[5] Hexasaccharide-containing fractions are pooled, evaporated, dissolved in a mixture (65:35, v/v) of acetonitrile and water (HPLC grade), and applied to an 8 mm × 30 cm MicroPak AX-5 (Varian, Palo Alto, CA) diaminopropyl-bonded silica HPLC column. The hexasaccharide is eluted by passing a linear gradient of water through the column. After washing the column for 15 min with the initial acetonitrile–water mixture, the gradient is initiated; it is formed by increasing the aqueous phase with 1.75% water/min at a flow rate of 1 ml/min. Fractions are evaporated and assayed by HPTLC, as above.

In the final purification step, hexasaccharide-containing fractions are dissolved in HPLC grade water and eluted with water (0.3 ml/min) from two Superose 12 (Pharmacia) columns in series. Fractions are evaporated and assayed as detailed above; hexasaccharide material is pooled for further analysis. Quantitative coaggregation–inhibition assays done by utilizing the

[22] B. N. Ames, this series, Vol. 8, p. 115.

hexasaccharide, polysaccharide, or simple sugars as inhibitors are important to determine the relative inhibitory capability of these molecules on both a weight and molar basis.[5] From 0.9 g crude cell walls, approximately 15 mg of purified hexasaccharide is obtained.

Preliminary Structural Characterization of Streptococcus oralis ATCC 55229 Hexa- and Polysaccharide

Chemical analysis of the purified polysaccharide by colorimetric methods[5] indicates that the majority of the sample is indeed carbohydrate, with the additional presence of organic phosphate, whereas protein and nitrogen are absent in any appreciable amounts. HPTLC analysis of the purified hexasaccharide hydrolyzed to its monosaccharide constituents indicates that it consists of rhamnose, galactose, and glucose; the mobility of the intact hexasaccharide is between that of maltopentaose and maltohexaose standards (Fig. 1). Gas chromatography of alditol acetate derivatives of the acid-hydrolyzed hexasaccharide confirmed[5] the constituents as rhamnose, galactose, and glucose, in the molar ratio 2:3:1. Additional gas chromatographic analysis of the trimethylsilylated butyl glycosides derived from the butanolyzed hexasaccharide established that the rhamnosyl residues are in the L configuration, whereas the glucose and all of the galactose residues

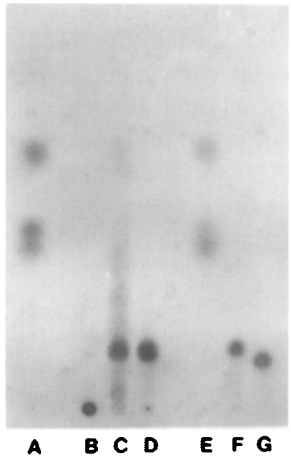

FIG. 1. Silica G HPTLC of *S. oralis* ATCC 55229 hexasaccharide (lane D), acid-hydrolyzed hexasaccharide (E), and intact polysaccharide (B). Lane C shows the purified polysaccharide after HF treatment (prior to purification of the hexasaccharide). For chromatography conditions, see Ref. 5. Standards: lane A, equimolar mixture of rhamnose, glucose, and galactose [fastest (top) to slowest migrating, respectively]; lane F, maltopentaose; lane G, maltohexaose.

are of the D configuration.[19] Californium^{252}- plasma desorption mass spectrometry of the hexasaccharide in its native, acetylated, and reduced and acetylated forms revealed its native mass to be 959, consistent with two rhamnose and four hexose residues; the two rhamnose residues were deduced to be linked to one another at the nonreducing terminus of the hexasaccharide (Fig. 2).[19]

Using the Hakomori methylation procedure described by York *et al.*,[23] glycosyl linkages of the receptor hexasaccharide and polysaccharide were determined by combined gas–liquid chromatography–mass spectrometry. Table I lists the linkage and terminal residue positions determined for each monosaccharide from the partially methylated alditol acetate derivatives. The single C4-linked glucopyranose (Glc*p*) and C2-linked rhamnopyranose (Rha*p*) residues are present in both the hexasaccharide and polysaccharide. The nonreducing terminal rhamnopyranose residue present in the hexasaccharide is seen in the polysaccharide as C3-linked, indicating that the residue is responsible for the interhexasaccharide linkage to the reducing-end galactose of the adjacent repeating unit in the polymer. The three C3-linked galactopyranose (Gal*p*) residues seen in the hexasaccharide (Table I) correspond to a single C3-linked galactopyranose and a single C3-linked galactofuranose (Gal*f*) in the methylation analysis of the polysaccharide. The two galactose derivatives coelute under the gas chromatographic conditions employed; however, the occurrence of C3-linked galactofuranose was verified from its characteristic electron-impact spectrum.[20] The presence of the galactofuranose in the polysaccharide and its absence in the hexasaccharide indicate that the galactofuranose is initially present in the intact polymer; on treatment with aqueous HF,[5] the acid-labile linkage between the galactofuranosyl and the rhamnosyl residues is cleaved, resulting in depolymerization of the polysaccharide and conversion of Gal*f* to Gal*p*.

Methylation analysis of the polysaccharide fails to reveal the presence of a third C3-linked galactose residue; owing to the presence of a covalently attached phosphate residue, the partially methylated alditol acetate derivative is not volatile under the gas chromatographic conditions employed. The presence of galactose phosphate in the polymer is demonstrated by partial acid hydrolysis of the polysaccharide followed by high-pH HPLC of the hydrolyzate. The resulting chromatogram shows both galactose 6-phosphate and glycerol 1-phosphate, indicating that one of the two internal galactose residues is present as glycerol-(1 → PO_4 → 6)-galactose. The HF cleavage of the polysaccharide results in the loss of glycerol phosphate.

[23] W. S. York, A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim, this series, Vol. 118, p. 3.

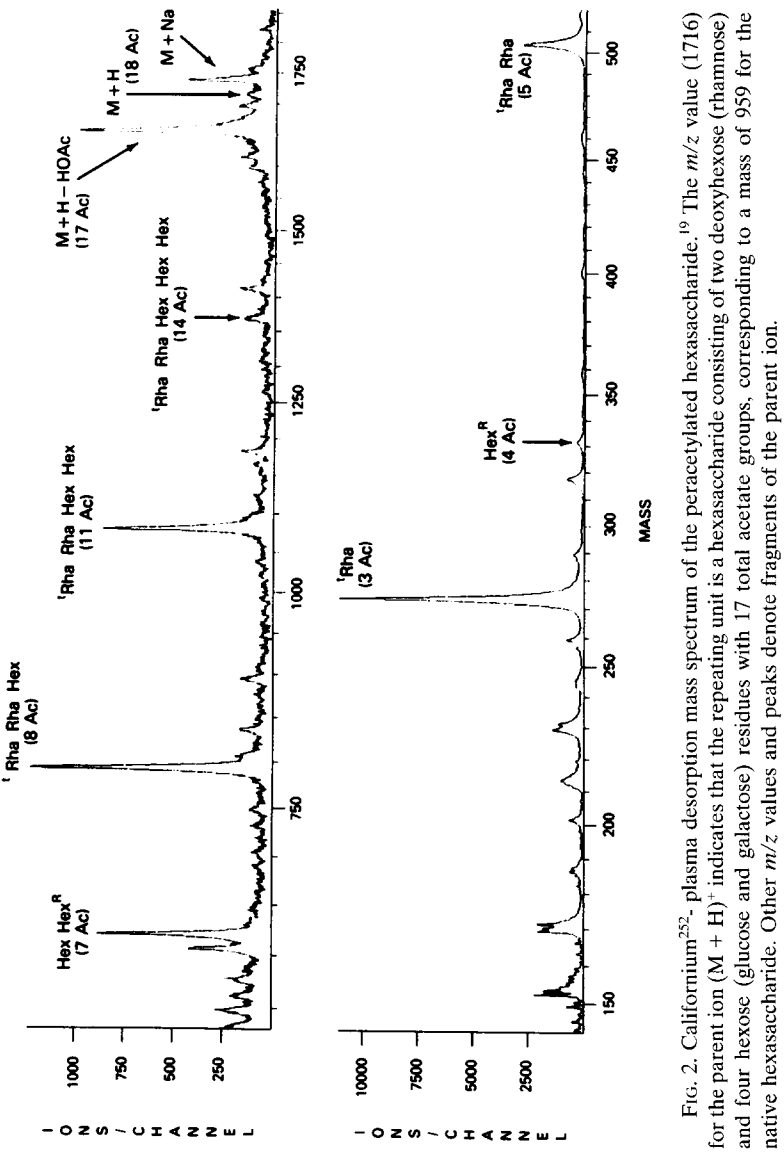

FIG. 2. Californium^{252}- plasma desorption mass spectrum of the peracetylated hexasaccharide.[19] The m/z value (1716) for the parent ion $(M + H)^+$ indicates that the repeating unit is a hexasaccharide consisting of two deoxyhexose (rhamnose) and four hexose (glucose and galactose) residues with 17 total acetate groups, corresponding to a mass of 959 for the native hexasaccharide. Other m/z values and peaks denote fragments of the parent ion.

TABLE I
GLYCOSYL LINKAGE[a] AND TERMINAL RESIDUE DETERMINATION IN RECEPTOR POLYSACCHARIDE[b] FROM *Streptococcus oralis* ATCC 55229 AND HEXASACCHARIDE REPEATING UNIT[c]

Glycosyl residue	Linkage position	Terminal position	Hexasaccharide	Polysaccharide
Rha*p*	C2	—	1.0[a]	1.0[a]
Rha*p*	—	Nonreducing	1.0	0.0
Rha*p*	C3	Nonreducing	0.0	1.0
Gal*p*	C3	—	2.0	*d*
Gal*p*	C3	Reducing	1.0	*d*
Gal*f*	C3	Reducing	0.0	*d*
Glc*p*	C4	—	1.0	1.2

[a] In moles relative to C2-linked Rha.
[b] From Ref 19.
[c] From Ref. 20.
[d] The experimentally determined total of C3-linked Gal*p* and Gal*f* in the polysaccharide is 1.9 mol/mol. Refer to text for further information.

The combined results from chemical, chromatographic, and mass spectrometric analysis indicate that the structure of the hexasaccharide repeating unit of the polysaccharide is

$$[\rightarrow 3)\text{-L-Rha}p(1 \rightarrow 2)\text{-L-Rha}p \rightarrow \text{D-Hex}p \rightarrow \text{D-Hex}p \rightarrow \text{D-Hex}p(1 \rightarrow 3)\text{-D-Gal}f(1 \rightarrow]$$

Nuclear Magnetic Resonance Spectroscopy Methods

Purified samples of the *S. oralis* ATCC 55229 hexasaccharide (14 mg) and polysaccharide (15 mg) are used for NMR analysis. The samples are dissolved in D_2O (99.8% D; Cambridge Isotope Laboratories, Wilmington, DE), freeze-dried, and reexchanged three times with high-grade D_2O (99.99% D; Cambridge Isotope Laboratories). The final solutions of the hexasaccharide and polysaccharide samples in 0.5 ml D_2O (pH 6–7) are then transferred into 5-mm NMR tubes (Wilmad, Buena, NJ). The NMR spectra are recorded on Bruker AM-500 and AMX-600 spectrometers at 27° and 23°, respectively. The NMR data are processed off-line using the FELIX software package, version 2.1 (BioSym Technologies, San Diego, CA), on a Silicon Graphics Personal Iris workstation. The ^1H and ^{13}C chemical shifts (δ) are reported in parts per million (ppm) downfield from internal 4,4-dimethyl-4-silapentane 1-sulfonate (DSS), with accuracies of

0.01 and 0.02 ppm, respectively. The ^{31}P chemical shifts are referenced to external 85% H_3PO_4.

Two dimensional (2D) COSY (scalar correlated spectroscopy),[24] TOCSY (total correlation spectroscopy),[25] NOESY (nuclear Overhauser effect (NOE) correlated spectroscopy),[26] HMQC (heteronuclear multiple-quantum coherence),[27] and HMBC (heteronuclear multiple-bond correlation)[28] experiments are performed in the phase-sensitive mode using the time-proportional phase incrementation[29] method. The TOCSY experiments use either MLEV-17 (for the hexasaccharide)[30] or DIPSI-2 (for the polysaccharide) spin-lock pulses[31] for isotropic mixing. The GARP-1 sequence[32] is used for ^{13}C decoupling during acquisition in the $^1H\{^{13}C\}$ HMQC experiment. In most ^1H-detected experiments, low-power presaturation is applied to the residual HDO signal. Additional experimental details can be found in the figure legends, and complete experimental protocols are provided in Refs. 19 and 20.

Carbohydrate Sequencing by Nuclear Magnetic Resonance Spectroscopy

Figure 3 shows the one-dimensional (1D) ^1H and ^{13}C NMR spectra of the purified hexasaccharide and intact polysaccharide, respectively, from *S. oralis* ATCC 55229. The ^1H and ^{13}C NMR spectra each represent unique "fingerprints" of the carbohydrates; mere comparison of the ^1H or ^{13}C NMR spectra of compounds, even without detailed interpretation, can demonstrate their identities. Thus, a 1 D ^1H NMR study may suffice for primary structure determination if the saccharide has been previously characterized.

The ^1H and ^{13}C NMR spectra of an oligosaccharide are essentially superpositions of the spectra of the individual glycosyl residues, modified only slightly from the spectra of loose individual residues because of the linkages to one another. Thus, the positions (chemical shifts) and patterns (scalar couplings, line widths, intensities) of the signals in the NMR spectrum of a saccharide are characteristic of the entire primary structure of the compound under investigation. Interpretation of carbohydrate ^1H and

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[25] L. Braunschweiler and R. R. Ernst, *J. Magn. Reson.* **53,** 521 (1983).
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[31] S. P. Rucker and A. J. Shaka, *Mol. Phys.* **68,** 509 (1989).
[32] A. J. Shaka, P. B. Barker, and R. Freeman, *J. Magn. Reson.* **64,** 547 (1985).

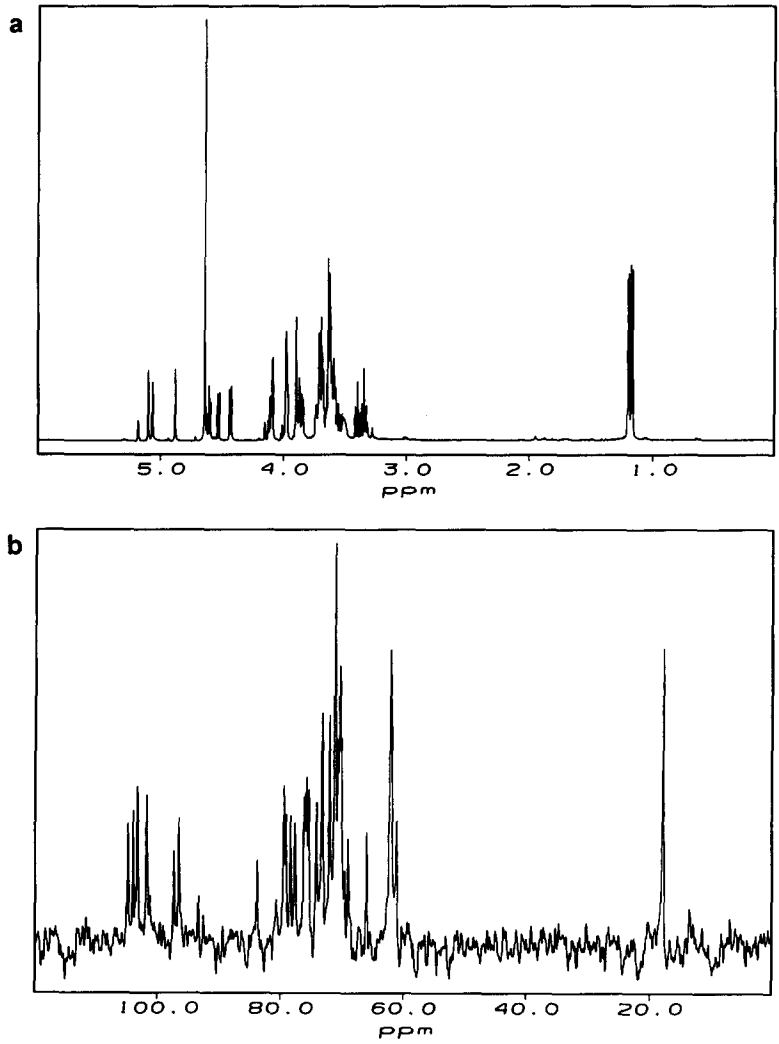

FIG. 3. (a) ^{1}H and (b) ^{13}C 1D NMR spectra of the hexasaccharide and (c) ^{1}H 1D NMR spectrum of the adhesin polysaccharide receptor of *Streptococcus oralis* ATCC 55229.

^{13}C NMR spectra requires the cataloging of signals by position and pattern; the chemical shifts are usually compiled as tabular data (compare Tables II and III). These values may then be compared with literature data on mono-, oligo-, and polysaccharides. Several carbohydrate ^{1}H- and ^{13}C-NMR

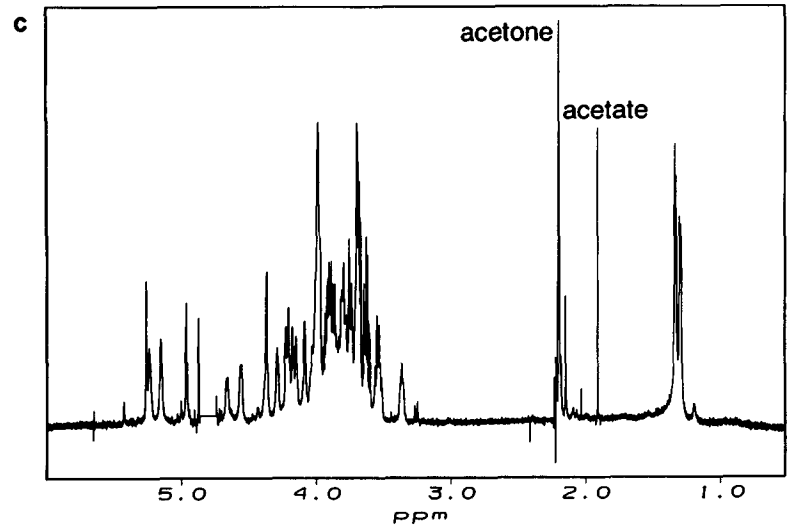

FIG. 3. (*continued*)

databases are now in existence,[33–35] and efforts aimed at the automation of NMR data processing and peakwise spectra comparison[34,35] are underway. A potentially faster way to achieve NMR-based structural identification of "known" saccharides is to use the entire spectrum (especially the ^{1}H spectrum with its crowded 3–4 ppm envelope region, high in information density) for pattern recognition. The NMR spectrum is no longer reduced to a list of chemical shifts. Artificial intelligence techniques are beginning to emerge for both automated NMR pattern recognition and spectral interpretation.[36,37] In the foreseeable future, NMR spectral databases will be linked to the complex carbohydrate structure database (CCSD).[38]

In cases such as the *S. oralis* ATCC 55229 saccharides, where the 1D ^{1}H and ^{13}C NMR spectra do not resemble those of previously encountered

[33] G. M. Lipkind, A. S. Shashkov, N. E. Nifant'ev, and N. K. Kochetkov, *Carbohydr. Res.* **237,** 11 (1992).
[34] P.-E. Jansson, L. Kenne, and G. Widmalm, *Anal. Biochem.* **199,** 11 (1991).
[35] P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.* **188,** 169 (1989).
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[37] J. P. Radomski, H. van Halbeek, and B. Meyer, *Nature (London) Struct. Biol.* **1,** 217 (1994).
[38] S. Doubet, K. Bock, D. Smith, A. Darvill, and P. Albersheim, *Trends Biochem. Sci.* **14,** 475 (1989).

TABLE II
^{1}H NMR CHEMICAL SHIFTS[a] FOR HEXASACCHARIDE AND POLYSACCHARIDE FROM *Streptococcus oralis* ATCC 55229

Residue	Sample[b]	Chemical shift					
		H1	H2	H3	H4	H5	H6, H6′
β-Gal*f*(**A**)	Poly	5.26	4.37	4.29	4.22	3.99	3.70, 3.74
α-Gal*p* (**A**α)	Hexa	5.18	3.88	3.86	4.15	4.00	3.58–3.65
β-Gal*p* (**A**β)	Hexa	4.53	3.54	3.70	4.09	3.62	3.58–3.65
β-Glc*p* (**B**)	Poly	4.66	3.37	3.70	3.69	3.65	3.87, 4.00
	Hexa	4.60, 4.61	3.33	3.58	3.58	3.50	3.72, 3.85
β-Gal*p* (**C**)	Poly	4.56	3.69	3.81	4.18	3.75	3.76, 3.81
	Hexa	4.42	3.58	3.70	4.09	3.63	3.58–3.65
α-Gal*p* (**D**)	Poly	5.16	4.01	4.03	4.15	4.38	3.99[c]
	Hexa	5.06	3.90	3.90	3.99	4.11	3.71, 3.62
α-Rha*p* (**E**)	Poly	5.23	4.09	3.98	3.53	3.84	1.29
	Hexa	5.10	3.97	3.87	3.40	3.71	1.20
α-Rha*p* (**F**)	Poly	4.97	4.21	3.90	3.54	3.79	1.32
	Hexa	4.88	3.96	3.69	3.34	3.62	1.18
Glycerol (**G**)	Poly[d]	3.87, 3.93	3.90	3.61, 3.68			

[a] Chemical shifts are referenced to internal DSS; data were acquired in D_2O at 23° (polysaccharide) or 27° (hexasaccharide) and pH 6.
[b] Poly, Polysaccharide; Hexa, hexasaccharide.
[c] Only the shift of H6 was determined.
[d] Glycerol is not present in the hexasaccharide.

structures, 2D NMR analysis is the method of choice. To decipher the primary sequence of a saccharide from NMR experiments *de novo*, one first needs to assign the ^{1}H NMR spectrum completely, that is, to map one-to-one the signals in the spectrum to protons in the constituent sugar residues as revealed by composition analysis (Table I). Following the assignment of every signal in the spectrum, homonuclear NOE experiments may be performed in order to gather information about the distance between pairs of protons; this, in turn, renders sequence information. A less ambiguous approach to sequencing relies on the detection of long-range ^{1}H, ^{13}C scalar couplings through glycosidic bonds. Known as HMBC (heteronuclear multiple-bond correlation), this approach requires the prior assignment of the ^{13}C NMR spectrum of the oligosaccharide. The latter is usually accomplished through a 2D $^1J_{CH}$ correlation experiment, either ^{1}H-detected [HMQC or HSQC (heteronuclear single-quantum coherence spectroscopy)] or ^{13}C-detected [HETCOR (heteronuclear correlation spectroscopy)], depending on the amount of carbohydrate material available), resulting in a one-to-one correlation of signals in the ^{1}H and ^{13}C spectra. The

TABLE III
^{13}C NMR CHEMICAL SHIFTS[a] FOR HEXASACCHARIDE AND POLYSACCHARIDE FROM *Streptococcus oralis* ATCC 55229

Residue	Sample[b]	Chemical shift					
		C1	C2	C3	C4	C5	C6
β-Gal*f* (**A**)	Poly	111.90	82.48	87.17	84.38	72.69	65.08
α-Gal*p* (**A**α)	Hexa	93.31	69.00	80.69	70.06	71.18	62.25
β-Gal*p* (**A**β)	Hexa	97.32	72.01	83.80	69.53	75.94	62.13
β-Glc*p* (**B**)	Poly	104.51	75.03	76.58	80.99	77.15	62.22
	Hexa	104.77	74.14	75.38	79.43	75.72	61.09
β-Gal*p* (**C**)	Poly	105.57	71.90	80.18	67.38	77.46	63.29
	Hexa	103.97	70.67	78.34	65.94	76.18	61.94
α-Gal*p* (**D**)	Poly	98.36	70.25	78.64	70.84	71.87	66.27
	Hexa	96.54	69.00	77.64	70.40	72.01	62.13
α-Rha*p* (**E**)	Poly	103.11	80.40	72.57	74.61	71.65	19.45
	Hexa	101.78	79.09	71.28	73.15	70.33	17.82
α-Rha*p* (**F**)	Poly	104.54	72.38	80.04	73.41	71.61	19.45
	Hexa	103.26	71.06	71.18	73.24	70.33	17.82
Glycerol (**G**)	Poly[c]	68.68	73.08	64.37			

[a] Chemical shifts are referenced to internal DSS; data were acquired at pH 6 in D_2O at 23° (for polysaccharide) and at 27° (for hexasaccharide).
[b] Poly, Polysaccharide; Hexa, hexasaccharide.
[c] Glycerol is not present in the hexasaccharide.

applicability of the HMBC experiment is somewhat limited by the molecular size of the saccharide: sensitivity is excellent for oligosaccharides (molecular mass <2 kDa) but borderline for polysaccharides (molecular mass >10 kDa). Given the availability of both the intact polysaccharide and the hexasaccharide (essentially the repeating unit of the polysaccharide, stripped of the glycerol phosphate) in amounts of 14–15 mg each, this chapter allows us to illustrate the complementarity of the HMBC and NOESY approaches to sequencing carbohydrates.

Assignment of ^{1}H Resonances by Tracing J-Coupling Networks through COSY and TOCSY. Strategies for obtaining sequential assignments in ^{1}H NMR spectra of carbohydrates generally involve two different types of NMR experiments. The various glycosyl residue spin systems of a particular carbohydrate are identified by a combination of 2D direct (COSY) and relayed [TOCSY, also known as HOHAHA (homonuclear Hartmann–Hahn)] *J*-correlated spectroscopy. The basic principles of using these techniques for assignment strategies are well documented (e.g., see Refs. 10 and 12).

Figure 4, depicting a schematic three-dimensional structure of

$$\underset{\mathbf{F}}{\text{Rha}p\alpha(1\rightarrow 2)}\underset{\mathbf{E}}{\text{Rha}p\alpha(1\rightarrow 3)}\underset{\mathbf{D}}{\text{Gal}p\alpha(1\rightarrow 3)}\underset{\mathbf{C}}{\text{Gal}p\beta(1\rightarrow 4)}\underset{\mathbf{B}}{\text{Glc}p\beta(1\rightarrow 3)}\underset{\mathbf{A}}{\text{Gal}}$$

serves to aid in the discussion of NMR connectivities. Owing to the consecutive allocation of protons around each glycosyl ring, a vicinal J-coupled connectivity exists for each residue so that each proton leads to the next proton in the ring. The majority of the nonanomeric ring proton signals of the glycosyl residues are superimposed on one another in the ^{1}H NMR spectra of the saccharides [see Fig. 3a, 3c]. However, the signals of the sugar anomeric ($4.5 < \delta < 6$ ppm) and deoxy protons ($1.0 < \delta < 1.3$ ppm) are well-resolved from the others. These instantly recognizable H1 and CH_3 signals provided convenient starting points for 2D spectroscopy. The

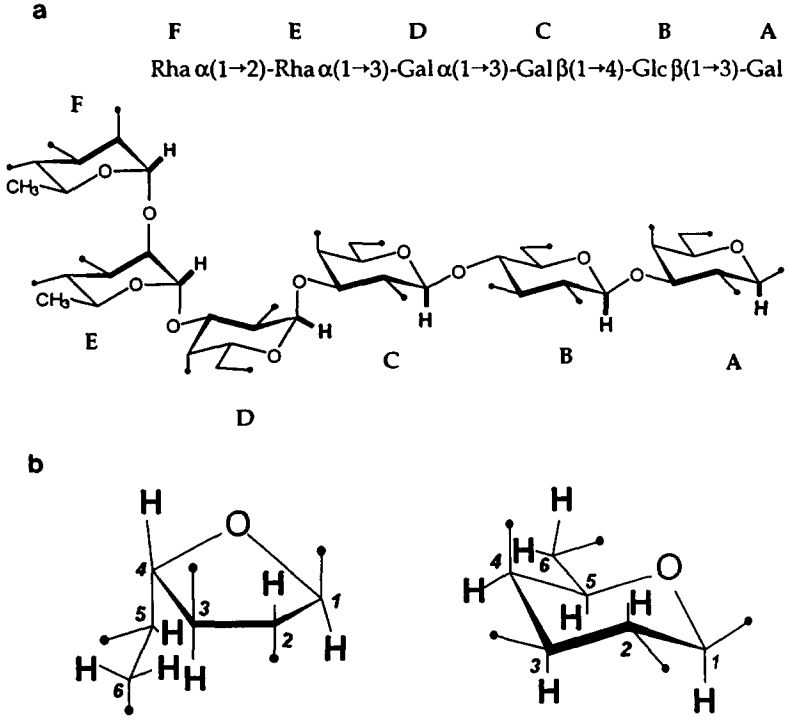

FIG. 4. (a) Schematic structure of the hexasaccharide obtained from the *S. oralis* ATCC 55229 adhesin polysaccharide receptor. The symbol ●— symbolizes an OH group. Sugar ring H atoms are not shown, except for the H1 atoms. (b) Schematic structures of β-galactofuranose (βGal$f$) and β-galactopyranose (βGal$p$).

J-connectivity trail for each of the six residues is readily initiated in these regions.

Not unexpectedly for molecules with ^{1}H NMR spectra as crowded as Fig. 3a, c (see the envelope regions at $3.0 < \delta < 4.0$ ppm), COSY fails to reveal the complete identification of the spin systems of the individual sugar residues. The TOCSY technique, however, permits subspectral editing of the ^{1}H spectrum for each constituting glycosyl residue and, consequently, the virtually complete assignment of all multiplet patterns in the ^{1}H NMR spectrum. The 2D TOCSY spectrum of the hexasaccharide is shown in Fig. 5a, and that of the polysaccharide is in Fig. 7. Subspectra are assigned to types of constituting residues based on the fact that the magnitudes of the *J* couplings reflect the stereochemistry and configuration at each carbon; the complete set of *J* couplings is therefore typical of the identity of the residue. Figure 5b shows the cross sections (slices) through the anomeric proton signals in Fig. 5a, clearly revealing the subspectra of the constituting residue types (Glc, Gal, Rha). In fact, the Glc residue is the only type of residue for which the subspectrum is complete. Cross sections through H1 of glycosyl residues with the *galacto* configuration (Gal) include only signals of H1–H4, not H5, H6, and H6′, owing to the small magnitude of $J_{4,5}$ in these residues.[39] The sensitivity of the cross sections through H1 of glycosyl residues with the *manno* configuration (Rha) suffers from the small magnitude of $J_{1,2}$ in these residues.[40] However, neither COSY nor TOCSY spectra reveal any information about the sequence or linkage positions of the six residues.

Sequencing Carbohydrates by Tracing $^3J_{CH}$ Couplings through HMBC. Primary structural analysis of complex carbohydrates via 2D NMR is ideally based on tracing through-bond *J*-coupling connectivities. Glycosyl residues are identified as homonuclear two- and three-bond *J*-coupling (intraring connectivities) networks (see above). Interring connectivities, however, cannot be made by {^{1}H, ^{1}H} scalar connectivities because J_{HH} couplings over four bonds are rarely observed. Therefore, it is necessary to make use of the heteronuclear coupling constants $^3J_{CH}$ across the glycosidic linkages. The hexasaccharide allows the detection of long-range $^3J_{CH}$ couplings over glycosidic linkages; they provide the key to its sequencing by 2D NMR spectroscopy.

The successful use of long-range {^{13}C, ^{1}H} shift-correlation spectroscopy for sequencing an oligosaccharide is dependent on the complete assignment of its 1D ^{13}C spectrum. Given the complete assignment of the ^{1}H spectrum

[39] L. Poppe and H. van Halbeek, *Magn. Reson. Chem.* **29,** 355 (1991).

[40] D. A. Powell, W. S. York, H. van Halbeek, J. T. Etse, A. I. Gray, and P. G. Waterman, *Can. J. Chem.* **68,** 1044 (1990).

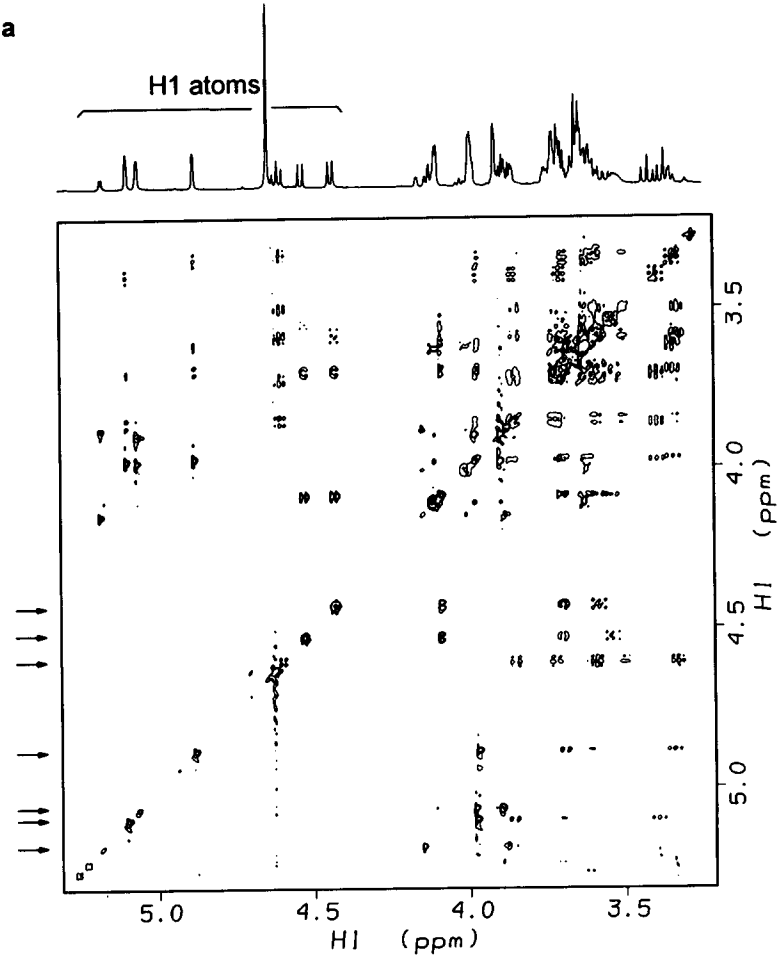

FIG. 5. (a) Two-dimensional TOCSY spectrum of the hexasaccharide isolated from the *S. oralis* ATCC 55229 polysaccharide. The corresponding region of the 1D ^1H spectrum is shown at the top. The spectrum was obtained on 14 mg of the oligosaccharide in D_2O (pH 6) at 500 MHz and 27°. Data matrix: 512 × 2048; 64 scans per t_1 value; spin locking by MLEV-17; mixing time 200 msec; sine-bell window functions applied in both dimensions prior to Fourier transformation. (b) Cross sections ("slices") through the anomeric proton resonances on the diagonal of the 2D TOCSY spectrum, taken at the positions of the arrows in (a), constituting the (partial) subspectra of the glycosyl residues of the hexasaccharide (indicated by capital letters). The numbers correspond to the location of the protons in the rings. The CH_3 signals of the α-rhamnosyl residues, outside of the narrow spectral width used to record the spectrum, were aliased into the spectrum (at δ of ~4.71).

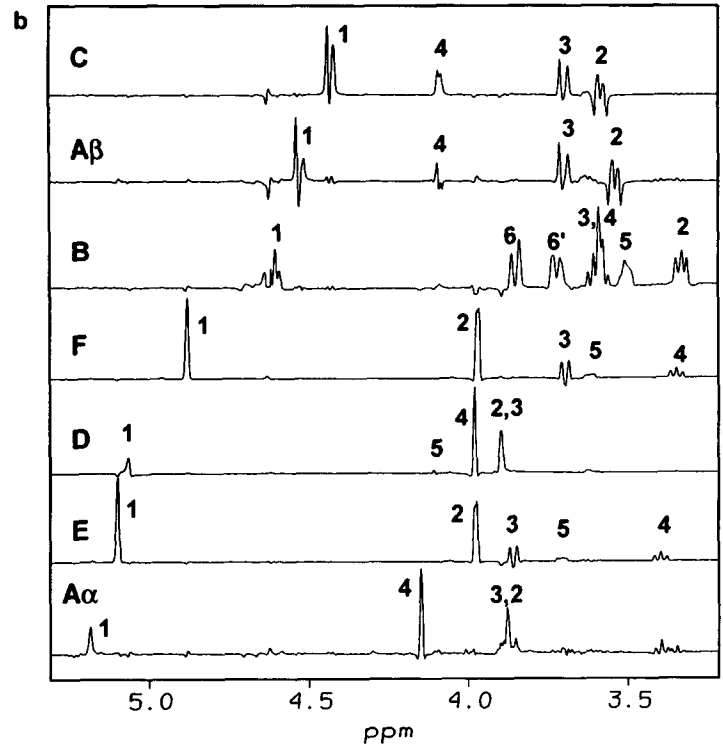

FIG. 5. (*continued*)

(Fig. 5), the latter task is accomplished by one-bond {^{13}C, ^{1}H} shift correlation spectroscopy. A conventional (i.e., ^{13}C-detected) HETCOR experiment yields connectivities between pairs of directly coupled (via ^{1}J) ^{13}C and ^{1}H nuclei. The observed nucleus in HETCOR spectroscopy is ^{13}C; therefore, the technique is only applicable if at least 10 to 15 mg of a medium-sized oligosaccharide is available. The sensitivity problem in the detection of ^{1}J connectivities between ^{13}C and ^{1}H nuclei for smaller amounts of carbohydrate (such as 15 mg of the *S. oralis* polysaccharide) can be overcome by so-called inverse (i.e., ^{1}H-detected) 2D {^{13}C, ^{1}H} shift-correlation experiments such as HMQC or HSQC spectroscopy. The HMQC experiment requires 1/20 to 1/30 as much sample as HETCOR to obtain {^{13}C, ^{1}H} one-bond shift-correlation maps. The additional advantage of HMQC over HETCOR lies in its increased (digital) resolution in the ^{1}H dimension. The ^{13}C assignments for the hexa- and polysaccharide, obtained by HMQC experiments, are listed in Table III.

Once the ^1H and ^{13}C spectra have both been completely assigned, long-range $^3J_{CH}$ couplings over glycosidic linkages may be used for sequencing of the saccharide by 2D NMR spectroscopy. $^3J_{CH}$ values are usually small (<10 Hz) compared with those of $^1J_{CH}$ (>100 Hz). Correlation of ^{13}C and ^1H nuclei, scalar-coupled to one another via long-range couplings ($^2J_{CH}$ and $^3J_{CH}$), was conventionally achieved by ^{13}C-detected COLOC (correlation through long-range couplings) spectroscopy. However, the COLOC experiment requires at least an order of magnitude more sample than conventional HETCOR. The ^1H-detected HMBC experiment is 50 to 100 times more sensitive than the COLOC experiment. The HMBC pulse sequence generates multiple-quantum coherence between ^{13}C and ^1H coupled through $^2J_{CH}$ and $^3J_{CH}$, which is detected by ^1H observation.

The sequencing of the *S. oralis* ATCC 55229 hexasaccharide by HMBC is illustrated in Fig. 6. The sequence information is provided by the connectivities between the anomeric protons and the carbons across the glycosidic linkages. With the assignment of the ^{13}C spectrum at hand, these can be readily distinguished from intraresidue long-range $^2J_{CH}$ and $^3J_{CH}$ connectivities. Starting from Rha H1 at δ 4.88, the sequence is traced as in Scheme I.

In summary, the one-bond {^{13}C, ^1H} correlation map provided by the HMQC experiment is generally sufficient to assign unambiguously all resonances in the ^{13}C NMR spectrum, provided that the entire ^1H spectrum was assigned by the COSY/TOCSY approach. The tracing of long-range interglycosidic $^3J_{CH}$ couplings from the HMBC spectrum completes the primary structural analysis by providing the linkage positions and the sequence of the glycosyl residues in the hexasaccharide.

Using ^1H NOEs to Assist in Sequencing. Seemingly the ideal tool for sequencing carbohydrates, the HMBC experiment suffers the drawback of dramatically decreasing sensitivity with increasing molecular size. During the interpulse delay used to sort multiple-bond from one-bond J_{CH} connectivities, much of the NMR signal of a polymer decays because of short relaxation times (T_2) for high molecular weight compounds. Therefore, it is not possible to complete the sequencing of the *S. oralis* ATCC 55229 polysaccharide by HMBC spectroscopy.

One may resort to the alternative method of sequencing the polysaccharide by tracing homonuclear through-space (dipolar) connectivities (NOEs) between protons on opposite sites of the glycosidic linkages. This type of NMR experiment allows us to probe which protons are close in space to one another (less than 4.5 Å away). A drawback of the method is that the interglycosidic NOE between H1 and the proton on the attachment site of the other glycosyl residue is not necessarily the largest interresidue NOE effect. Nuclear Overhauser effects should therefore not be used as the sole

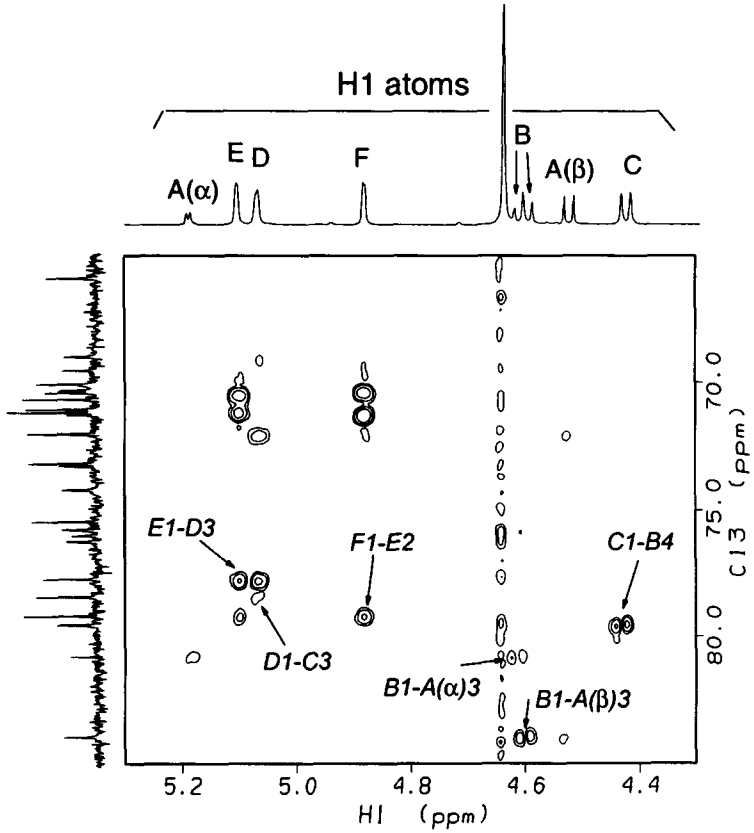

FIG. 6. Heteronuclear multiple-bond correlation (HMBC) spectrum of the hexasaccharide isolated from the adhesin polysaccharide receptor of *S. oralis* ATCC 55229. The selected region ($4.3 < \delta < 5.3$ ppm in the ^{1}H domain; $65 < \delta < 85$ ppm in the ^{13}C domain) shows the long-range ^{1}H–^{13}C connectivities of the anomeric protons. The corresponding regions of the 1D ^{1}H and ^{13}C spectra are shown at the top of the contour map and beside the vertical axis, respectively. Cross-peaks marked by capital letters indicate three-bond couplings ($^3J_{CH}$) across the glycosidic linkages between pertinent residues; cross-peaks that are not marked refer to $^2J_{CH}$ and $^3J_{CH}$ couplings within the glycosyl rings. The HMBC spectrum was obtained on 14 mg of the oligosaccharide in D_2O (pH 6) at 500 MHz and 27°. Data matrix: 200 × 2048; 400 scans per t_1 value; squared sine-bell multiplication applied in the ^{1}H dimension, Gaussian line broadening in the ^{13}C dimension, prior to Fourier transformation.

	F	E	D	C	B	A
	Rha*p*(1→2)Rha*p*(1→3)Gal*p*(1→3)Gal*p*(1→4)Glc*p*(1→3)Gal					
H1	4.88	5.10	5.06	4.42	4.61/4.60	
	↘	↑ ↘	↑ ↘	↑ ↘	↑ ↘	
C*x*		79.1	77.6	78.4	79.4	80.8/83.8
x		2	3	3	4	3

SCHEME I

source of evidence for the position of a glycosidic linkage and, thus, the sequence of glycosyl residues.

Given the structure of the hexasaccharide repeating unit, structural analysis of the *S. oralis* ATCC 55229 polysaccharide backbone is completed by detecting NOE effects involving H1 of the Gal-**A** residue (reducing-end residue in the hexasaccharide, now glycosidically linked). The TOCSY and NOESY spectra of the polysaccharide are combined in Fig. 7. The TOCSY spectrum through H1 at δ 5.26 revealed that Gal-**A** in the polysaccharide occurred in its furanosyl form. The H1 chemical shift, in conjunction with the coupling constant J_{12} of approximately 1 Hz, is indicative of this ring form.[41] The interresidue NOE effects between the protons of δ 5.26 and 3.90 (H3 Rha-**F**) shown in Fig. 7 pointed to a Gal-**A**(1 → 3)Rha-**F** linkage. Thus, NMR spectroscopy, in conjunction with the results from methylation analysis (Table I), allows us to unambiguously establish the primary structure of the polysaccharide backbone. A Gal*f* residue is found to provide the "bridge" between adjacent repeating unit moieties. The linkage configuration is inferred as β-L from the chemical shift (δ 111.9) for C1 of Gal*f*-**A**.[42,43] The NMR data are compatible with the following structure:

$$[\rightarrow 3)\text{Rha}p\alpha(1 \rightarrow 2)\text{Rha}p\alpha(1 \rightarrow 3)\text{Gal}p\alpha(1 \rightarrow 3)\text{Gal}p\beta(1 \rightarrow 4)\text{Glc}p\beta(1 \rightarrow 3)\text{Gal}f\beta(1\rightarrow]_n$$

Locating Phosphate Ester Group. In addition to the glycosyl residues mentioned above, composition analysis of the *S. oralis* ATCC 55229 polysaccharide revealed the presence of glycerol and phosphate. NMR spectroscopy can be successfully applied for locating the site of this naturally occurring phosphate group in the complex carbohydrate.

[41] K. Bock and H. Thøgersen, *Annu. Rep. NMR Spectrosc.* **13,** 1 (1982).
[42] K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.* **41,** 27 (1983).
[43] K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.* **42,** 193 (1984).

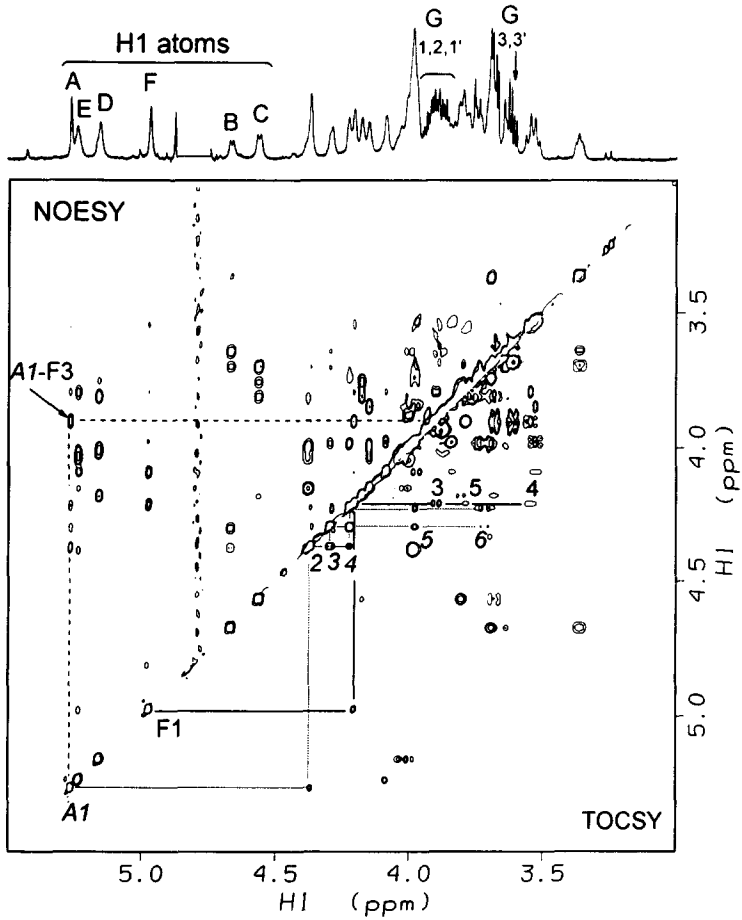

FIG. 7. Corresponding regions ($3.0 < \delta < 5.5$ ppm) of 2D TOCSY and NOESY NMR spectra of the *S. oralis* ATCC 55229 polysaccharide (15 mg) in D_2O at pH 6, recorded at 600 MHz and 23°. (Top left) NOESY spectrum (mixing time 80 msec); (bottom right) TOCSY spectrum (mixing time 54 msec). The dotted lines in the TOCSY spectrum indicate the scalar connectivity trail for Gal*f*-**A**; the solid lines indicate the spin system of Rha*p*-**F**. The italicized and normal-face numbers in the indicated assignments refer to the protons in the glycosyl residues **A** and **F**, respectively. The A1 to F3 NOESY connectivity is indicative of the occurrence of the structure entity (→ 3)Gal*fβ*(1 → 3)Rha*pα*(1 →). (Parenthetically, the relatively sharp signals marked as G1 through G3′ in the 1D projection are attributed to the glycerol residue.)

Figure 3c shows the 1D ^1H NMR spectrum of the polysaccharide. The signals arising from the glycerol protons in the spectrum have noticeably narrower line widths than those from the glycosyl protons in the polysaccharide backbone (see also Fig. 7, top). This is consistent with the terminal position of glycerol in a side chain of the hexasaccharide repeating unit. The 1D ^{31}P NMR spectrum of the polysaccharide (not shown) consisted of a single signal at δ −1.7 ppm, characteristic of a phosphodiester.[44] This signal does not shift significantly for two different pH values (pH 6 and 10), confirming the presence of the phosphate group as a diester, not a monoester. A $^1H\{^{31}P\}$ HMQC spectrum[20] provides correlations between the ^{31}P signal and three proton signals. One correlation originates from the overlapping H6 and H6′ protons of galactosyl residue **D**; the other two originate from two geminal protons of glycerol. Despite the overlap of the Gro H1, H1′, and H2 resonances (see Fig. 7), the location of the phosphate group is identified as attached to C1 by virtue of the downfield shift of the glycerol C1 in the $^1H\{^{13}C\}$ HMQC spectrum of the polysaccharide (see Table III).

Summary

The procedure for the purification of the adhesin polysaccharide receptor and its hexasaccharide repeating unit from whole *S. oralis* ATCC 55229 by chemical, enzymatic, and chromatographic techniques has been described. Chemical, chromatographic, and mass spectrometric procedures allow preliminary structural characterization of the hexasaccharide repeating unit and polysaccharide. The structural characterizations of the hexasaccharide and polysaccharide are completed using several 1D and 2D NMR techniques. Identification of the anomeric ^1H and ^{13}C signals of the glycosyl residues permits, by virtue of their chemical shifts and coupling constants ($^3J_{HH}$ and $^1J_{CH}$), the determination of the configurations of the glycosidic linkages. The HMBC connectivities permit the establishment of the hexasaccharide sequence as Rha*p*α(1 → 2)Rha*p*α(1 → 3)Gal*p*α(1 → 3)Gal*p*β(1 → 4)Glc*p*β(1 → 3)Gal. The ^1H NMR chemical shifts of the polysaccharide, as determined by the combination of COSY and TOCSY experiments, and the observed interglycosidic NOESY cross-peaks reveal the structure of the polysaccharide to be

$$\begin{array}{l} \qquad\qquad\qquad\quad \text{Gro}(1 \rightarrow \text{PO}_4 \rightarrow 6)\searrow \\ [\rightarrow 3)\text{Rha}p\alpha(1 \rightarrow 2)\text{Rha}p\alpha(1 \rightarrow 3)\text{Gal}p\alpha(1 \rightarrow 3) \\ \qquad\qquad\qquad\qquad\qquad\quad \text{Gal}p\beta(1 \rightarrow 4)\text{Glc}p\beta(1 \rightarrow 3)\text{Gal}f\beta(1 \rightarrow]_n \end{array}$$

[44] J. G. Verkade and L. D. Quin (eds.), "Phosphorus-31 NMR Spectroscopy in Stereochemical Analysis," p. 33. VCH, New York, 1987.

where the position of the glycerol (Gro) phosphate moiety has been determined by $\{^{1}H, ^{31}P\}$ NMR spectroscopy.

Acknowledgments

Research was supported in part by National Institutes of Health (NIH) Grant P41-RR-05351 from the National Center for Research Resources (to H. v. H.) and by a research award from the Warner-Lambert Company (to F. J. C.). The authors thank Mr. Tim McClure of the Complex Carbohydrate Research Center (CCRC, University of Georgia) for preparation of the figures, Ms. Traci Swanson (CCRC) for editing the manuscript, and Drs. Jack London and Paul Kolenbrander (National Institute of Dental Research, NIH, Bethesda, MD) for critically reading the manuscript and for continued support and encouragement.

[8] Identification of Receptors for Bacterial Lectins by Blotting Techniques

By NATHAN SHARON and ITZHAK OFEK

Introduction

Gel electrophoresis of proteins (or glycoproteins) followed by blotting of the gel and probing the resultant blot with suitable reagents is an extremely useful technique for the detection and characterization of cell surface receptors for antibodies, lectins, growth factors, or intact cells, as well as for other molecules of biological interest. It is also employed to an increasing extent for the investigation of receptors for bacterial adhesins, in particular those that are sugar-specific (i.e., lectins) (Table I). Glycolipids that serve as receptors for bacterial adhesion are identified and characterized after separation by thin-layer chromatography[1,2] (see also [12] in this volume).

A survey of the basic concepts of the protein blotting technique and examples of selected applications can be found in the review by Gershoni.[3] A typical experiment starts with separation of the protein mixture (a cell extract or membrane preparation) into its constituents, most commonly by electrophoresis on a sodium dodecyl sulfate (SDS)–polyacrylamide gel. After electrophoresis, a part of the gel may be stained for protein (by Coomassie Brilliant Blue) and carbohydrate (by the periodic acid–Schiff reagent) to serve as a reference, and the remainder is used for blotting. A

[1] K. A. Karlsson and N. Stromberg, this series, Vol. 138, p. 220.
[2] K. A. Karlsson, *Annu. Rev. Biochem.* **58,** 309 (1989).
[3] J. M. Gershoni, *Methods Biochem. Anal.* **33,** 1 (1988).

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TABLE I
RECEPTORS FOR BACTERIAL ADHESINS IDENTIFIED BY BLOTTING TECHNIQUE

Species	Type of adhesin	Specificity	Source of receptors	Probe	Method of detection[a,b]
Escherichia coli	Type 1	Mannose	Human granulocytes	Bacteria	IM (1)
	Type S	NeuAcα(2,3)	Human erythrocytes	^{125}I-Labeled bacteria	AU (2)
	CFA I and CFA II	NeuAcα(2,3)	Rabbit intestinal cells and HT-29 cells	CFA I and CFA II	IM (3)
	CFA II		HT-29 cells	^{125}I-Labeled CFA II	AU (4)
	K88	Galα1,3 ?	Porcine intestinal cells	Bacteria and K 88 fimbriae[c]	IM (5)
	K88ac		Porcine intestinal cells	^{35}S-Labeled bacteria	AU (6)
				Biotin-K88ac	HS (6)
Fusobacterium nucleatum	—	Galactose	Human saliva	^{3}H- or ^{125} I-labeled bacteria	AU (7)
Pseudomonas aeruginosa	PAK	NeuAc, L-fucose	Human buccal cells	PAK fimbriae[d]	IM (8,9)
	PAK		Mouse corneal cells	PAK fimbriae[d]	IM (10)
Staphylococcus aureus	—	Fibronectin	Human saliva	^{3}H-Labeled bacteria	AU (7)
Streptococcus sanguis	—	NeuAcα(2,3) Gal(β1,4)	Human saliva	^{35}S-Labeled bacteria	AU (11)
Streptococcus suis	—	NeuAcα(2,3) Gal(β1,4) GlcNAc(β1,3)	Human erythrocytes	^{125}I-Labeled bacteria	AU (12)

[a] IM, immunoblot; AU, autoradiography; HS, horseradish peroxidase streptavidin.

[b] *Key to references:* (1) A. Gbarah, C. G. Gahmberg, I. Ofek, U. Jacobi, and N. Sharon, *Infect. Immun.* **59,** 4524, (1991); (2) J. Parkkinen, G. N. Rogers, T. Korhonen, W. Dahr, and J. Finne, *Infect. Immun.* **54,** 37 (1986); (3) C. Wenneras, J. Holmgren, and A.-M. Svennerholm, *FEMS Microbiol. Lett.* **66,** 107 (1990); (4) J. R. Neeser, A. Chambaz, M. Golliard, H. Link-Amster, V. Fryder, and E. Kolodziejczyk, *Infect. Immun.* **57** 3727 (1989); (5) P. T. J. Willemsen, and F. K. deGraaf, *Microb. Pathog.* **12,** 367 (1992); (6) A. K. Ericson, J. A. Willgohns, S. Y. McFarland, D. A. Benfield, and D. H. Francis, *Infect. Immun.* **60,** 983 (1992); (7) A. Prakobphol, P. A. Murray, and S. J. Fisher, *Anal. Biochem.* **164,** 5 (1987); (8) R. T. Irvin, P. Doig, K. K. Lee, P. A. Sastry, W. Paranchych, T. Todd, and R. S. Hodges, *Infect. Immun.* **57,** 3720 (1989); (9) P. Doig, W. Paranchych, P. A. Sastry, and R. T. Irvin, *Can. J. Microbiol.* **35,** 1141 (1989); (10) X. L. Rudner, Z. Zheng, R. S. Berk, R. T. Irvin, and L. D. Hazlett, *Invest. Ophthalmol. Visual Sci.* **33,** 2185 (1992); (11) P. A. Murray, A. Prakobphol, T. Lee, C. I. Hoover, and S. J. Fisher, *Infect. Immun.* **60,** 31 (1992); (12) J. Liukkonen, S. Haataja, K. Tikkanen, S. Kelm, and J. Finne, *J. Biol. Chem.* **267,** 21105 (1992).

[c] Human colonic cell line.

[d] Also probed with a synthetic peptide, PAK 128-144.

piece of membrane filter, usually nitrocellulose, is applied to the gel, and the assembly is then secured in a cassette which is placed into a transfer apparatus, consisting of a plexiglass tank equipped with two electrode arrays capable of producing an electrical field perpendicular to the slab gel. Electrotransfer is performed for several hours, and then the gel and filter are removed.

The blotted gel may be stained to determine the efficiency of protein

elution, while the blot is quenched in a buffer containing an inert protein (e.g., bovine serum albumin) and/or nonionic detergents; the purpose of this step is to block all unoccupied potential binding sites on the membrane so as to prevent nonspecific staining. Quenching is followed by reacting the blot with a suitable probe. There are two major techniques to visualize the binding of the probe to glycoprotein bands on the blot. One approach, which is based on tagging the probe by either radiolabeling or biotinylation prior to its addition to the blot, is widely used (Table I) and is not described here. The other method is based on reacting the blot with the unmodified probe and staining the bound probe with an enzyme-linked immunosorbent assay (ELISA)-based system. For this purpose, the blot is treated with a quench solution containing an antibody against the probe (first antibody), and then reacted with a solution of a second antibody, derivatized with horseradish peroxidase or alkaline phosphatase. The bound second antibody is revealed by incubating the blot in a solution of an appropriate chromogenic substrate.

Alternative means to reveal the bound probe, which are not described here (see Table I), include radiolabeling or biotinylation of either the first or second antibody. The bound labeled antibodies are revealed by autoradiography or enzyme-derivatized avidin (or streptavidin), respectively.

In the following a procedure is described for the identification of the glycoprotein(s) which serves as a receptor on human polymorphonuclear leukocytes (PMNL) for binding *Escherichia coli* via its mannose-specific type 1 fimbrial adhesin. The procedure employs whole bacteria as the probe, but isolated fimbriae may also be used.

Materials

Phenylmethylsulfonyl fluoride, Triton X-100, Tween 20, lactoperoxidase, dextran (average molecular weight 488,000), sodium deoxycholate, bovine serum albumin (BSA), sodium fluoride, ATP, concanavalin A, horseradish peroxidase, and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) are from Sigma (St. Louis, MO); Ficoll-Paque is obtained from Pharmacia (Uppsala, Sweden); methylα-D-mannoside (MeαMan) and D-galactose from Pfanstiehl (Waukegan, IL); nitrocellulose paper, BA-8S, 0.45-μm pore size, from Schleicher & Schuell (Dassel, Germany); *Saccharomyces cerevisiae*, baker's yeast, from Standard Brands (New York, NY); human immunoglobulin G (IgG) from Travenol (Lessines, Belgium); anti-rabbit IgG developed in donkeys and linked to horseradish peroxidase from BDH Chemicals (Poole, UK); anti-rabbit IgG developed in goats and linked to horseradish peroxidase from BioMakor (Rehovot, Israel); anti-rabbit IgG

developed in goats and conjugated to alkaline phosphatase, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolylphosphate from Merck (Darmstadt, Germany); endoglycosidase H (endo H) from Seikagaku Kogy (Tokyo, Japan); and endoglycosylase F-N-glycosidase (endo-F/N) from Boehringer (Mannheim, Germany). All other chemicals are of the highest purity available commercially.

Rabbit polyclonal antibody to *Escherichia coli* strain 346 is prepared by intravenous injection of whole bacteria as described elsewhere.[4] The serum obtained after immunization is stored, and only sera that give a titer of at least 1:600, as determined by slide agglutination of a bacterial suspension (10^9 bacteria/ml), are used in the experiments.

Bacteria

To obtain a bacterial suspension expressing mannose-specific type 1 fimbrial adhesin, a uropathogenic isolate of *E. coli* (strain 346) is grown in brain–heart infusion broth (Difco, Detroit, MI) under static conditions.[4] After 24 hr at 37°, the organisms are harvested, washed in phosphate-buffered saline (PBS) (0.15 *M* NaCl in 50 m*M* phosphate buffer, pH 7.4), and suspended in this buffer to a density of 10^9 bacteria/ml, as determined by counting in a Petroff–Hauser chamber and corresponding to an optical density (OD) of 1.0 at 550 nm. The mannose-binding activity of the bacteria is determined by the yeast aggregation assay,[5] and only suspensions exhibiting a yeast aggregation rate of 5 to 10 U/min are used for further study. To avoid day-to-day variations in the adhesin activity, the bacterial suspensions are routinely stored at −70° in PBS containing 10% (w/v) glycerol in small batches. Before use, the suspensions are thawed, washed in PBS, and suspended to the desired density in PBS. To obtain the nonfimbriated phenotype lacking adhesin activity, the bacteria are grown on agar plates, harvested, and stored as described.[4]

Preparation of Leukocyte Lysates

The PMNL are isolated and purified from fresh human blood obtained from healthy donors by methods described elsewhere.[6,7] Briefly, 25 ml of buffy coat suspension is mixed with an equal volume of 5% dextran in PBS and sedimented at unit gravity for 30 min at 37°. The supernatant is collected, washed twice in PBS, and suspended in 50 ml of the same buffer.

[4] A. Perry, I. Ofek, and F. J. Silverblatt, *Infect. Immun.* **39,** 1334 (1983).
[5] I. Ofek, and E. H. Beachey, *Infect. Immun.* **22,** 247 (1978).
[6] J. S. Gravery, N. E. Cremer, and D. H. Sussdorf, *Anal. Biochem.* **144,** 32 (1977).
[7] R. Hjorth, A. K. Jonsson, and P. Vretblad, *J. Immunol. Methods* **43,** 95 (1981).

The leukocyte-rich suspension is further purified on a discontinuous Percoll gradient.[8] A yield of 10^9 cells is obtained with a purity of 98% of PMNL as determined by Giemsa staining.

Cell lysates of PMNL are prepared as described by De Maio *et al.*[9] A suspension of 2×10^8 cells/ml is lysed for 30 min at 0° in a solution containing 50 m*M* Tris-HCl (pH 8), 110 m*M* NaCl, 5 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, 0.5% (w/v) Nonidet P-40 (NP-40), and the following protease inhibitors: leupeptin (20 μg/ml), pepsatin (5 μg/ml), and phenylmethylsulfonyl fluoride (10 μg/ml). The cell lysates are centrifuged at 100,000 *g* for 1 hr (Beckman 60 Ti rotor) in the cold. The yield of protein is 1.5 mg per 2×10^8 cells.

Procedures

Electrophoretic Separation, Protein Blotting, and Bacterial Overlay

Samples of PMNL lysates containing 10 μg protein are separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (9% acrylamide, 1.5 mm thick) according to Laemmli.[10] Each sample is loaded in at least 5 lanes. Immediately after electrophoresis the separated glycoproteins are transferred to a nitrocellulose sheet using a Trans-Blot electrophoretic transfer cell (Bio-Rad, Richmond, CA) according to the procedures described in the instruction manual. The nitrocellulose sheets are cut into strips (1.8 by 5 cm). The strips are incubated in PBS containing 5% (w/v) BSA at 4° for 16 hr, washed with PBS containing 0.05% (v/v) Tween, and each strip incubated for 16 hr at 4° in 15 ml of PBS containing 10^8 bacteria/ml and BSA (2.5–5%, w/v) with constant gentle shaking. To test the activity of potential inhibitors of bacterial binding, the bacterial suspensions are preincubated with various concentrations of the test inhibitor. After incubation (in the absence or presence of potential inhibitors) the strips are freed of unbound bacteria by washing four times with PBS containing 0.05% Tween 20.

Visualization of Bacteria Bound to Blotted Glycoprotein Bands

The washed strips are incubated for 1 hr at 37° with 15 ml of Tris buffer (pH 7.2) containing BSA (1%, w/v) and rabbit anti-*E. coli* polyclonal antibodies as first antibody. The antibody is used at 1 : 500 dilution for sera, giving an agglutination titer with whole bacteria of 1 : 600; sera with higher agglutinating titers may be used at appropriate dilutions. Excess antibody

[8] A. Boyum, *J. Clin. Lab. Invest.* **21**(Suppl 97), 77 (1968).
[9] A. De Maio, H. Lis, J. M. Gershoni, and N. Sharon, *FEBS Lett.* **194,** 28 (1986).
[10] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).

is removed by washing the strips four times with Tris buffer containing 0.05% (v/v) Tween 20. The strips are then incubated with anti-rabbit IgG antibody conjugated with alkaline phosphatase diluted 1 : 2000 in Tris buffer containing 1% BSA (second antibody). After three washes to remove unbound second antibody, the strips are incubated with substrate solution (0.33 mg of nitro blue tetrazolium and 0.16 mg of 5-bromo-4-chloro-3-indolylphosphate per milliliter of a buffer composed of 10 m*M* Tris, 100 m*M* NaCl, and 5 m*M* $MgCl_2$, pH 9.5). Bands that are stained blue after 15–30 min represent bound bacteria. Negative controls consisting of strips incubated with buffer solution devoid of either the first or the second antibody, or containing normal rabbit serum instead of first antibody, must be included. A parallel set of strips is subjected to staining with Coomassie blue in order to visualize total protein bands transferred to the nitrocellulose.[11] Certain glycoproteins, notably glycophorin, do not stain well with Coomassie blue; to detect such glycoproteins a parallel set of strips is overlaid with ^{125}I-labeled wheat germ agglutinin (or concanavalin A) after incubation in 2% (w/v) polyvinylpyrrolidone as described elsewhere.[12]

Role of Carbohydrates in Binding of Bacteria

To ascertain that the binding of bacteria results from the interaction of oligosaccharide side chains of glycoproteins with the mannose-specific type 1 fimbrial lectin, the nitrocellulose strips may be pretreated with various carbohydrate-modifying reagents before overlay with the bacterial suspension. The treatments include (a) periodate oxidation of vicinal hydroxyl groups of carbohydrate[13,14] by incubating the strips with 10 m*M* sodium metaperiodate in PBS at 37° for 1 hr, (b) desialylation by incubating the strips with *Vibrio cholerae* sialidase (neuraminidase, 50 mU/ml) in 50 m*M* sodium acetate buffer (pH 5.5) containing 5 m*M* $CaCl_2$ and 0.1% (w/v) BSA at 37° for 16 hr,[14] and (c) deglycosylation by incubating the strips with either endo H (0.015 U/ml) in 0.15 *M* citrate buffer (pH 5) or endo F/N (0.075 U/ml) in PBS at 37° for 20 hr. All treated strips are washed with PBS and then overlaid with bacteria as described above.

Comments

Blotting should preferably be done immediately after electrophoresis. Generally, it should proceed for at least 2 hr, but more time may be required,

[11] W. N. Burnette, *Anal. Biochem.* **112,** 195 (1981).
[12] J. R. Bartles and A. L. Hubbard, *Anal. Biochem.* **140,** 284 (1984).
[13] C. Giampapa, S. N. Abraham, T. M. Chiang, and E. H. Beachey, *J. Biol. Chem.* **263,** 5362 (1988).
[14] I. Ofek, D. Mirelman, and N. Sharon, *Nature* (*London*) **265,** 623 (1977).

depending on the buffer conditions, type of gel used, the intensity of the electrical field, and the molecular weight of the protein to be eluted. Some protocols prescribe 22 hr of blotting as a routine.[15] For quenching, proteins other than bovine serum albumin may be used, such as nonfat dried milk powder (5% w/v) or bovine hemoglobin (1% w/v). Blots may be stored for prolonged times prior to probing, preferably at −20°.

Although the method is described for PMNL and *E. coli* expressing type 1 fimbriae, the same procedure can be applied to other bacteria and animal cells. Owing to the phenomenon of phase variation in the production of adhesins, however, the bacteria must be grown under conditions that are optimal for phenotypic expression of the adhesin. Also, for application to the nitrocellulose strips, the bacteria must be suspended in buffer that is optimal for adhesin activity. The latter can be determined by testing the ability of the bacteria to adhere to suitable target cells.

Autoradiography is the most sensitive means to detect the receptors on the blots.[3] Moreover, exposures can be repeated until optimal signals are obtained. However, the resolution of enzyme-linked assays appears to be superior to that of autoradiography.

Whenever possible, the specificity of the binding should be ascertained. In the case of the bacterial surface lectins, this is readily done by establishing whether binding of the probe to the blotted glycoproteins is inhibited by the mono- or oligosaccharides for which the bacteria are specific. Another way is to test the effect of treatment of blots *in situ* with carbohydrate-modifying reagents, such as glycosidases or periodate. Such experiments provide also information on the structure of the carbohydrate moieties of the receptors. It is important to emphasize the advantage of *in situ* treatment, because removal of sugar groups from glycoproteins alters their electrophoretic mobilities. For example, desialylated glycoproteins migrate at different rates than the parental sialylated ones. Treating the blot after electrophoresis permits comparison of the same bands.[3]

It should be noted that identification of glycoproteins isolated from animal cell membranes that bind whole bacteria on blots does not provide absolute proof that the glycoproteins act as receptors for the bacteria in intact cells. To clarify this, it is advisable to prepare antibodies against the bands that bound the bacteria and to test if they inhibit the binding of the bacteria to appropriate target cells. As a control, antibodies prepared against bands that do not bind the bacteria should also be tested. Antibodies are prepared as follows. The bands are excised from the gel, cut into small pieces, suspended in 0.5 ml of Freund's complete adjuvant, and injected subcutaneously into rabbits at two sites, 0.2 ml for each site. In preliminary

[15] J. Parkkinen, G. N. Rogers, T. Korhonen, W. Dahr, and J. Finne, *Infect. Immun.* **54,** 37 (1986).

studies employing this technique it was shown that only antibodies against glycoproteins migrating on SDS–PAGE similar to CD11 inhibited binding of the type 1 fimbriated bacteria to PMNL, whereas antibodies against other membrane glycoproteins bound to PMNL but did not inhibit bacterial binding.[16]

[16] I. Ofek, U. Jakobi, A. Perry, and N. Sharon, *Isr. J. Med. Sci.* **28,** 77 (1992).

[9] Purification and Characterization of Galactose- and *N*-Acetylgalactosamine-Specific Adhesin Lectin of *Entamoeba histolytica*

By WILLIAM A. PETRI, JR., and RONALD L. SCHNAAR

Introduction

Parasite recognition of glycoconjugates plays an important role in the pathogenesis of amebiasis. Amebiasis is a common parasitic infection that results annually in 40 to 50 million cases of amebic colitis and liver abscess and 40,000 to 110,000 deaths worldwide. An initial step in parasite colonization of the large bowel is adhesion to colonic mucins. Trophozoites adhere to human colonic mucins and colonic epithelial cells *in vitro* via a surface lectin that binds to terminal galactose (Gal) and *N*-acetylgalactosamine (GalNAc).[1,2] Contact-dependent killing of host cells also requires the activity of the Gal/GalNAc lectin: *Entamoeba histolytica* kills human macrophages, monocytes, neutrophils, and T lymphocytes in a contact-dependent process. Mere apposition of amebic and target cell plasma membranes, as can be achieved by centrifuging target cells and amebas together into a pellet, will not lead to cytolysis if the amebic lectin is inhibited with galactose, indicating that the lectin either signals the initiation of cytolysis or directly participates in the cytolytic event.

The Gal/GalNAc lectin is apparently also involved in the evasion of lysis by serum on invasion of the colon that is critical for survival of the extracellular trophozoite. *Entamoeba histolytica* trophozoites are resistant to the complement C5b–9 complexes which form the membrane attack complex. Monoclonal antibodies (MAb) directed against amino acids 895–

[1] J. I. Ravdin and R. L. Guerrant, *J. Clin. Invest.* **68,** 1305 (1981).
[2] K. Chadee, W. A. Petri, Jr., D. J. Innes, and J. I. Ravdin, *J. Clin. Invest.* **80,** 1245 (1987).

studies employing this technique it was shown that only antibodies against glycoproteins migrating on SDS–PAGE similar to CD11 inhibited binding of the type 1 fimbriated bacteria to PMNL, whereas antibodies against other membrane glycoproteins bound to PMNL but did not inhibit bacterial binding.[16]

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1082 of the cysteine-rich domain (epitopes 6 and 7) of the lectin heavy subunit greatly increase the sensitivity of *E. histolytica* to lysis by human sera and by purified human C5b–9. The lectin binds to purified human C8 and C9, and the binding is inhibited by antilectin MAb which block serum resistance. The purified lectin confers C5b–9 resistance when reconstituted into C5b–9-sensitive amebas, a direct demonstration of its C5b–9 inhibitory activity.[3]

The amebic lectin responsible for these activities is a 260-KDa heterodimeric glycoprotein consisting of heavy (170 kDa) and light (35 and 31 kDa) subunits linked by disulfide bonds.[4] The 170-kDa subunit is encoded by a gene family of which three members (89–95% identical) from *E. histolytica* strain HM1 : IMSS have been sequenced.[5] The 170-kDa subunit sequences contain a carboxyl-terminal putative cytoplasmic and transmembrane domain followed by an extensive extracellular cysteine-rich domain. The cysteine-rich domain is recognized by adhesion-inhibitory antilectin monoclonal antibodies.[6]

The light subunit has been resolved into 35- and 31-kDa isoforms by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The 35- and 31-kDa isoforms have nearly identical amino acid compositions and CNBr-fragmented peptide patterns. Two genes have been identified that encode the 35-kDa isoform, both of which encode proteins (80.6% identical) with potential glycosylphosphatidylinositol (GPI) anchor addition sequences. The 31-kDa isoform contains a GPI anchor, as demonstrated by the release of phosphatidylinositol with nitrous acid.[4]

The locations of the carbohydrate and complement recognition sites of the lectin are unknown, despite all the structural work on the protein completed to date. The amino acid sequences of the 170- and 35-kDa lectin subunits do not share sequence identity with the conserved carbohydrate-binding regions of the eukaryotic C or S type lectins, the *Escherichia coli* Gal-Gal binding lectin, or plant lectins sequenced to date. Direct assignment of galactose-binding activity to either the 170- or 35-kDa subunits of the native lectin has not been possible, as the reduction in disulfide bonds required to separate the subunits results in loss of galactose-binding activity (W. A. Petri, Jr., unpublished results). However, the ability of MAb directed

[3] L. L. Braga, H. Ninomiya, J. J. McCoy, S. Eacker, T. Wiedmer, C. Pham, S. Wood, P. J. Sims, and W. A. Petri, Jr., *J. Clin. Invest.* **90,** 1131 (1992).

[4] J. J. McCoy, B. J. Mann, T. S. Vedvick, Y. Pak, D. B. Heimark, and W. A. Petri, Jr., *J. Biol. Chem.* **268,** 24223 (1993).

[5] J. E. Purdy, B. J. Mann, E. C. Shugart, and W. A. Petri, Jr., *Mol. Biochem. Parasitol.* **62,** 53 (1993).

[6] B. J. Mann, C. Y. Chung, J. M. Dodson, L. S. Ashley, L. L. Braga, and T. L. Snodgrass, *Infect. Immun.* **61,** 1772 (1993).

against the cysteine-rich domain of the 170-kDa subunit (amino acids 596–1082) to enhance and inhibit the galactose-binding activity of the protein suggests that the 170-kDa subunit contains the carbohydrate-binding region.[6]

Hemagglutination–Inhibition Assay to Test Carbohydrate Specificity of Lectin

Trophozoite Cultures

The starting material is amebic trophozoites, strain HM1 : IMSS, grown in axenic culture at 37° in TYI-S33 medium with 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate (Pfizer, New York, NY) in sealed plastic tissue culture flasks.[7] Amebas are harvested in the stationary phase of growth, at 72 hr after subculture, from four 250-ml tissue culture flasks. Amebas are pelleted at 150 *g* for 5 min at 4° and washed once by gentle suspension in 75 m*M* Tris (Sigma, St. Louis MO)–65 m*M* NaCl, pH 7.2 (wash buffer), and repelleting at 150 *g* for 5 min.

Preparation of Amebic Membranes

Trophozoites after washing (above) are lysed osmotically at 37° for 5 min in 10 m*M* sodium phosphate buffer, pH 8.0, containing 0.1 *M* 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, Calbiochem, San Diego, CA), 2 m*M* phenylmethylsulfonyl fluoride (PMSF, Sigma), 5 m*M* EDTA, and 2 m*M* *p*-hydroxymercuribenzoate (PHMB, Sigma). The lysed trophozoites are then placed on ice and sonicated (three to five 10-sec bursts at maximum setting with a microtip sonicator) (Sonifier Cell Disruptor, Model 140D, Ultrasonics–Heat Systems, Plainview, NY). The membranes are then pelleted at 50,000 *g* for 1 hr at 4° and the supernatant discarded. The membrane pellet is suspended in fresh osmotic lysis buffer, and the membranes are pelleted at 100,000 *g* for 1 hr at 4°. The membrane pellet is then resuspended for use in the hemagglutination assay.

Hemagglutination Assay with Amebic Membranes

Human type A blood is drawn into heparinized tubes. Whole blood is diluted 1 : 1 with Dulbecco's phosphate-buffered saline (PBS), and 4 ml of the suspension is pipetted over 3 ml of Ficoll-Paque (Pharmacia LKB,

[7] L. S. Diamond, D. R. Harlow, and C. C. Cunnick, *Trans. R. Soc. Trop. Med. Hyg.* **72,** 431 (1978).

Piscataway, NJ) in a centrifuge tube. Erythrocytes are collected by centrifugation at 400 *g* for 25 min at 4°, then are washed twice in 10 ml PBS by centrifugation at 400 *g* for 5 min at 4°. The cells are counted and diluted to 2.4×10^9 cells/ml in PBS for storage at 0° for up to 30 days. For use in the hemagglutination assay, a suspension of 2×10^7 cells/ml in PBS containing 2 mg/ml bovine serum albumin is prepared.

Hemagglutination is performed in V-bottom 96-well polystyrene plates (Costar, Cambridge, MA, Seroclusters, Cat. No. 3897). Test samples containing the desired concentration of membranes (~4 μg protein) in 100 μl of PBS are placed in replicate microwells. Hemagglutination is initiated by adding 100 μl of the above erythrocyte suspension. Plates are incubated at 4° for 6–16 hr (there is no change in appearance after the erythrocytes settle). Hemagglutination is indicated by a uniform distribution of erythrocytes in the well, rather than a point concentration of cells at the well bottom (Fig. 1).

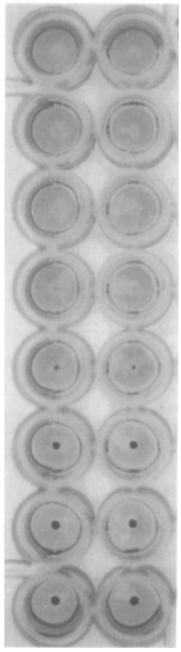

FIG. 1. Hemagglutination of human type A erythrocytes by isolated *E. histolytica* membranes. Hemagglutination was performed as described in the text. The top pair of duplicate wells contained 40 μg *E. histolytica* membrane protein, whereas each subsequent pair of wells contained half the concentration of the preceding set. Hemagglutination, indicated by a uniform erythrocyte distribution, is supported by membranes in the top five pairs of wells (≥2.5 μg *E. histolytica* membrane protein).

Hemagglutination is a threshold phenomenon, suggesting cooperative, multivalent interactions between membranes and erythrocytes. A membrane concentration–hemagglutination curve is determined for each *E. histolytica* membrane preparation, and a membrane concentration approximately 2-fold above the threshold is chosen for subsequent glycoconjugate inhibition experiments.

Purification of Galactose- and *N*-Acetylgalactosamine-Specific Lectin

Solubilization of Trophozoites

The amebic trophozoites, after pelleting from the wash buffer, are solubilized in 10 ml of 50 m*M* Tris, 150 m*M* NaCl, containing 0.5% (v/v) Nonidet P-40 (Sigma), 5 m*M* EDTA, and freshly added 0.1 *M* AEBSF, 2 m*M* PMSF, and 2 m*M* PHMB, pH 8.3. The solubilized amebas are microcentrifuged at 10,000 *g* for 10 min and the supernatant applied at 4° to an affinity column.

Buffers

Trophozoite washing buffer: 75 m*M* Tris (Sigma)–65 m*M* NaCl, pH 7.2

Osmotic lysis buffer: 10 m*M* sodium phosphate buffer, pH 8.0, containing 0.1 *M* AEBSF (Calbiochem), 2 m*M* PMSF (Sigma), 5 m*M* EDTA, and 2 m*M* PHMB (Sigma)

Trophozoite solubilization buffer: 50 m*M* Tris, 150 m*M* NaCl, 0.5% Nonidet P-40 (Sigma), 5 m*M* EDTA, 0.1 *M* AEBSF, 2 m*M* PMSF, and 2 m*M* PHMB, pH 8.3

Galactose affinity column buffer: 50 m*M* Tris, 200 m*M* NaCl, 10 m*M* $CaCl_2$, pH 7.35

Monoclonal antibody column elution buffer: 0.2 *N* acetic acid, pH 3.0

Monoclonal antibody column elution neutralization buffer: 1.5 *M* Tris, pH 8.8

Alternative A: Lectin Purification by Galactose Affinity Chromatography. Glutaraldehyde-activated silica beads (Boehringer-Mannheim, Indianapolis, IN) (1 g) are derivatized overnight at 4° with 5 ml of 20 mg/ml *p*-aminophenyl-1-thio-β-D-galactopyranoside (Sigma) in 0.1 *M* bicarbonate buffer, pH 8.0. The silica beads are washed in 50 m*M* Tris, 200 m*M* NaCl, 10 m*M* $CaCl_2$, pH 7.35 (column buffer), to remove unbound galactose before the application of solubilized trophozoites with a peristaltic pump overnight at 4°. The column is then extensively washed (50–100 ml) with solubilization buffer, first with and then without Nonidet P-40. The bound amebic lectin is eluted with 0.2 *N* acetic acid, pH 2.5, into test tubes con-

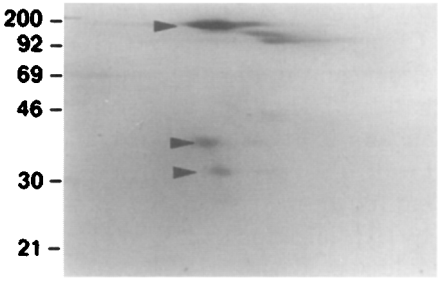

FIG. 2. Two-dimensional SDS–PAGE of affinity-purified lectin. The purified lectin was electrophoresed in a 6% tube gel under nonreducing conditions in the horizontal dimension and in a 10% slab gel under reducing conditions in the vertical dimension. Staining was performed with Coomassie blue. Arrowheads indicate the 170-kDa heavy subunit and the 31- and 35-kDa isoforms of the light subunit. Molecular mass markers (in kDa) are indicated on the left-hand side for the second dimension. (Reprinted with permission from McCoy *et al.*[4])

taining 1.5 *M* Tris, pH 8.3, to neutralize the acid rapidly.[8] The yield of lectin is lower with this method than with the monoclonal antibody affinity purification scheme below.

Alternative B: Lectin Purification by Monoclonal Antibody Affinity Chromatography. Two milligrams each of protein A-purified antilectin monoclonal antibodies (MAb) H85, 7F4, 5B8, 3F4, and 6D2 are immobilized on 1–2 ml of Affi-Gel 10 (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. The solubilized amebas are recirculated through the MAb column with a peristaltic pump overnight at 4°. The column is then extensively washed (50–100 ml) with solubilization buffer, first with and then without Nonidet P-40. The bound amebic lectin is eluted with 0.2 *N* acetic acid, pH 3.0, into test tubes containing 1.5 *M* Tris, pH 8.8, to neutralize the acid rapidly.[9]

Assay of Yield and Purity from Monoclonal Antibody Affinity Column. A monoclonal antibody-based radioimmunoassay is used to follow the yield of purification. The supernatant of solubilized amebas applied to the MAb affinity column contains approximately 2 μg of lectin/mg protein, with a typical yield of 300 μg of purified lectin from 200 mg of solubilized amebic proteins, representing a 400- to 500-fold purification. The purity of the MAb affinity-purified lectin is assessed by laser densitometry of Coomassie blue-stained heavy and light subunits by SDS–PAGE, with an average purity of 80% (Fig. 2).

[8] W. A. Petri, Jr., R. D. Smith, P. H. Schlesinger, C. F. Murphy, and J. I. Ravdin, *J. Clin. Invest.* **80,** 1238 (1987).

[9] W. A. Petri, Jr., M. D. Chapman, T. Snodgrass, B. J. Mann, J. Broman, and J. I. Ravdin, *J. Biol. Chem.* **264,** 3007 (1989).

Assay of Galactose-Binding Activity of Purified Lectin. The binding of the lectin to Lec2 Chinese hamster ovary (CHO) cells is measured. The Lec2 CHO cells are deficient in the attachment of sialic acid to complex N-linked oligosaccharides.[10] The Lec2 CHO cells are grown in monolayer culture in a CO_2 incubator in MEM alpha medium (GIBCO, Grand Island, NY) supplemented with 10% (w/v) fetal bovine serum (GIBCO) with 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate (Pfizer). The CHO cells are harvested by trypsinization (0.25% w/v) and suspended in medium M199 (GIBCO) supplemented with 5.7 m*M* cysteine, 25 m*M* HEPES, 0.5% bovine serum albumin (all from Sigma), and 1% fetal bovine serum.

The Lec2 CHO cells (0.3 ml of a 2.5×10^6 cells/ml suspension) and the MAb affinity-purified lectin (20 μg) are incubated together in Dulbecco's phosphate-buffered saline (DPBS, Mediatech, Washington, DC) in the presence or absence of 110 m*M* galactose for 1 hr on ice. The unbound lectin is removed from the CHO cells by 3–5 cycles of centrifugation of the CHO cells (at 150 *g* for 5 min) followed by suspending the CHO cells in DPBS. The CHO cells are then resuspended in DPBS in a final volume of 0.3 ml. Bound lectin is determined by adding 5×10^6 counts/min (cpm)/ml of ^{125}I-labeled antilectin MAb 7F4 to the CHO cells and separating bound from free ^{125}I-labeled MAb 7F4 by pelleting the CHO cells through a 4:1 silicone oil (Accumetric, Elizabeth, KY)/mineral oil (Sigma) solution. The tips of the tubes containing the cellular pellet are cut off with a razor blade, and the ^{125}I radioactivity is determined in a gamma counter (Beckman Instruments, Fullerton CA). Specific binding (determined by subtracting the background binding in the presence of 110 m*M* galactose) averages 90% under these conditions, with approximately 1 ng of lectin bound per 10^3 CHO cells.[11]

[10] P. Stanley, *Annu. Rev. Genet.* **18,** 525 (1984).

[11] W. A. Petri, Jr., T. L. Snodgrass, T. F. H. G. Jackson, V. Gathiram, A. E. Simjee, K. Chadee, and M. D. Chapman, *J. Immunol.* **144,** 4803 (1990).

[10] Specificity Mapping of Bacterial Lectins by Inhibition of Hemagglutination Using Deoxy and Deoxyfluoro Analogs of Receptor-Active Saccharides

By GÖRAN MAGNUSSON, SCOTT J. HULTGREN, and JAN KIHLBERG

Introduction

Many bacteria use their surface lectins for attachment to saccharide moieties on the surface of eukaryote cells.[1] Such adhesion is often the first step in an infectious process. Detailed knowledge of the lectin–saccharide interaction would make it possible to interfere with bacterial binding and thereby provide an opportunity to develop antiadhesive agents for treating or preventing infections. Information on the molecular details of the interactions can be obtained by inhibition of lectin–saccharide binding using either whole bacteria or the purified lectins. Hydrogen bonds between saccharide and lectin seem to be the most important type of bond in the lectin–saccharide complex. Consequently, the use of a complete set of monodeoxysaccharide analogs as inhibitors of hemagglutination reveals which hydroxyl groups in the natural saccharide are necessary for complex formation. In addition, deoxyfluorosaccharide analogs reveal the directionality of the hydrogen bond, because the fluoro group is a hydrogen bond acceptor but not a donor. The use of deoxysaccharides and deoxyfluorosaccharides to map the combining site of plant lectins and antibodies is well-known,[2–4] unlike the case of bacterial lectins.[5]

In addition to deoxysaccharides and deoxyfluorosaccharides, derivatives carrying other functional groups are also valuable for the detailed mapping of receptor sites. For example, substitution of hydroxyl groups for alkyl and ether groups may reveal steric requirements for a good fit between saccharide and protein.[5,6]

It is important that different saccharide analogs used for receptor mapping have very similar conformations, ensuring that the only difference

[1] D. Mirelman, (ed.), "Microbial Lectins and Agglutinins." Wiley, New York, 1986.
[2] U. Spohr, E. Paszkiewitz-Hnatiew, N. Morishima, and R. U. Lemieux, *Can. J. Chem.* **70,** 254 (1992), and references therein.
[3] C. P. J. Glaudemans, *Chem. Rev.* **91,** 25 (1991).
[4] D. R. Bundle, *Pure Appl. Chem.* **61,** 1171 (1989).
[5] J. Kihlberg, S. J. Hultgren, S. Normark, and G. Magnusson, *J. Am. Chem. Soc.* **111,** 6364 (1989).
[6] P. V. Nikrad, H. Beierbeck, and R. U. Lemieux, *Can. J. Chem.* **70,** 241 (1992).

between the saccharide structures is the presence or absence of specific hydroxyl groups. Conformational analyses based on nuclear magnetic resonance (NMR) spectroscopy and computer calculations of galabioside derivatives and larger galabiose-containing saccharides revealed that the conformation of the galabiose moiety was practically independent of structural modifications.[7,8]

Normal methods of glycoside synthesis[9] can be used for the preparation of deoxysaccharides. A deoxy function makes the saccharide more sensitive to hydrolysis, especially at low pH. Kept dry and cold, deoxysaccharides normally have a shelf-life of many years. This means that it is possible to create large and long-lasting banks of deoxysaccharide compounds despite the fact that the synthetic work is quite time-consuming (the syntheses of compounds **1–14** in Fig. 1 required about 3 years). In contrast, many deoxyfluorosaccharides decompose within 1 year even at −20°. The organic synthesis of deoxysaccharides and deoxyfluorosaccharides has been reviewed as part of a chapter on the synthesis of neoglycoconjugates.[10]

Hemagglutination is based on aggregation of erythrocytes. Specific bacterial adhesins presented in fibers called pili or fimbriae on the bacterial surface bind to saccharides on the erythrocyte surface, causing hemagglutination. Inhibition of aggregate formation causes the erythrocytes to sediment, thereby enabling the identification of efficient inhibitors. Plastic particles coated with an active saccharide can be used as an alternative to erythrocytes.[11] Expression of saccharides and adhesins on the surface of erythrocytes and bacteria, respectively, may vary, and therefore the absolute values of inhibition may vary between experiments. Consequently, the fine dissection of binding epitopes, using deoxysaccharides and deoxyfluorosaccharides, is only possible if the complete series of saccharides is employed in experiments using a single batch of erythrocytes and bacteria.

An illustration of the use of deoxysaccharides and deoxyfluorosaccharides in epitope mapping is our investigation of the galabiose (Galα1→4Galβ) binding specificity of uropathogenic *Escherichia coli* bacteria.[5] The specific details for preparing erythrocytes and bacteria, as well as the hemagglutination studies, are described below.

[7] K. Bock, T. Frejd, J. Kihlberg, and G. Magnusson, *Carbohydr. Res.* **176,** 253 (1988).
[8] G. Grönberg, U. Nilsson, K. Bock, and G. Magnusson, *Carbohydr. Res.* **257,** 35 (1994).
[9] K. Toshima and K. Tatsuta, *Chem. Rev.* **93,** 1503 (1993).
[10] G. Magnusson, A. Y. Chernyak, J. Kihlberg, and L. O. Kononov, *in* "Neoglycoconjugates: Preparation and Application" (Y. C. Lee and R. T. Lee, eds.), p. 53 Academic Press, San Diego (1994).
[11] P. de Man, B. Cedergren, S. Enerbäck, A.-C. Larsson, H. Leffler, A.-L. Lundell, B. Nilsson, and C. Svanborg-Edén, *J. Clin. Microbiol.* **25,** 401 (1987).

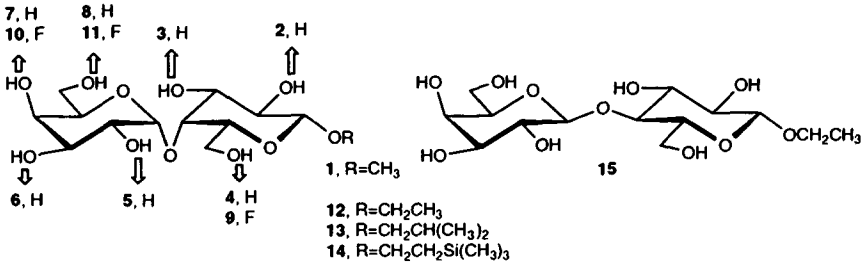

FIG. 1. Galabioside analogs **1–14** and ethyl lactoside **15** used as hemagglutination inhibitors. Arrows indicate which hydroxyl group has been replaced by a hydrogen or fluorine atom in compounds **1–14**.

Synthesis of Deoxysaccharides and Deoxyfluorosaccharides

The synthesis of compounds **1–14** (Fig. 1) has been previously described: **1**,[12] **2**,[13] **3**,[14] **4**,[13] **5**,[15] **6**,[15] **7**,[15] **8**,[15] **9**,[13] **10**,[15] **11**,[15] **12**,[16] **13**,[5] and **14**.[17]

Preparation of Erythrocytes

Erythrocytes (obtained from humans that are blood group A^+) are introduced into ACD Solution B Vacutainer tubes (Becton Dickinson, Fairview Lakes, NJ) in order to prevent clotting. The surface of these erythrocytes presents the P antigen (globoside) which contains the Galα(1-4)Gal disaccharide moiety recognized by P piliated bacteria. The erythrocytes are then washed and suspended in phosphate-buffered saline (PBS): 120 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* phosphate buffer salts, pH 7.4 (Sigma, St. Louis, MO).

Pilus Architecture and Preparation of Bacterial Strains HB101/pPAP5

The *E. coli* strain HB101[18] is not piliated and does not bind to human erythrocytes or other galabiose-containing surfaces. pPAP5 contains the

[12] P. J. Garegg and S. Oscarsson, *Carbohydr. Res.* **137,** 270 (1985).
[13] J. Kihlberg, T. Frejd, K. Jansson, A. Sundin, and G. Magnusson, *Carbohydr. Res.* **176,** 271 (1988).
[14] J. Kihlberg, T. Frejd, K. Jansson, and G. Magnusson, *Carbohydr. Res.* **152,** 113 (1986).
[15] J. Kihlberg, T. Frejd, K. Jansson, S. Kitzing, and G. Magnusson, *Carbohydr. Res.* **185,** 171 (1988).
[16] J. Dahmén, T. Frejd, G. Grönberg, T. Lave, G. Magnusson, and G. Noori, *Carbohydr. Res.* **118,** 292 (1983).
[17] K. Jansson, S. Ahlfors, T. Frejd, J. Kihlberg, G. Magnusson, J. Dahmén, G. Noori, and K. Stenvall, *J. Org. Chem.* **53,** 5629 (1988).
[18] H. W. Boyer and D. Roulland-Dussoix, *J. Mol. Biol.* **41,** 459 (1969).

entire *pap* gene cluster cloned from the human urinary tract *E. coli* isolate J96.[19] The DNA sequence of the entire gene cluster has been studied by extensive mutant analyses (Fig. 2).[20] Transformation of pPAP5 into the nonpiliated laboratory *E. coli* strain HB101 confers on the resulting strain HB101/pPAP5 the ability to produce P pili.[21] P pili are composite heteropolymeric fibers consisting of flexible adhesive fibrillae joined end to end to pilus rods.[22] The pilus rod is composed of repeating PapA protein subunits arranged in a right-handed helical cylinder. Tip fibrillae, which extend from the distal ends of each pilus rod, are composed mostly of repeating subunits of PapE arranged in an open helical conformation. The PapG adhesin which binds galabiose is localized to the distal ends of tip fibrillae that seemingly are flexible; by electron microscopy they often appear to be bent in various orientations. The distal location of PapG in the tip fibrillum probably maximizes its ability to recognize glycolipid receptors on eukaryotic cells. The composite architecture of the P pilus fiber reveals the strategy used by pyelonephritic *E. coli* to present the PapG adhesin to eukaryotic receptors. The rigid PapA rod distances the adhesin from lipopolysaccharide and other potentially interfering components at the bacterial cell surface, whereas the flexible fibrillum allows PapG steric freedom to recognize and bind to the digalactoside moiety on the uroepithelium (see model in Fig. 2).

Expression of P pili is induced by growing the HB101/pPAP5 bacteria overnight at 37° on a solid medium such as CFA (20 g/liter casamino acids, 3 g/liter yeast extract, 1 m*M* $MgSO_4$, 0.1 m*M* $MnCl_2$, and 20 g/liter agar). To activate the transcription of the *pap* operon, it is sometimes necessary to passage the cells several times. The hemagglutination titer is determined for the agar-grown *E. coli* (HB101/pPAP5) bacteria as described below.

Hemagglutination Reactions

Procedures for hemagglutination (HA) reactions are adapted from Ref. 23.

Bacterial Suspension

1. Suspend bacteria (fresh, overnight culture) in PBS to an A_{540} of 1.0.
2. Spin down 1 ml of the cell solution in a microcentrifuge for 1 min.
3. Suspend the cell pellet in 100 μl PBS.

[19] R. A. Hull, R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow, *Infect. Immun.* **33,** 933 (1981).
[20] S. J. Hultgren, S. Abraham, M. Caparon, J. St. Geme, P. Falk, and S. Normark, *Cell,* (Cambridge, Mass.) **73,** 887 (1993).
[21] F. P. Lindberg, B. Lund, and S. Normark, *EMBO J.* **3,** 1167 (1984).
[22] M. J. Kuehn, J. Heuser, S. Normark, and S. J. Hultgren, *Nature* (London) **356,** 252 (1992).
[23] S. J. Hultgren, W. R. Schwan, A. S. Schaeffer, and J. L. Duncan, *Infect. Immun.* **54,** 613 (1986).

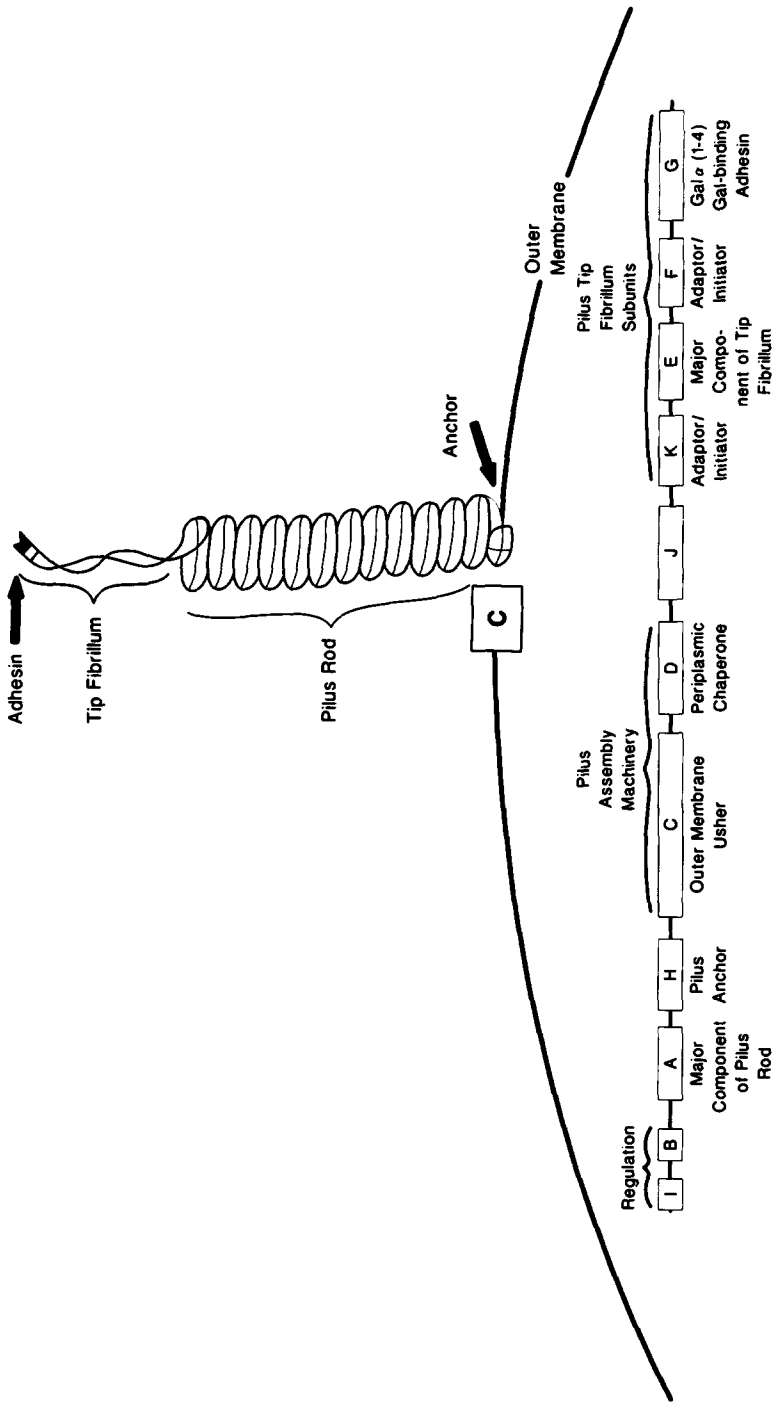

FIG. 2. Model of P pilus architecture and the *pap* gene cluster. The *pap* gene cluster and the function of each of the gene products are indicated. The architecture of the pilus reveals a strategy used by pyelonephritic *E. coli* to present an adhesin in an accessible configuration that allows it to recognize Galα(1-4)Gal-containing saccharides.

Red Blood Cell Suspension

1. Wash blood in PBS by repeated mixing and centrifugation until the supernatant is clear (no lysed cells).
2. Suspend red blood cells (RBC) in PBS to an A_{640} of 1.9.

Hemagglutination Assay and Determination of Titer

1. In a microtiter plate with pointed well bottoms (Costar, Cambridge, MA), dispense 25 μl PBS/well.
2. Add 25 μl bacterial suspension to the first well. Mix. Make serial dilutions by taking 25 μl from the first well, transferring to the next well, mixing, taking 25 μl from the second well, and so on.
3. Add 25 μl RBC suspension to all wells.
4. Mix suspension gently by tapping the side of the plate. Cover with Parafilm and place at 4°.
5. Read the HA titer as the maximum dilution of bacteria which yields 50% hemagglutination. In the absence of hemagglutination the RBC are able to settle into a pellet at the bottom of the well. In the presence of hemagglutination the red blood cells and bacteria form a diffuse sheet of clumped cells covering the entire well.

Inhibition of Hemagglutination

The bacterial suspension is diluted to an HA titer of 64 (i.e., to a bacterial density which gives 50% hemagglutination after 64-fold dilution). The saccharide inhibitors **1–15** are dissolved in PBS buffer to give 100 m*M* solutions. Each inhibitor solution (25 μl) is serially diluted in microtiter plate wells containing 25 μl of PBS each. The bacterial suspension (25 μl; HA titer of 64) is added to each well, and the plates are incubated for around 15 min at room temperature. The P erythrocytes (1% suspension, 25 μl) are added, and the plates are shaken gently, then incubated at 4° for 16 hr. The end point (IC_{50}) is defined as the inhibitor concentration that gives 50% inhibition (seen as an incomplete formation of an erythrocyte pellet) of hemagglutination. The IC_{50} values for the different inhibitor saccharides, shown in Table I, are mean values from two or three consecutive determinations.

It is important to realize that in experiments such as these, where rather small differences in inhibitory power need to be measured, all experiments with a series of deoxysaccharide inhibitors must be performed with the same batch of erythrocyte and bacterial suspensions. As shown in Table II, experiments performed with different batches can give rather different

TABLE I
INHIBITION OF AGGLUTINATION OF HUMAN P ERYTHROCYTES BY *Escherichia coli* (HB101/pPAP5) USING COMPOUNDS **1–15**

Compound	IC_{50}[a] (m*M*)	Inhibitory power[b] (%)	$\Delta\Delta G$[c] (kJ/mol)
1	0.18	100	0.0
2	0.30	61	1.1
3	0.98	19	3.9
4	4.2	4.4	7.2
5	2.3	7.6	5.9
6	6.4	2.7	8.3
7	10	1.9	9.1
8	3.6	5.4	6.7
9	9.2	1.9	9.1
10	3.3	6.2	6.4
11	0.33	50	1.6
12	0.13	140	−0.8
13	0.082	200	−1.6
14	0.046	400	−3.2
15	>25	<1.0	>11

[a] The 95% confidence limits are as follows: [0.930 × IC_{50}; 1.07 × IC_{50}] m*M*.
[b] The 0.95% confidence limits are [0.904 × inhibitory power; 1.11 × inhibitory power] %.
[c] The 95% confidence limits are $\Delta\Delta G$ ± 0.25 kJ/mol.

absolute IC_{50} values but the relative values for a series of inhibitors are fairly constant.

Data Analysis

The relative equilibrium constant (K_{rel}, equal to the ratio of IC_{50} values) for each of the saccharides is derived using saccharide **1** as a reference

TABLE II
INHIBITION OF AGGLUTINATION OF HUMAN P ERYTHROCYTES AND *Escherichia coli* (HB101/pPAP5)[a]

	IC_{50} (m*M*)		
Compound	Experiment 1[b]	Experiment 2	Experiment 1/experiment 2
1	0.18	0.043	4.2
12	0.13	0.022	5.9
13	0.082	0.026	3.2
14	0.046	0.011	4.2

[a] Using two different batches of blood and bacteria.
[b] See Ref. 5.

inhibitor. Inhibitory power is defined as $100K_{rel}$. The K_{rel} values[24] are used to calculate the difference in free energies ($\Delta\Delta G = -RT \ln K_{rel}$). The natural logarithms of the IC_{50} values and inhibitory powers (logarithmation is performed in order to obtain normal distributed sets of data) together with the $\Delta\Delta G$ values (which are already normal distributed) are subjected to analyses of variance with two sources of variation (inhibitor and run). The calculated mean values (and the 95% confidence limits) for IC_{50}, inhibitory powers, and $\Delta\Delta G$ values for saccharides **1–15** are presented in Table I.

The saccharide inhibitors **2–15** were categorized according to their $\Delta\Delta G$ values: (1) $\Delta\Delta G < 0$ kJ/mol: more potent than the reference saccharide **1**; (2) $\Delta\Delta G = 0$–2 kJ/mol: marginal loss of potency; (3) $\Delta\Delta G = 4$–10 kJ/mol: significant loss of potency, indicating either loss of a hydrogen bond or introduction of a steric repulsion; and (4) $\Delta\Delta G > 11$ kJ/mol: noninhibitory, indicating the loss of more than one important interaction. The loss of a hydrogen-bonding site in a ligand-binding enyme has been found to cause decreased binding strength by 2.1–6.3 kJ/mol.[25] It is recommended that conclusive argumentation based on borderline $\Delta\Delta G$ values (1.5–3.0 kJ/mol) be avoided.

The following conclusions can be drawn, based on the inhibitory data presented in Table I. The hydroxyl group at C-2 (HO-2) is not involved in hydrogen bonding to the adhesin because saccharide **2** ($\Delta\Delta G$ of 1.1 kJ/mol) shows only a marginal reduction of the inhibitory power. Saccharide **3** ($\Delta\Delta G$ of 3.9 kJ/mol) shows intermediate reduction of inhibitory power, indicating that HO-3 is involved in weak hydrogen bonding to the adhesin or that the mere size of HO-3 is important for binding. The two alternatives were discriminated by replacement of HO-3 by a methyl group.[5] Deoxysaccharides **4–8** ($\Delta\Delta G$ of 7.2, 5.9, 8.3, 9.1, and 6.7 kJ/mol) show significant reduction of inhibitory power, indicating that the remaining hydroxyl groups HO-6, -2′, -3′, -4′, and -6′ are involved in important hydrogen bonding to the adhesin. HO-3′ was found to be a proton acceptor because the CH_3O-3′ analog ($\Delta\Delta G$ of -1.6 kJ/mol) was a stronger inhibitor than **1**.[5] Deoxyfluorosaccharides **9** and **10** ($\Delta\Delta G$ of 9.1 and 6.4 kJ/mol) also show significant reduction of inhibitory power, indicating that HO-6 and -4′ donate a proton to the adhesin. Deoxyfluorosaccharide **11** ($\Delta\Delta G$ of 1.6 kJ/mol) shows only marginal reduction of inhibitory power, indicating that HO-6′ accepts a proton from the adhesin. The glycosides **12–14,** carrying hydrophobic aglycones, were stronger inhibitors than **1**, indicating possibilities for improve-

[24] D. Pressman and A. L. Grossberg, *in* "The Structural Basis of Antibody Specificity," p. 16. Benjamin, New York, 1968.

[25] A. R. Fersht, J.-P. Shi, J. Knill-Jones, D. M. Lowe, A. J. Wilkinson, D. M. Blow, P. Brick, P. Carter, M. M. Y. Waye, and G. Winter, *Nature* (*London*) **314,** 235 (1985).

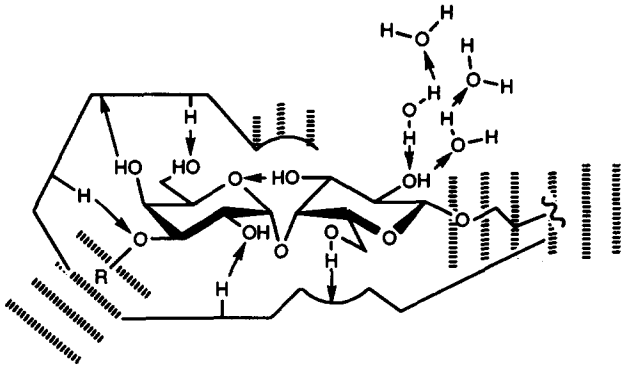

FIG. 3. Schematic model of the galabiose-specific binding site of the PapG adhesin of *E. coli.* (Reprinted with permission from Kihlberg *et al.*[5] Copyright 1989 American Chemical Society.)

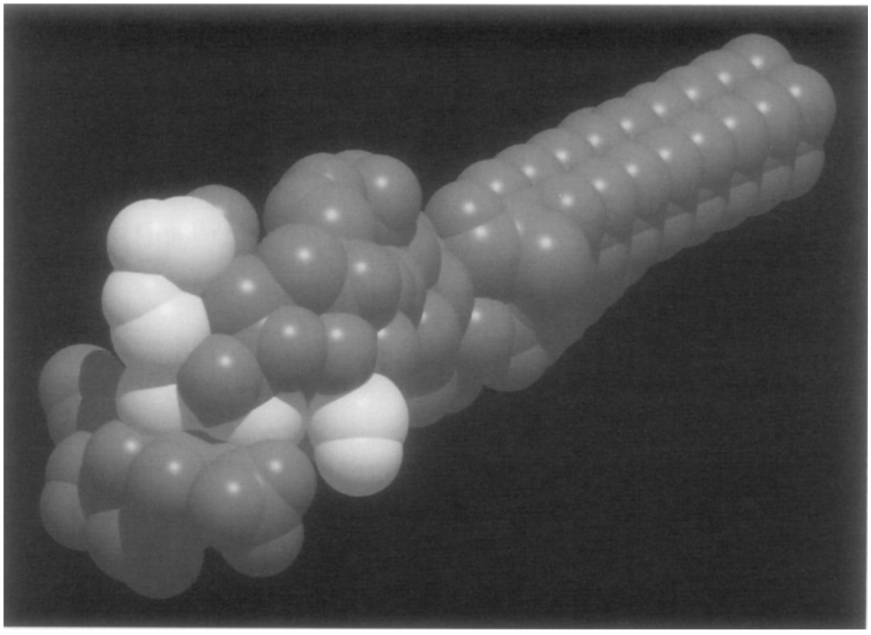

FIG. 4. Space-filling model of globotetraosylceramide. The oxygen functionalities of Galα(1-4)Gal that are involved in hydrogen bonding to the PapG adhesin (HO-6, HO-2′, O-3′, HO-4′, and HO-6′) are highlighted.

ment of the binding strength between saccharide and lectin. Finally, saccharide **15** ($\Delta\Delta G > 11$ kJ/mol) is a lactoside and is a noninhibitor.

The data and interpretations are summarized in the schematic model of the galabiose-specific binding site shown in Fig. 3. Galabiose is the central disaccharide of globoside, which is the natural glycolipid present on human erythrocytes. Figure 4 is a three-dimensional representation of globoside, with the hydrogen-bonded hydroxyl groups highlighted. It seems as if the PapG adhesin recognizes the convex side of membrane-bound globoside, which is well presented to the environment. This is a probable mechanism for bacterial adhesion in connection with infection and disease. It has been suggested that the constitution of the saccharide part of galabiose-containing glycolipids influences their lectin accessibility, which would explain the preference of certain *E. coli* strains for human tissue, whereas other strains prefer dog tissue for colonization.[26]

Perspectives

The rather detailed structural information obtained from receptor mapping with deoxy and deoxyfluoro analogs of saccharides is potentially useful for the design of antiadhesive drugs against bacterial infection. Such analogs may well have improved metabolic stability and uptake characteristics, which are important for their use as drugs. In addition, the saccharide analogs may be used as haptens on affinity chromatography gels for lectin purification.[27] Attenuated binding between the protein and the saccharide hapten can be useful in cases where the protein binds its natural counterpart too strongly to permit efficient protein recovery. Such affinity chromatography gels have been used for the preparation of the PapG/PapD complex[27], which is currently being crystallized for X-ray analysis.

[26] N. Strömberg, P. G. Nyholm, I. Pascher, and S. Normark, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 9340 (1991).

[27] S. J. Hultgren, F. Lindberg, G. Magnusson, J. Kihlberg, J. M. Tennent, and S. Normark, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 4357 (1991).

[11] Use of Synthetic Peptides in Characterization of Microbial Adhesins

By KOK K. LEE, WAH Y. WONG, HASMUKH B. SHETH, ROBERT S. HODGES, WILLIAM PARANCHYCH, and RANDALL T. IRVIN

Introduction

The attachment of microorganisms to host receptors is critical for the successful colonization and initiation of an infection. The mechanisms by which bacteria adhere to mammalian epithelial cell surface receptors have been extensively studied, but only relatively recently have inroads been made in determining the molecular basis of microbial adhesin function. The use of synthetic peptides has greatly facilitated this type of study. In this chapter, the *Pseudomonas aeruginosa* pilus adhesin is utilized to demonstrate the application of synthetic peptides to delineate the receptor-binding domain of the pilus adhesin, to characterize antigenic epitopes of that region of the pilus adhesin, and to begin structure–activity relationship studies of the pilus adhesin.

Pseudomonas aeruginosa is a significant opportunistic pathogen that initiates an infection by binding to the mucosal surface of a susceptible patient by means of polar pili.[1] The *Pseudomonas* pilus is composed of a single structural protein termed pilin that is assembled into a vectorial helical array to form a hollow tube or fimbrial structure.[1-3] The pilus adhesin function resides in the pilin structural protein.[1] The pilins of *P. aeruginosa* strains vary from 14 to 17 kDa. There are as many as seven different prototypes, with each strain expressing only one of the seven pilin genes.[4] The pilin can be roughly divided into three regions: a conserved hydrophobic N-terminal region; a hypervariable central region; and a semiconserved C-terminal region. The model strain that has been used in our studies is *P. aeruginosa* strain PAK. The PAK pilin gene has been cloned and sequenced, and the protein has been shown to consist of 144 amino acid residues.[5] There are two conserved cysteine residues in the C-terminal

[1] R. T. Irvin, *in* "*Pseudomonas aeruginosa* as an Opportunist Pathogen" (M. Campa, M. Bendinelli, and H. Friedman, eds.), p. 19. Plenum, New York, 1993.
[2] T. H. Watts, C. M. Kay, and W. Paranchych, *Biochemistry* **22,** 3640 (1983).
[3] W. Paranchych and L. S. Frost, *Adv. Microb. Physiol.* **29,** 53 (1988).
[4] W. Paranchych, B. L. Pasloske, and P. A. Sastry, *in* "*Pseudomonas:* Biotransformations, Pathogenesis and Evolving Biotechnology" (S. Silver, A. M. Chakrabarty, B. H. Iglewski, and S. Kaplan, eds.), p. 343. American Society for Microbiology, Washington, D.C., 1990.
[5] P. A. Sastry, J. R. Pearlstone, L. B. Smillie, and W. Paranchych, *FEBS Lett.* **151,** 253 (1983).

region of all pilin proteins, and the two cysteines form a disulfide bond between positions 129 and 142 in the PAK pilin.

Characterization of Antigenic Epitopes Associated with Adhesins

Prediction and Synthesis of Epitopes

Pseudomonas pili have been previously demonstrated to mediate adhesion of nonmucoid strains to buccal epithelial cells[6] (BECs) and tracheal epithelial cells[7] (TECs). Paranchych *et al.*[8] have purified *P. aeruginosa* pili from strains PAK and PAO. Polyclonal anti-PAK pili antisera were produced against purified PAK pili, and the immunodominant epitope was found in the central region of the pilin subunit. Extensive work has been carried out to determine the antigenic regions on the surface of the PAK pilin protein and to locate the receptor-binding domain of the adhesin. The delineation of the receptor-binding domain could be derived if one could obtain antibodies which block pilus adhesin binding to cell surface receptors. Both monoclonal antibodies and monospecific polyclonal antipeptide antibodies could be utilized to achieve this objective.

With regard to the monoclonal antibodies and polyclonal antipeptide antibodies, only a brief description is mentioned to provide readers with a more comprehensive understanding of other corroborative studies detailed below. Determination of antigenic determinants on the *Pseudomonas* PAK pili has been previously described by Lee *et al.*[9,10] and Doig *et al.*[11] The surface regions of *Pseudomonas* PAK pilin could be postulated theoretically by using prediction algorithms based on the hydrophilicity, accessibility, or mobility parameters. A prediction algorithm developed by Parker *et al.*[12] which combines all three parameters to predict the possible surface regions on *P. aeruginosa* PAK pilin is available. Peptides corresponding to predicted regions were synthesized and conjugated to protein carriers as previously described.[9] Eight antipeptide antisera specific to predicted surface regions

[6] D. E. Woods, D. C. Straus, W. G. Johanson, Jr., V. K. Berry, and J. A. Bass, *Infect. Immun.* **29,** 1146 (1980).

[7] R. Ramphal, J. C. Sadoff, M. Pyle, and J. D. Silipigni, *Infect. Immun.* **44,** 38 (1984).

[8] W. Paranchych, P. A. Sastry, L. S. Frost, M. Carpenter, G. D. Armstrong, and T. H. Watts, *Can. J. Microbiol.* **25,** 1175 (1979).

[9] K. K. Lee, P. Doig, R. T. Irvin, W. Paranchych, and R. S. Hodges, *Mol. Microbiol.* **3,** 1493 (1989).

[10] K. K. Lee, W. Paranchych, and R. S. Hodges, *Infect. Immun.* **58,** 2727 (1990).

[11] P. Doig, P. A. Sastry, R. S. Hodges, K. K. Lee, W. Paranchych, and R. T. Irvin, *Infect. Immun.* **58,** 124 (1990).

[12] J. M. R. Parker, D. C. Guo, and R. S. Hodges, *Biochemistry* **25,** 5425 (1986).

were assayed against native PAK pili using enzyme-linked immunosorbent assays (ELISA). Of the eight predicted surface regions, five were positively identified on the basis of ELISA data. When those antisera were subsequently assayed for antiadhesive capabilities, only antibodies against the peptide representing the C-terminal disulfide-bonded region (PAK128–144) could block the adhesion of *Pseudomonas* pili to cell surface receptors in whole-cell ELISA. This result indicated that the C-terminal region of *P. aeruginosa* pilin could be important in binding to receptors.

Monoclonal antibodies to the *P. aeruginosa* PAK pilin have been developed and typed by Doig *et al.*[11] Four different monoclonal antibodies (PK3B, PK34C, PK41C, and PK99H) have been assayed for the ability to bind to synthetic peptides (corresponding to the PAK pili sequence) and chymotrypsin-generated PAK pilin fragments by competitive ELISA. The protocols are based on the principles of Voller *et al.*[13] and have been modified as previously described.[11] Polystyrene cuvettes (Gilford Instrument Laboratories, OH) are coated by the addition of aliquots of 100 μl PAK pili (10 μg/ml in 10 m*M* carbonate coating buffer, pH 9.5) and incubation for 6 hr at room temperature. Cuvettes are washed 3 times (250 μl/cuvette) with 10 m*M* phosphate, pH 7.4, containing 150 m*M* NaCl (phosphate-buffered saline, PBS) and 0.05% (w/v) bovine serum albumin (BSA) (buffer A) and are blocked with 5% (w/v) BSA in PBS by incubation overnight at 4°. Fixed concentrations of the antibodies are mixed with serially diluted competing peptides. The reactants are brought up to 100 μl with buffer A. The mixtures are incubated for 1 hr at room temperature before addition into precoated cuvettes. The incubation time between the immobilized PAK pili and the primary and secondary antibodies [goat antiimmunoglobulin G (anti-IgG, heavy and light chains)–horseradish peroxidase purchased from Jackson ImmunoResearch Laboratories, PA] is 2 hr, at room temperature. The reaction is allowed to proceed for 2 hr at room temperature. Each cuvette is washed 3 times with 250 μl of buffer A between incubations. The enzyme substrate, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) in citrate buffer, pH 4.2, containing 0.03% (v/v) hydrogen peroxide, is added (250 μl/cuvette). The reaction is stopped by the addition of 250 μl of 4 m*M* sodium azide. The absorbance is monitored at 405 nm. These studies have also been replicated with standard 96-well microtiter plates using identical conditions.

Results from the studies[11] show that both PK34C and PK99H bound to peptides that correspond to the C-terminal disulfide-looped region. PK41C recognizes an epitope that is located in the N-terminal region of the PAK pilin sequence. PK3B fails to bind to any of the synthetic peptides available.

[13] A. Voller, D. Bidwell, G. Huldt, and E. Engvall, *Bull. WHO* **51,** 209 (1974).

Doig *et al.*[11] have shown that PK3B, PK41C, and PK99H demonstrate strain specificity, whereas PK34C is able to recognize a number of *P. aeruginosa* strains. Both PK99H and PK34C inhibit whole-cell *P. aeruginosa* binding to BECs. The data are in agreement with those obtained with the polyclonal antipeptide antibodies. This points to the possible involvement of the C-terminal region in the mediation of *Pseudomonas* pilus adhesion to receptors.

Epitope Mapping of Antipili Monoclonal Antibodies

The sequences recognized by antibodies may represent the actual sequence that confers receptor-binding properties or may represent sequences that are located near the receptor-binding region. Hence, the determination of the epitope of the antiadhesive monoclonal antibodies could provide important data for understanding the mechanism of bacterial adhesion to cell surface receptors and for the development of antiadhesive vaccines. The epitope of PK99H consists of a linear 7-residue sequence, DEQFIPK, within the C-terminal disulfide-looped region (residues 134–140) of PAK pilin.[14] We have obtained another monoclonal antibody, PAK-13, which is similar to PK99H except that it is cross-reactive with a number of *P. aeruginosa* pili (R. T. Irvin, 1993, unpublished data).

The epitope of PAK-13 is determined by competitive ELISA in which monoclonal antibody binding to immobilized PAK pili on microplates is competed with synthetic peptides corresponding to the last 17 residues of PAK pilin [PAK(128–144)]. N^{α}-AcPAK(128–144)$_{ox}$-OH peptide corresponds to residues 128–144 of PAK pilin: the amino terminus is acetylated (N^{α}-Ac), ox denotes the oxidation of the two cysteines to form a disulfide bond, and -OH indicates that the C (carboxyl) terminus exists in the free acid form. The peptides are synthesized on an Applied BioSystems (Foster City, CA) automated peptide synthesizer, Model 430A. Peptide analogs have a single alanine substitution along the 17-residue sequence with the exception that the two cysteine residues are not substituted as they are required in the formation of a disulfide bond in the peptide. A residue is deemed to be important in antibody binding if that substitution results in a loss of binding (hence, a lack of competitive binding to the antibody). The solid-phase peptide synthesis protocols, purification, and characterization of the peptides have been described.[14] PAK pili are coated onto the microtiter wells (0.2 μg/well). Nonspecific adsorption sites in the wells are blocked with BSA. Varying concentrations of peptides diluted with buffer A are mixed with PAK-13, diluted 1:1000 (the diluted antibody has a

[14] W. Y. Wong, R. T. Irvin, W. Paranchych, and R. S. Hodges, *Protein Sci.* **1,** 1308 (1992).

titer of 10^8 by ELISA), and incubated for 1 hr at room temperature. The incubation time between the primary and secondary antibodies and immobilized PAK pili is 2 hr at 37°. The wells are washed five times with buffer A (250 μl/well) between incubation periods. Enzyme substrate is added to the wells (125 μl/well). The color reaction is quenched with the addition of 4 m*M* NaN_3 (125 μl/well), and the absorbance readings at 405 nm are recorded.

The data in Table I derived from competitive ELISA with PAK-13 and synthetic peptide analogs show that the epitope of PAK-13 also resides in a linear 7-residue sequence like the epitope of PK99H, but only F137, I138, and K140 can be considered critical residues. It is interesting that PAK-13 is more cross-reactive than its PK99H counterpart even though they seem to share the same basic epitope. The epitope of a polyclonal anti-PAK(128–144)$_{ox}$-OH cross-reactive IgG has also been mapped to this region.[15a] The epitope mapping data are in agreement with structural data obtained from two-dimensional proton nuclear magnetic resonance spectroscopy, which show that the polypeptide backbone of the residues of the region forms the most structurally defined region of the peptide.[15b] These observations indicate that antibody specificity or antibody binding diversity is defined not only by the amino acid sequence of the antigenic epitope but also by the relative contribution each residue makes to the binding energetics. Thus, a strain-specific antibody and a cross-reactive antibody can recognize an identical region of an antigen with each antibody employing a different set of critical amino acid residues for binding. These results also suggest that cross-reactive antigenic epitopes need not always have extensive sequence similarity.

Identification of Epithelial Cell-Binding Domain

Peptide Inhibition of PAK Pili Binding to Buccal Epithelial Cells

Identification of an antigenic determinant on the PAK pilin does not provide direct proof that the epitope in question has a receptor-binding function. It is possible that it is merely located sterically close to the receptor-binding region. The importance of peptide sequences in the mediation of bacterial adhesion could be demonstrated by the ability of the peptide to inhibit pilus/bacterium binding to cell surface receptors.

[15a] W. Y. Wong, H. B. Sheth, R. T. Irvin, and R. S. Hodges, *Pediatric Pulmon.* **S10,** 157 (1994).
[15b] C. McInness, F. D. Sonnichsen, C. M. Kay, R. S. Hodges, and B. D. Sykes, *Biochemistry* **32,** 13432 (1993).

TABLE I
MAPPING EPITOPE OF ANTI-PAK PILI MONOCLONAL ANTIBODY PAK-13[a]

Peptides[a]	I_{50}[b] (nM)	PAK-13 (Kn/Ks)[c]	PK99H[d] (Kn/Ks)
K128A	5.0	0.5	0.6
T130A	4.0	0.4	0.6
S131A	10.0	1.0	1.4
D132A	9.0	0.9	1.5
Q133A	30.0	3.0	1.7
D134A	450	45.0	64.0
E135A	200	20.0	9.6
Q136A	10.0	1.0	1.6
F137A	>100,000	10,000	4,000
I138A	7,000	700	600
P139A	750	75	76
K140A	>10,000	1000	880
G141A	2.0	0.2	0.3
S143A	8.0	0.8	0.4
K144A	4.5	0.5	0.6
Native-amide	10.0	1.0	0.9

[a] The epitope is represented by the shaded portion. The epitope mapping involved the use of competitive ELISA with the synthetic peptides competing for the antibody binding with immobilized *P. aeruginosa* PAK pili.

[b] The native amide peptide represents the native 17-residue sequence of the C-terminal region of PAK pilin that has been amidated. The remainder of the peptide analogs listed indicate the natural residue (represented by a single capital letter) that has been substituted and its position in the sequence.

[c] I_{50} is the concentration of the competitor which gave a 50% inhibition of binding.

[d] K_n/K_s ratio indicates the effect of an alanine substitution at the given position on the apparent affinity constant (K_s) in relation to the native unsubstituted peptide (K_n).

[e] Data for monoclonal antibody PK99H are from W. Y. Wong, R. T. Irvin, W. Paranchych, and R. S. Hodges, *Protein Sci.* **1,** 1308 (1992). The shaded area indicates the epitope of the antibody.

In the competitive binding assays,[16] BECs are collected by gently rubbing the inner cheek linings of 10 healthy nonsmoking males with wooden applicator sticks (Fisher Scientific, Pittsburgh, PA). The BECs are removed from the wooden sticks by gentle agitation in 20 ml PBS, pH 7.2. The cells

[16] R. T. Irvin, P. Doig, K. K. Lee, P. A. Sastry, W. Paranchych, T. Todd, and R. S. Hodges, *Infect. Immun.* **57,** 3720 (1989).

are washed three times by centrifugation (2000 *g*) with 10 ml PBS. Washed BECs are passed through a nylon mesh (70 μm pore size). The cell number is determined with a hemacytometer and adjusted to 2.0×10^5 cells/ml with PBS. Aliquots of synthetic N^{α}-AcPAK(128–144)$_{ox}$-OH peptide (0.1 ml is added such that a final assay concentration of 0, 40, 80, or 120 nmol/ml is obtained) and BECs (0.2 ml at 2.0×10^5 cells/ml) are preincubated for 30 min at room temperature. Pili (0.1 ml at 0 to 100 μg/ml) are added to the above BECs. The mixtures are then incubated at 37° for 2 hr while being agitated at 300 rpm on a gyratory shaker (New Brunswick Instruments). The BECs together with bound pili are collected by centrifugation for 2 min at room temperature at 13,000 *g*. The cells are washed five times with PBS in this manner. Monoclonal antibody PK3B (0.1 ml of a 10^{-4} dilution in PBS), which recognizes PAK pili but does not react with the synthetic N^{α}-AcPAK(128–144)$_{ox}$-OH peptide, is then added to the BECs and incubated for 1 hr as described above. Cells are collected by centrifugation, washed as described above, and incubated with secondary antibody–enzyme conjugates. The BECs are collected by centrifugation and washed as described above. In the final wash step, the pellet is transferred to a clean tube and suspended in 0.2 ml of ABTS solution. The enzymatic reaction is stopped by the addition of 0.2 ml of 4 m*M* NaN_3. The number of BECs in each tube is determined with a hemacytometer. The cells are then removed by centrifugation and the supernatant distributed into microtiter wells (200 μl/well). The absorbance of the supernatant at 405 nm is recorded. Results from these studies[16] demonstrate that the synthetic peptides competitively inhibit PAK pili binding to BECs, suggesting that the N^{α}-AcPAK(128–144)$_{ox}$-OH peptides contain the receptor-binding domain in PAK pilin.

Peptide Inhibition of PAK Pili Binding to Human Lung Cell Line A549

Biotinylated PAK pili are employed to bind to human lung A549 cells in order to demonstrate the ability of synthetic peptides to complex to the same cell surface receptors as *Pseudomonas* pili. Biotinylated PAK pili are prepared by pipetting 300 μl of purified pili (4.8 mg/ml) into a dialysis tubing with a molecular weight cutoff of 12,000–14,000 (Spectrum Medical Industries, CA). A 10-μl aliquot of biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, St. Louis, MO) (20 mg/ml in dimethyl sulfoxide) is added to the PAK pili, and the reaction is allowed to proceed for 45 min at room temperature in a 50-ml conical tube under constant shaking in a gyroshaker. Excess biotin esters are removed by extensive dialysis at 4° with four changes of PBS, pH 7.4. Biotinylated PAK pili are removed and the dialysis tubing rinsed with PBS to maximize the recovery. The biotinylated PAK pili (3.0 mg/ml, final concentration) is stored at −20°.

Human lung pneumocytes, cell line A549 [ATCC (Rockville, MD) CCL185, Batch F-8669], are passaged and cultured in 25-ml cell culture flasks (Corning, Corning, NY) in Waymouth's MB 752/1 medium containing 10% (v/v) fetal calf serum (FCS), sodium pyruvate (1 m*M*), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). All medium components are obtained from GIBCO–BRL (Life Technologies, New York, NY). The flask is placed in an incubator kept at 37° and maintained at 5% (v/v) carbon dioxide. The cell monolayer is removed by the addition of 1 ml prewarmed EGTA solution (5 m*M*) and 5 ml PBS (10 m*M* phosphate buffer, pH 7.4) to the flask. The flask is then incubated for 30 min. After the flask is shaken gently to assist the detachment of the A549 cells, the cells are removed from the flask with a disposable sterile pipette into a 10-ml centrifuge tube. The tube is centrifuged at 2000 *g* for 2 min. The supernatant is discarded, the cell pellet is suspended in 10 ml plain medium, and the centrifugation is repeated. The cells are washed twice in this manner.

The cells are then suspended in 5 ml Waymouth's medium (same as culture medium except that the FCS has been heat-inactivated for 30 min at 56°). A 50-μl aliquot of the cell suspension is mixed with 50 μl of trypan blue solution and the number of viable cells determined. The cell density is adjusted to 4×10^5 cells/ml with the growth medium. Cells are then dispersed into a 96-well tissue culture plate (100 μl/well). Another 100-μl of medium is added to each well, and the cells are allowed to grow overnight in the incubator mentioned above. The medium in each well is very gently removed by aspiration with a multichannel pipettor fitted with tips (Bio-Rad, Richmond, CA) designed for electrophoresis in order to minimize the loss of cells. In the assays, it is critical to minimize shear forces to prevent cell detachment. The cell monolayers in the wells are washed with 200-μl portions of Hanks' balanced salt solution (HBSS) with supplement (NaCl, 140 m*M*; KCl, 5 m*M*; $CaCl_2 \cdot 2H_2O$, 2.2 m*M*; $MgSO_4$, 0.2 m*M*; $MgCl_2 \cdot 6H_2O$, 2.5 m*M*; Na_2HPO_4, 0.35 m*M*; KH_2PO_4, 40 μ*M*; $NaHCO_3$, 4 m*M*; and glucose, 5.5 m*M*) and 0.05% (w/v) BSA (buffer B). Buffer B is delivered slowly into the wells to prevent detachment of the A549 cells. Biotinylated PAK pili (1:2000) and serially diluted peptides (with buffer B) are premixed, and aliquots (75 μl/well) of the mixture are added to the microtiter plate and incubated for 2 hr at 37°. Wells are washed three times (150 μl/well) with buffer B. Streptavidin–alkaline phosphatase (GIBCO–BRL) which has been diluted 1:3000 with HBSS containing 1% (w/v) BSA is added (75 μl/well), and the incubation is allowed to proceed for 1 hr at room temperature. Wells are washed twice with buffer B and another two times with 50 m*M* tris(hydroxymethyl)aminomethane hydrogen chloride (Tris-HCl), pH 7.4, containing 150 m*M* NaCl, 2.2 m*M* $CaCl_2$, 2 m*M* $ZnCl_2$

and 0.05% BSA (w/v). *p*-Nitrophenyl phosphate [1 mg/ml in 10% (w/v) aqueous diethanolamine, pH 9.6] is added (100 μl/well), and the plate is incubated at room temperature for 2 hr. The reaction is quenched by the addition of 2 *N* NaOH (100 μl/well), and the absorbance at 405 nm is recorded.

Synthetic N^{α}-AcPAK(128–144)$_{ox}$-OH competitively inhibits biotinylated PAK pili binding to A549 cells (Fig. 1). Peptides which correspond to the C-terminal region of the pilin from *P. aeruginosa* strains PAO and KB7 also compete with PAK pili in binding to cell surface receptors. The data suggest that the C-terminal disulfide-looped region of *P. aeruginosa* pilin may possess the receptor-binding domain of the pilus adhesin and that there may be a common receptor for the binding of all *P. aeruginosa* strains.

Immunofluorescence Localization of Cell Surface Receptors

Synthetic peptides that include the receptor-binding domain could be employed in fluorescence spectroscopy to visualize the presence of pilus receptors on human TECs and BECs. Human ciliated TECs are obtained from patients in the surgical intensive care unit by bronchoscopic brushing

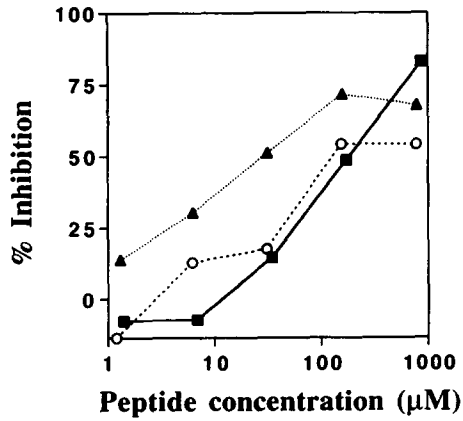

FIG. 1. Peptide inhibition of biotinylated PAK pili to A549 cells. Competitive ELISA were used to test the ability of synthetic peptides corresponding to the C-terminal region of *P. aeruginosa* pilins from strains PAK (■), PAO (▲), and KB7 (○) to compete with PAK pili for receptor sites on the cell surface of A549 cells immobilized onto wells of microtiter plates. The percent inhibition of biotinylated PAK pili binding to A549 cells is a measure of the absorbance in the presence of competing peptides versus that in the absence of peptides under the same conditions.

of the bronchial mucosa by the method of Franklin *et al.*[17] The cells are fixed with 1% (v/v) formaldehyde in PBS for 1 hr at room temperature. The cells are washed twice with PBS, and the cell number is determined with a hemacytometer. The density of TECs is adjusted to 10^5 cells/ml. The TECs (0.1 ml) are mixed with an equal volume of PAK pili (345 μg/ml), N^{α}-AcPAK(128–144)$_{ox}$-OH (10 or 50 μM), or PBS. The mixtures are incubated at 37° for 1 hr with agitation at 300 rpm. Cells are collected and washed three times with PBS by centrifugation at room temperature (6000 *g* for 1 min). Volumes (0.1 ml) of monoclonal antibody PK99H (working titer of 10^6) are added to the respective TECs previously incubated with pili or peptides. The mixtures are incubated for 1 hr at 37° and agitated at 300 rpm. Cells are collected by centrifugation and washed three times with PBS as described above. Affinity-purified rabbit anti-mouse IgG (heavy and light chains)–fluorescein isothiocyanate conjugates (Jackson ImmunoResearch Laboratories) diluted 1:100 with PBS are added to the TECs (0.1 ml) and incubated for 30 min at 37° with agitation at 300 rpm. The TECs are washed three times as described above and suspended in 0.1 ml of PBS. Wet mounts are prepared and examined by epifluorescence and phase-contrast microscopy by using a Leitz Laborlux microscope equipped with an MPS4 camera system. Photographs are recorded on Kodak (Rochester, NY) T-Max film.

The results in Fig. 2 show that the synthetic peptides have a preference in binding to the luminal portion of the cytoplasmic membrane and the basal two-thirds of the cilia of the TECs. Similar results are obtained when PAK pili are used to localize cell-surface receptors on TECs.[16] A control of TECs incubated with PBS shows a lack of epifluorescence.

Whole-Cell Binding Assay

The adhesin property of N^{α}-AcPAK(128–144)$_{ox}$-OH can be assessed by using the peptide in direct binding assays to epithelial cells utilizing either an immunological method or biotinylated peptides. Evidence for the direct binding of N^{α}-AcPAK(128–144)$_{ox}$-OH peptides to BECs has been published. The BECs (0.2 ml at 2.0×10^5 cells/ml) are added to an equal volume of synthetic peptide (0 to 120 μM) in PBS and incubated for 1 hr at 37° with agitation at 300 rpm. Cells are collected by centrifugation for 2 min at room temperature at 13,000 *g* and then washed five times with PBS. Purified PK99H (0.2 ml of a 1:1000 dilution with a working titer of 10^6) is added to the BECs and incubated for 1 hr as described above. Cells are collected by centrifugation and washed as described above. Goat anti-

[17] A. L. Franklin, T. Todd, G. Gurman, D. Black, P. M. Mankinen-Irvin, and R. T. Irvin, *Infect. Immun.* **55,** 1523 (1987).

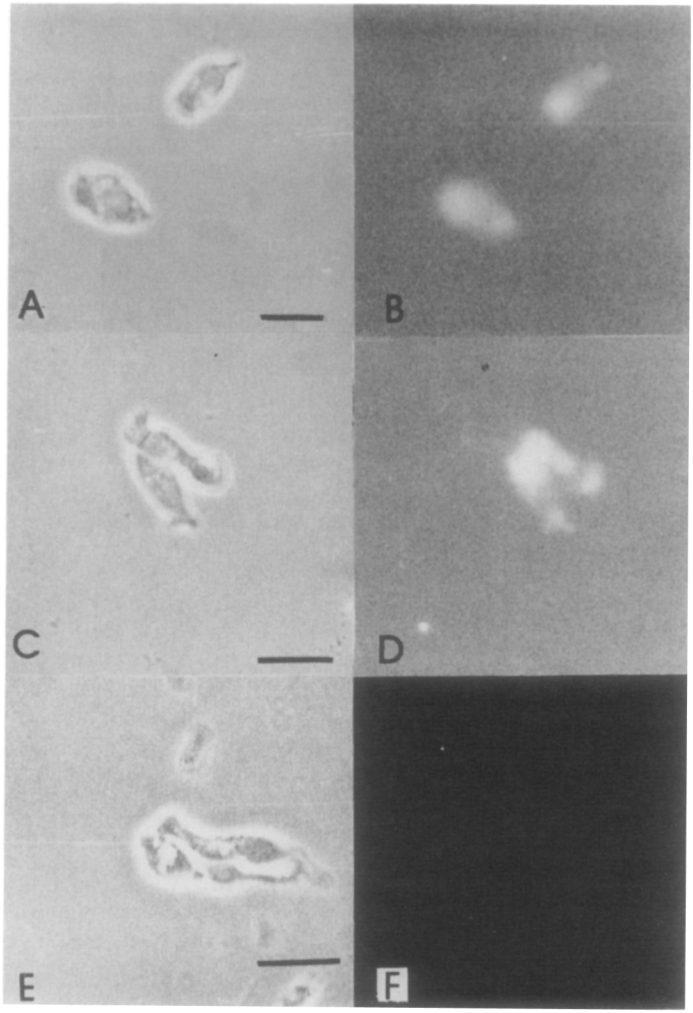

FIG. 2. Indirect immunofluorescence localization of binding of synthetic peptides PAK(128–144)-OH in oxidized (A, B) and reduced states (C, D) to human ciliated TECs. (E, F) Control preparations that were not exposed to synthetic peptides during the incubation. (A, C, E) Phase-contrast micrographs of the same cells visualized by immunofluorescence microscopy in B, D, and F. (Reprinted with permission from Irvin *et al.*[16])

mouse heavy and light (H+L) IgG–peroxidase conjugate (Jackson ImmunoResearch Laboratories) diluted 1 : 10,000 with PBS is added to the BECs (0.2 ml) and incubated as described above. Cells are collected by centrifugation and washed as described above. The pellet is transferred to a clean

tube and suspended in 0.2 ml of ABTS (1 m*M*). The enzymatic reaction is stopped by the addition of 0.2 ml of 4 m*M* NaN_3. The number of BECs in each tube is determined with a hemacytometer, and the absorbance at 405 nm is determined after removal of the BECs by centrifugation.

An investigation of synthetic peptide adhesin binding to receptors by using biotinylated PAK(128–144)$_{ox}$-OH to bind to A549 cells has been performed. These biotinylated PAK peptides are synthesized in the same manner as for N^{α}-AcPAK(128–144)$_{ox}$-OH using automated solid-phase peptide synthesis protocols[14] with the exception that the N terminus is coupled to four glycine residues (used as a larger spacer owing to the nature of the biotin-binding domain of avidin) followed by a biotin molecule. A549 cells are cultured and plated onto microtiter wells as described above with the biotinylated PAK pili. Biotinylated PAK(128–144)$_{ox}$-OH (614 μM) is serially diluted with buffer B and distributed in the microtiter plate (75 μl/well). The reaction is allowed to proceed at 37° for 2 hr. The remainder of the protocol is identical to those described above until the detection of the second antibody. An ELISA amplication kit (GIBCO–BRL, Life Technologies) is utilized to amplify the alkaline phosphatase signal as the signals obtained with the biotinylated peptides are not very strong. The final washes of the wells are performed with Tris-buffered saline (50 m*M* Tris-HCl, pH 7.5, and 0.15 *M* NaCl). Samples of reconstituted Substrate (obtained with the kit) are added to the wells (50 μl/well) and incubated for 15 min at room temperature. Aliquots of reconstituted Amplifier (obtained with the kit) are then added to the wells (50 μl/well) and incubated for another 15 min at room temperature. The color development is stopped by the addition of 0.3 *M* H_2SO_4 (50 μl/well). The absorbance readings are recorded at 492 nm.

Biotinylated PAK(128–144)$_{ox}$-OH binds to the A549 cells in a saturable and concentration-dependent manner (Fig. 3). A control biotin-GGGGKAGFAGDDAPR-amide peptide fails to bind to the A549 cells. The data demonstrate that the 17-residue PAK peptide contains the receptor-binding domain.

Peptide Binding to Asialo-G_{M1}

Synthetic peptides can be used to demonstrate interactions with glycoconjugate receptors in solid-phase binding assays. *Pseudomonas aeruginosa* pili have been shown to bind to the glycosphingolipid asialoganglioside-G_{M1} [Galβ(1-4)GalNAcβ(1-4)Galβ(1-4)Glc(1-1)-ceramide].[18] Monosialoganglioside G_{M1} and asialo-G_{M1} (Sigma) are dissolved in 10% (v/v) methanol in chloroform to a concentration of 1 mg/ml. Glycosphingolipids are dis-

[18] K. K. Lee, H. B. Sheth, W. Y. Wong, R. Sherburne, W. Paranchych, R. S. Hodges, C. A. Lingwood, H. C. Krivan, and R. T. Irvin, *Mol. Microbiol.* **11,** 705 (1994).

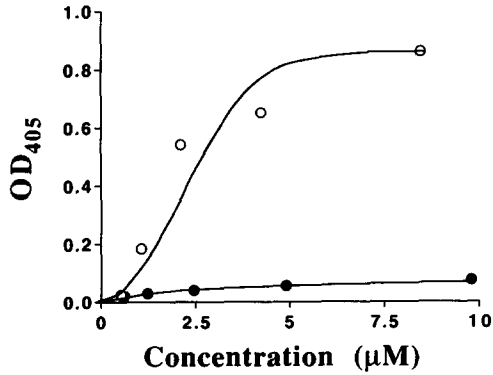

FIG. 3. Direct binding of biotinylated PAK(128–144)$_{ox}$-OH peptides (○) and biotinylated GGGGKAGFAGDDAPR-amide (●) to immobilized A549 cells. The alkaline phosphatase signals have been amplified using an ELISA amplification kit (GIBCO–BRL), and the absorbance at 492 nm was recorded.

solved in methanol to 5 μg/ml and coated onto microtiter wells (100 μl/well). The methanol is allowed to evaporate at room temperature in a fume hood. Excess sites in the wells are blocked with a solution containing 5% (w/v) BSA in PBS (150 μl/well). The microtiter plate is incubated for 1 hr at 37°. Excess BSA is removed by aspiration, and the coated wells are then employed in binding assays.

Biotinylated PAK(128–144)$_{ox}$-OH peptides (307 μM) are diluted to required concentrations with PBS, pH 7.4. Portions (in triplicate) of the diluted peptides are added to precoated wells (100 μl/well) and incubated for 1 hr at 37°. The wells are washed 5 times with buffer A (250 μl/well). Streptavidin–alkaline phosphatase (Jackson ImmunoResearch Laboratories) is diluted 1 : 5000 with buffer A and added to the wells (100 μl/well), and the incubation is repeated as described above. Wells are washed five times with buffer A (250 μl/well). *p*-Nitrophenyl phosphate (Sigma) is dissolved in 10% (w/v) aqueous diethanolamine (Fisher Scientific), pH 9.6, to a concentration of 1 mg/ml and is added to the wells (100 μl/well). The absorbance at 405 nm is recorded. The results indicate that the biotinylated peptides bind to asialo-G_{M1} in a concentration-dependent manner.[18] The data also show that the PAK(128–144)$_{ox}$-OH peptide can bind to glycosphingolipid receptors.

Peptide Binding to GalNAcβ(1-4)Gal

Krivan *et al.*[19] proposed that many microbial pathogens capable of binding to asialoganglioside-G_{M1} and asialoganglioside-G_{M2} recognize a minimal

[19] H. C. Krivan, D. D. Roberts, and V. Ginsburg, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6157 (1988).

disaccharide sequence consisting of GalNAcβ(1-4)Gal. The method below examines the binding of biotinylated peptides to synthetic GalNAcβ (1-4)Gal.[20] The disaccharide is first synthesized and conjugated to BSA. The synthesis of GalNAcβ(1-4)Gal-O$(CH_2)_8COOCH_3$ has been previously described by Sabesan and Lemieux.[21] The conjugation of the GalNAcβ (1-4)Gal to BSA has been described by Pinto and Bundle.[22] A stock solution (1 mg/ml) is prepared by dissolving the BSA–GalNAcβ(1-4)Gal conjugate in PBS. The conjugate is diluted to 5 μg/ml with coating buffer (10 m*M* carbonate/bicarbonate, pH 9.5). Samples of the conjugate are added to the wells (100 μl/well) and incubated overnight at 4° to coat the conjugates onto the wells. Excess unbound conjugates are removed by aspiration, and the wells are blocked with BSA as described above. The rest of the protocol is similar to that for the binding assays with the glycosphingolipids. The binding of biotinylated PAK$(128–144)_{ox}$-OH peptide (Fig. 4A) demonstrates that the peptide contains the disaccharide-binding domain. The corresponding synthetic peptide mimicking the PAO pilin sequence is also able to bind to the disaccharide–BSA conjugates.[20]

Competition between Pili and Synthetic Peptide for GalNAcβ(1-4)Gal

The wells of microtiter plates are coated with GalNAcβ(1-4)Gal–BSA (5 μg/ml) as described above. A fixed concentration of biotinylated PAK$(128–144)_{ox}$-OH is selected on the basis of the data from the direct binding assays above. The selection is based on the concentration of biotinylated peptide which gives a sufficiently high A_{405} signal (between 0.5 and 1.0) with a minimal amount of receptor coated (5 μg/ml) on the microtiter plate for a reasonable signal. The competitors, PAK pili or N^{α}-AcPAK$(128–144)_{ox}$-OH peptides, are serially diluted. The biotinylated PAK$(128–144)_{ox}$-OH peptides are premixed with each of the diluted competitors and subsequently added to the wells precoated with the GalNAcβ(1-4)Gal–BSA. The reaction is incubated for 1 hr at 37°. The remainder of the protocol is similar to that for the direct binding assays described above. The binding of biotinylated PAK$(128–144)_{ox}$-OH peptide to the disaccharide is competitively inhibited by native PAK pili as shown in Fig. 4B.

Although use of biotinylated peptides constitutes a fairly straightforward approach to assessing whether a given peptide sequence can interact with a receptor, it is prudent to interpret the results cautiously as biotinylated peptides are generally more hydrophobic than the native sequence.

[20] H. B. Sheth, K. K. Lee, W. Y. Wong, G. Srivastava, O. Hindsgaul, R. S. Hodges, W. Paranchych, and R. T. Irvin, *Mol. Microbiol.* **11,** 715 (1994).
[21] S. Sabesan and R. U. Lemieux, *Can. J. Chem.* **62,** 644 (1984).
[22] B. M. Pinto and D. R. Bundle, *Carbohydr. Res.* **124,** 313 (1983).

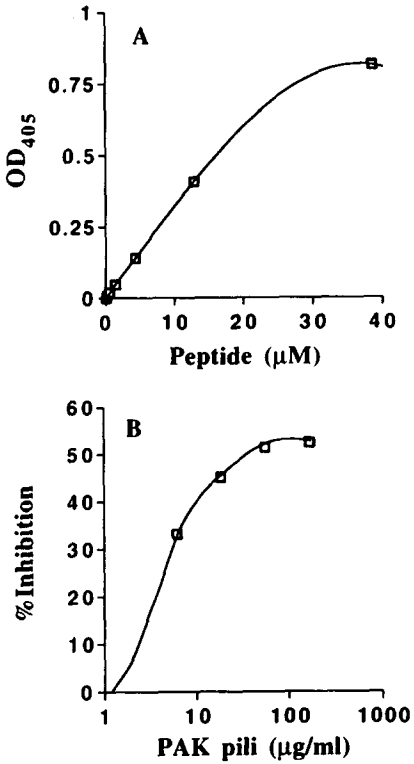

FIG. 4. Interactions between PAK(128–144)$_{ox}$-OH peptide and GalNAcβ(1-4)Gal. The biotinylated PAK(128–144)$_{ox}$-OH peptides were assayed for binding to GalNAcβ(1-4)Gal-BSA immobilized onto microtiter wells (A) as indicated by the absorbance recorded at 405 nm. The specificity of the peptide–GalNAcβ(1-4)Gal interactions was demonstrated by the ability of PAK pili to compete with the biotinylated PAK(128–144)$_{ox}$-OH peptides (B). The percent inhibition of biotinylated peptide binding to the GalNAcβ(1-4)Gal–BSA conjugate is a measure of the absorbance in the presence of competing native PAK pili versus that in the absence of competing pili under the same experimental conditions. (Reprinted with permission from Sheth *et al.*[20])

The increased hydrophobicity of biotinylated peptides can result in a variety of nonspecific interactions, particularly when glycoconjugates are employed as receptors. Direct binding studies should be confirmed by competition studies with native peptide sequences and with the native adhesin. The increased hydrophobicity of biotinylated peptides frequently enhances the binding affinity of a peptide for its receptors by one or two orders of magnitude. Subsequently, it is frequently difficult to obtain good inhibition curves with free peptides when biotinylated peptides are employed as the reporting ligand.

Determination of Structure–Activity Relationships in Microbial Adhesins

Relatively little progress has been made toward the determination of the molecular basis of microbial adhesin function. One approach to defining structure–activity relationships is to use a series of peptide analogs with a sequential single alanine substitution along the native sequence to define which residues are critical for peptide function. We have been able to identify amino acid residues that are critical or required for adhesin function by examining the binding of biotinylated PAK pili to human A549 cells in the presence of competing peptide analogs. The methods utilized for the studies are identical to those described above for the inhibition of PAK pili binding to A549 cells with native peptide. The results of the competition between two alanine-substituted peptide analogs and the native N^{α}-Ac-PAK(128–144)$_{ox}$-OH with biotinylated PAK pili (Fig. 5A) and biotinylated PAK(128–144)$_{ox}$-OH (Fig. 5B) are representative of the data obtained. The residues in positions 138 and 139 are critical for antibody interactions.[14]

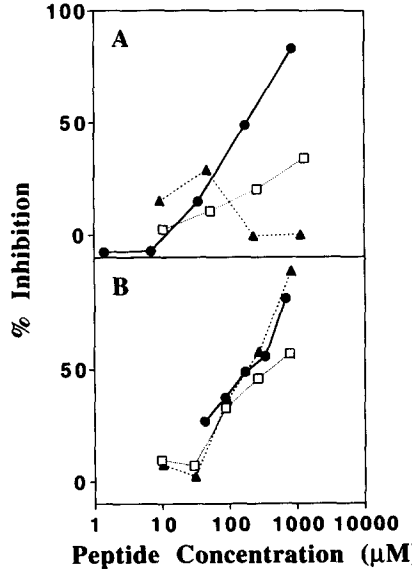

FIG. 5. Inhibition of biotinylated PAK pili (A) and biotinylated PAK(128–144)$_{ox}$-OH peptide (B) to A549 cells with native N^{α}-AcPAK(128–144)$_{ox}$-OH (●) and two single-alanine-substituted peptide analogs [P139A (□) and I138A (▲)]. The percent inhibition binding of biotinylated PAK pili and biotinylated PAK(128–144)$_{ox}$-OH to A549 cells is a measure of the absorbance in the presence of competing peptides versus that in the absence of peptides under the same conditions.

The I138A and P139A peptide analogs have few effects on biotinylated PAK pili binding to the A549 cells compared with the native PAK peptide. Interestingly, the same analogs are as effective as the native PAK peptide in the inhibition of biotinylated PAK(128–144)$_{ox}$-OH binding to the A549 cells. Clearly, utilizing the native adhesins (PAK pili) in the competition assay allows one to identify residues that are normally required for adhesin function, whereas utilizing a biotinylated peptide in a similar study does not. These results suggest that biotinylated peptides may be too flexible and/or too hydrophobic to be useful in determining the structure–activity relationship of an adhesin.

Once a residue critical for adhesin function is identified, it remains to be established whether the residue is required because it mediates a direct interaction with the receptor or is required in order to generate a functional conformation of the peptide. Differentiating the two possibilities requires extensive structural studies as computational predictions based on sequence data are not yet reliable.

Summary

The characterization of microbial adhesins is greatly facilitated by the use of synthetic peptides. Synthetic peptides can be used to identify specific antigenic epitopes, to delineate receptor-binding domain of adhesins, and to facilitate the characterization of the adhesin, and they allow for a direct examination of structure–binding relationships.

Acknowledgments

Research was supported by grants from the Medical Research Council of Canada, Canadian Cystic Fibrosis Foundation, Canadian Bacterial Diseases Network, and Protein Engineering Network of Centers of Excellence. We also acknowledge the technical assistance of Thorsten Schmidt in mapping the epitope of PAK-13.

[12] Identifying Bacterial Receptor Proteins and Quantifying Strength of Interactions They Mediate

By AKRAPORN PRAKOBPHOL, HAKON LEFFLER, and SUSAN J. FISHER

Introduction

One of the most difficult problems encountered in studying the molecular basis of bacterial adhesion is determining which component(s) of a complex mixture, such as a biological fluid or a cell membrane, can act as a receptor. The limited availability of receptor-containing host material, combined with the large number of low-abundance candidate molecules, can make this a daunting process. However, strategies have emerged for overcoming these problems. A significant advancement was the discovery that glycolipids, separated by thin-layer chromatography, could be overlaid with radiolabeled bacteria and components to which the bacteria bind identified by autoradiography.[1,2] Because bacteria frequently express one or more lectinlike proteins (adhesins) that interact with host mucosal glycoconjugates,[3] it is no surprise that the overlay technique has frequently shown that the carbohydrate portions of lipids are critical for bacterial receptor activity. For example, uropathogenic *Escherichia coli* bind to Galα1→4Gal-containing glycolipids,[1,4] and sequences containing GalNAc serve as receptors for two potential oral pathogens, *Actinomyces viscosus* and *A. naeslundii*.[5] Nevertheless, microorganisms that bind via specific aspects of nonglycosylated lipid receptor molecules have also been identified. For example, it has been shown that *Candida tropicalis* interacts with lysophospholipids, but not with intact phospholipids.[6]

We have developed an analogous assay in which proteins are separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), blotted onto nitrocellulose, and overlaid with radiolabeled bacteria; components to which the bacteria bind are then identified by autoradiography.[7] This technique has been used to study interactions between sali-

[1] K. Bock, M. E. Breimer, A. Brignole, G. C. Hansson, K.-A. Karlsson, G. Larson, H. Leffler, B. C. Samuelsson, N. Stromberg, C. Svanborg-Eden, and J. Thurbin, *J. Biol. Chem.* **260,** 8545 (1985).
[2] K.-A. Karlsson, *Annu. Rev. Biochem.* **58,** 309 (1989).
[3] D. Mirelman, "Microbial Lectins and Agglutinins." Wiley, New York, 1986.
[4] H. Leffler and C. Svanborg-Eden, *FEMS Microbiol. Lett.* **8,** 127 (1980).
[5] N. Stromberg and K.-A. Karlsson, *J. Biol. Chem.* **265,** 11251 (1990).
[6] A. Prakobphol, H. Leffler, and S. J. Fisher, *Biochemistry* **33,** 9496 (1994).
[7] A. Prakobphol, P. Murray, and S. J. Fisher, *Anal. Biochem.* **164,** 5 (1987).

vary glycoproteins and various species of bacteria that colonize the oral cavity. In several cases components of highly specific interactions have been identified. For example, *Fusobacterium nucleatum* binds only to the highly glycosylated proline-rich glycoprotein,[8] whereas *Streptococcus sanguis* 72-40 interacts only with the low molecular weight salivary mucin.[9] In other cases, a bacterium (e.g., *S. sanguis* 10556) may interact with multiple receptors.[9] As with the thin-layer chromatogram overlay assay, this technique appears to be especially useful for identifying carbohydrate receptors. The adhesion of *F. nucleatum* is mediated by unsubstituted lactosamine units on the 6-antenna of the major oligosaccharide,[8] and that of *S. sanguis* 72-40 by the terminal sialic acid residues of the mucin.[9]

Once an interaction is identified, its biological significance depends on whether structurally related endogenous receptors are present at the site of infection *in vivo*[10] and whether the strength of adhesion is greater than that of distractive forces. Because *in vivo* studies in humans are not usually possible, an approach for measuring the adhesive strength of the bacteria–receptor interactions as one means of determining whether they actually occur *in vivo* has been devised. To do so, we adapted an adhesion assay that has been used to quantify the strength of eukaryotic cell–cell and cell–extracellular matrix adhesive interactions.[11-13] Bacteria are centrifuged onto receptor-coated microtiter wells. The plates are sealed with pressure-sensitive, double-sided tape so that they can be inverted and are centrifuged again. In the assay, the centrifugal force required to remove the bacteria is directly related to the adhesive strength of the interaction. Importantly, the design of the assay avoids liquid shear forces, generally 10^{-4} dynes, which are inherent in many adhesion assays[14] and which prevent the measurement of binding strengths that are weaker than the liquid shear forces. This technique has been used to measure the adhesive strengths of interactions between bacteria, many of whose receptors had been characterized by overlay assays, and parotid and submandibular/sublingual (SM/SL) salivas. Often the measured strength of the interactions suggested that they could withstand the distractive force produced as a result of salivary flow (e.g.,

[8] B. L. Gillece-Castro, A. Prakobphol, A. L. Burlingame, H. Leffler, and S. J. Fisher, *J. Biol. Chem.* **266,** 17358 (1991).

[9] P. A. Murray, A. Prakobphol, T. Lee, C. I. Hoover, and S. J. Fisher, *Infect. Immun.* **60,** 31 (1992).

[10] H. Leffler and C. Svanborg-Eden, *Am. J. Respir. Cell Mol. Biol.* **2,** 409 (1990).

[11] D. R. McClay, G. M. Wessell, and R. B. Marchase, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4975 (1981).

[12] M. M. Lotz, C. A. Burdsal, H. P. Erickson, and D. R. McClay, *J. Cell Biol.* **109,** 1795 (1989).

[13] C. A. Burdsal, M. C. Alliegro, and D. R. McClay, *Dev. Biol.* **144,** 327 (1991).

[14] G. I. Bell, *Science* **200,** 618 (1978).

6.1×10^{-8} dyne/streptococcus). Together, the nitrocellulose overlay and centrifugal force assays, which are described in the following sections, can be used to determine rapidly the identity of a receptor and the strength of an adhesive interaction, respectively.

Nitrocellulose Overlay Assay for Identifying Bacterial Receptor Proteins

Materials

Bovine serum albumin fraction V and prestained molecular weight standards for SDS–PAGE are purchased from Sigma (St. Louis, MO). Nitrocellulose membrane (0.45 μm) is obtained from Schleicher & Schuell (Keene, NH). Trypticase soy broth is purchased from Becton Dickinson Co. (Cockeysville, MD) and yeast extract from Difco Laboratories (Detroit, MI). [^{35}S]Methionine (1159 Ci/mmol) is obtained from New England Nuclear (Wilmington, DE). Na^{125}I (15.7 Ci/μg iodine) and Hyperfilm (X-ray film) are purchased from Amersham (Arlington Heights, IL). *N*-succinimidylhydroxyl[^{125}I]iodophenyl propionate (Hunter reagent, 2723 Ci/mmol) is purchased from ICN Biomedical (Irvine, CA). Gelatin is obtained from Allied Chemical (Morristown, NJ).

Procedure

The assay is summarized in Fig. 1. The first step is to run duplicate SDS–polyacrylamide gels. One gel is stained as a reference, and the other is transferred to nitrocellulose. Samples (e.g., cells, biological fluids) are solubilized in SDS–PAGE loading buffer, consisting of 0.1 *M* phosphate

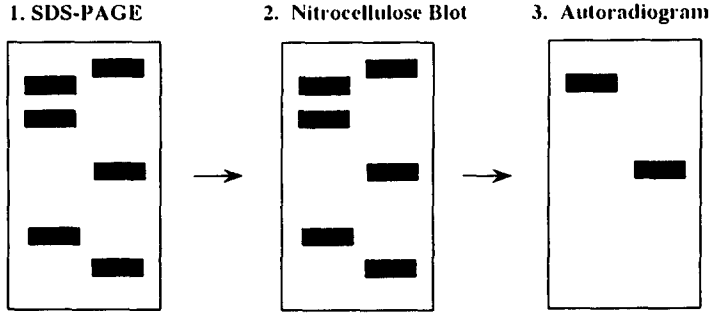

FIG. 1. Diagrammatic summary of the nitrocellulose blot assay for detecting bacterial receptor proteins.

buffer (pH 7.0) containing 6 *M* urea, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 0.015% (w/v) bromphenol blue. For screening purposes samples are adjusted to a concentration of 100 μg protein/lane. Once an interaction is detected, sample concentration is adjusted (usually downward) to give optimum resolution of receptor bands. Molecular weight standards are as follows: M_r 26,600, triose-phosphate isomerase; 36,500, lactate dehydrogenase; 45,000, ovalbumin; 58,000, pyruvate kinase; 84,000, fructose-6-phosphate kinase; 116,000, β-galactosidase; 180,000, α_2-macroglobulin. The samples are then separated on 7.5% slab gels[15]; proteins are detected with silver[16] and highly glycosylated components with periodic acid–Schiff staining (PAS).[17] Proteins are transferred to nitrocellulose membranes according to the procedure of Towbin *et al.*[18] After blotting, gels are routinely stained as described above to estimate the efficiency of transfer.

To label bacteria metabolically, batch cultures of the organisms are grown at 37° to late exponential phase (16–18 hr) in the appropriate medium containing 10 μCi/ml [^{35}S]methionine. For the bacteria we have studied (streptococci, actinomyces, fusobacteria) radiolabel incorporation is usually $2–6 \times 10^3$ bacteria/cpm (counts per minute). Bacteria are harvested by centrifugation and washed three times in 10 m*M* phosphate buffer (pH 7.0) containing 0.154 *M* sodium chloride (phosphate-buffered saline, PBS). Cells are suspended in Tris-buffered saline (50 m*M* Tris-HCl, 0.15 *M* NaCl, pH 7.4) containing 5% bovine serum albumin (TBS–BSA) and briefly sonicated on ice (<10 sec) to disperse aggregates. If BSA interferes with the binding, then nonspecific interactions can be blocked by using TBS containing 0.2% (w/v) gelatin. Bacteria are then adjusted to a final density of 5×10^8 cells/ml by A_{620} determinations previously related to cell number by direct counting in a Petroff–Hauser chamber.

If radiolabel incorporation is low, chemical labeling is also possible. Oxidation–reduction methods (chloramine-T; lactoperoxidase-catalyzed iodination) can be used. Bacteria can also be radiolabeled by using ^{125}I-labeled Bolton–Hunter reagent.[19] Two hundred fifty microcuries of the ^{125}I-labeled reagent is dried in a conical vial under a stream of nitrogen. Then, 0.5 ml containing 10^{10} cells suspended in PBS is added to the vial, and the reaction is allowed to proceed for 30 min at 4°. After the labeling procedure, the cells are washed four times in PBS. Typical incorporations of radioactivity for *Staphylococcus aureus* and *Escherichia coli* are 1000 and 100 bacteria/cpm, respectively. If a bacterium-specific antibody is available,

[15] U. K. Laemmli, *Nature (London)* **277,** 680 (1970).
[16] J. H. Morrissey, *Anal. Biochem.* **117,** 307 (1981).
[17] H. G. Wada, P. E. Haas, and H. H. Sussman, *J. Biol. Chem.* **254,** 12629 (1979).
[18] H. Towbin, T. Staehelin, and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350 (1979).
[19] A. E. Bolton and W. M. Hunter, *Biochem. J.* **133,** 529 (1973).

then unlabeled cells can be used and the attached cells visualized as described below.

The final steps are attachment of labeled bacteria to nitrocellulose blots and autoradiography. To prevent nonspecific attachment of the cells to the nitrocellulose blots, the replicas are soaked for 1 hr at 37° in TBS–BSA. Cells are suspended in that buffer and allowed to attach to the replicas for 2 hr at room temperature or for 18 hr at 4°. Then the blots are washed with PBS at least four times, 1 min each, to remove cells that are nonspecifically bound to the membrane. For visualization of bands to which radiolabeled bacteria are attached, the nitrocellulose membranes are air-dried and exposed to Hyperfilm. Use of a bacterium-specific antibody to detect bound cells is also possible.

An example of the outcome of a typical experiment is shown in Fig. 2. Figure 2A shows an SDS–PAGE gel that was stained with silver to demonstrate the protein components of human parotid and submandibular/sublingual saliva, the source of receptors in this particular experiment. Figure 2B shows an autoradiogram that was made after transferring an identical gel to nitrocellulose and overlaying it with radiolabeled *S. sanguis* 72-40. In this case, only one receptor, the low molecular weight salivary mucin, was detected.

Centrifugal Force Assay for Quantifying the Strength of Bacteria–Receptor Interactions

Materials

Unless otherwise indicated, the source of materials is the same as in the preceding section. Anisaldehyde and molybdenum blue reagent are purchased from Sigma. Fibronectin is obtained from Collaborative Research (Lexington, MA). Falcon flexible flat-bottomed polyvinyl chloride 96-well microtiter plates (Microtest III flexible assay plates) are obtained from Becton Dickinson (Oxnard, CA). Pressure-sensitive, double-sided tape is purchased from Scotch 3M (St. Paul, MN). The microcentrifuge tube cutter is obtained from Research Products International (Mount Prospect, IL). Alternatively, a pet toenail clipper can be used. In both cases the guard at the end of the retracting blade is replaced with a custom-made brass fitting with a 7-mm circular opening and a depth that can accommodate the bottom 3 mm of the microtiter well.

Procedure

The routine assay has been adapted from that devised by McClay *et al.*[11] The major steps are diagrammed in Fig. 3. Microtiter wells are coated

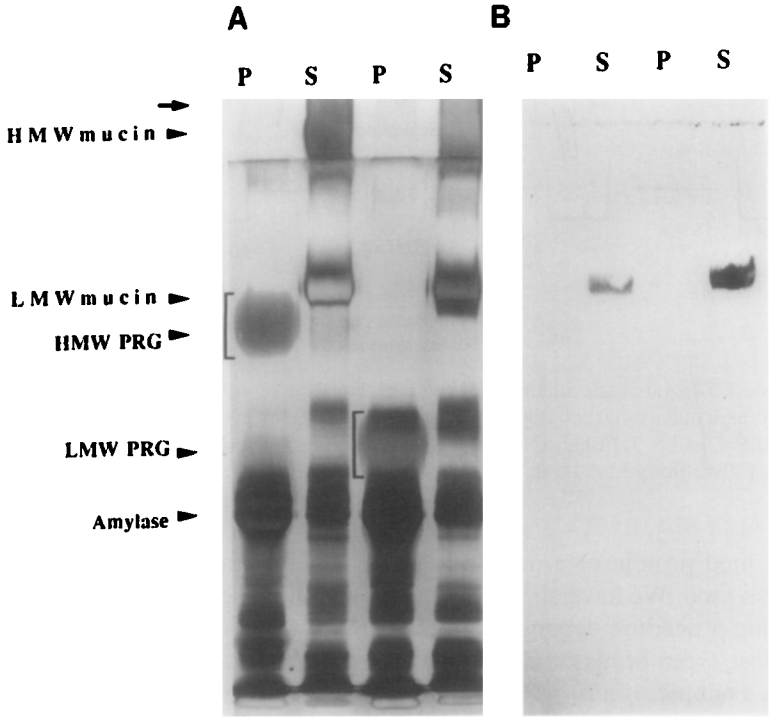

FIG. 2. (A) Analysis by SDS–PAGE of parotid (P) and submandibular/sublingual (S) salivas collected from subject 1 (first 2 lanes) and subject 2 (second 2 lanes). The components were separated on 7% gels and visualized by staining with silver. Nitrocellulose blots of the samples were used in all the adhesion assays. HMW, High molecular weight; LMW, low molecular weight; PRG, highly glycosylated proline-rich glycoprotein; →, origin. (B) Binding of ^{35}S-labeled *S. sanguis* 72-40 to the low molecular weight mucin. The salivary glycoproteins were separated by SDS–PAGE, transferred to nitrocellulose, and overlaid with the metabolically labeled bacteria. Adhesion to purified protein was detected by autoradiography. (Reprinted by permission from Murray *et al.*[9])

with protein and radiolabeled bacteria are added. The plates are sealed with pressure-sensitive tape and centrifuged to bring the bacteria rapidly into contact with the coated wells (spin on). After incubation, the plates are inverted and spun for 10 min over a range (10–450 *g*) of centrifugal forces (spin off). The wells are immediately frozen in a slurry of dry ice and ethanol, thus halting the adhesion process. The bottom 3 mm of the well is cut out, and the number of organisms bound is calculated from the radioactivity per well, as determined by scintillation spectroscopy, and the specific activity of the labeled bacteria.

The first step is to coat microtiter wells with a receptor protein. Either

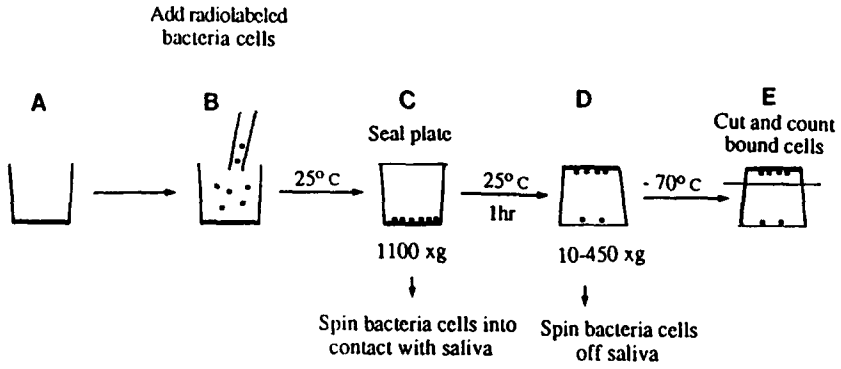

FIG. 3. Diagrammatic summary of the centrifugal force adhesion assay for determining the strength of bacterial adhesive interactions. [Reprinted by permission from A. Prakobphol, C. Burdsal, and S. J. Fisher, Quantifying the strength of bacterial adhesive interactions with salivary glycoproteins, *J. Dent. Res.* (1995) in press.]

a purified protein or a mixture such as a biological fluid or a cell extract can be used. We have also coated wells with lipid receptors. As the specific coating procedure depends on the receptor, we describe a method for each of these types of materials. The protocol for coating wells with fibronectin is an example of a procedure that can be used for a purified molecule. In this case 50 μl of a 10 μg/ml solution of fibronectin in PBS is added to each well. After incubation for 2 hr at room temperature or overnight at 4°, the fibronectin solution is removed and the wells are washed three times with PBS and used immediately. The protocol employed for coating wells with saliva is an example of a procedure that can be applied when receptors are components of complex protein mixtures. In this case microtiter plates are incubated overnight at 4° with 50 μl of fresh, undiluted parotid saliva or 50 μl of a 1:2 (v/v) mixture of SM/SL saliva, diluted with PBS to reduce the viscosity. The coated wells are washed three times with PBS and used immediately. Finally, to coat microtiter wells with lipids, the sample is first dissolved in methanol (20 μg/ml). A sample (50 μl) is added to the microtiter well and allowed to evaporate to dryness by standing at room temperature for 1 hr. Nonspecific binding to the wells is blocked by incubation with TBS–BSA or TBS containing 0.2% gelatin for 1 hr at room temperature, after which the protein solution is removed.

Next, it is important to determine the efficiency of the coating procedure. Again, there are many possible approaches depending on the reagents available (e.g., antibodies). If a purified molecule is used, then the amount of protein that is adsorbed to the well can be measured directly. When a mixture of proteins is used, additional experiments are necessary to deter-

mine if certain components are preferentially adsorbed. One approach is to coat several wells, remove the adsorbed proteins by solubilizing in SDS–PAGE loading buffer, and separate them by SDS–PAGE.[15] The separated proteins can then be detected by staining the gels with silver[16] or, in the case of highly glycosylated molecules, with PAS.[17] In this way the relative abundance of the adsorbed components can be compared with their abundance in the sample that was used to coat the wells. When the wells are coated with lipid receptors, the adsorbed material is recovered by redissolving in methanol and separated by thin-layer chromatography. The detection method depends on the nature of the lipid. For example, glycolipids are visualized by staining with anisaldehyde[1] and phospholipids with molybdenum blue reagent.[20] In this way the relative abundance of the adsorbed lipid can be estimated by comparing its staining intensity with that of standards of known concentration.

Once a procedure has been devised, every other row of wells is coated so that during the subsequent centrifugation steps overflow runs into empty wells. After the wells have been blocked, 125 μl PBS, 50 μl radiolabeled bacteria (1–2.5 $\times$ 10^8/ml), prepared as described in the previous section, and another 125 μl PBS are added. This volume (300 μl total) produces a slight positive meniscus in the well which helps to eliminate trapping of air. The plate is then carefully sealed with pressure-sensitive tape. To bring the bacteria into contact with the bottom of the well, the plates are centrifuged in a microtiter plate carrier at room temperature for 10 min (spin on). The speed depends on the organism size. For example, we use 1100 *g* for streptococci and 10 *g* for *C. tropicalis.* After incubation at room temperature for 1 hr, the plates are inverted and centrifuged again for 10 min at room temperature (spin off); duplicate plates are subjected to different centrifugal forces ranging from 10 to 450 *g*. The plates are then removed from the centrifuge in the inverted position and submerged in ethanol/dry ice to freeze the contents of each well. To determine the total number of cells that came into contact with the substrates, some plates are frozen immediately after the spin-on step. The bottom 3 mm of each well is clipped off with a modified microcentrifuge tube cutter and transferred to a scintillation vial. The number of bacterial cells that remained bound is then determined by quantifying the radioactivity in each well.

The percentage of cells bound is equal to the number of cells bound after the spin-off step divided by number of cells brought into contact by the spin on step times 100. The data are plotted as percentage of cells bound as a function of relative centrifugal force. Dyne force per cell is calculated by the expression F = (specific density of the cell − specific

[20] J. C. Dittmer and R. L. Lester, *J. Lipid Res.* **5,** 126 (1964).

density of the medium) × volume of the cell × relative centrifugal force (g, where g is 980 dynes/g). For the bacterial strains tested thus far the specific density of the cells (1.07 g/cm^3) was calculated by Percoll density gradient centrifugation. The specific density of the PBS was 1.0 g/cm^3. The cell volumes were calculated based on measurements taken from *Bergey's Manual of Systematic Bacteriology*.[21,22] For example, the average cell volume calculated as a sphere for the streptococci was 0.52 μm^3. The cell volumes calculated as rods are 3.5 μm^3 for *A. viscosus* and 1.5 μm^3 for *F. nucleatum*.

Three control experiments are routinely conducted. In the first, the spin-on centrifugal force is varied to determine the point at which the maximum number of cells is brought into contact with the substrate. Second, the time course of initial bacterial adhesion must be established. Bacteria are spun onto the substrate and incubated for 10–120 min before the spin-off step. Third, it is necessary to determine if the spin-on step changes the strength of the bacterium–receptor interactions. In this case bacteria are allowed to attach to coated microtiter wells for 1 hr at 1 g. Some of the wells are frozen immediately to determine the total number of cells that came into contact with the substrates, whereas others are subjected to the spin-off step to measure the strength of adhesion.

Figure 4 illustrates the outcome of a typical experiment. In this case we measured the strength of adhesive interactions between two strains of *A. viscosus* and saliva. One strain (T14 V) causes rapid tooth decay and alveolar bone loss in rats, whereas the other (T14 AV) is not pathogenic.[23] At the lowest centrifugal force tested, T14 V (Fig. 4A) adhered well to microtiter wells coated with either SM/SL or parotid saliva, whereas T14 AV (Fig. 4B) adhered less well to both substrates and better to SM/SL-coated than to parotid-coated wells. The avirulent strain was removed from saliva-coated wells by a force of 2.2×10^{-8} dyne/cell. In contrast, at all centrifugal forces tested, substantially more of the virulent organisms remained bound to both parotid and SM/SL saliva. For example, at a centrifugal force of 1.1×10^{-7} dyne/cell, 40–50% of the virulent organisms remained attached to both saliva substrates. The data are consistent with previously described differences in the ability of the two strains to bind to saliva-coated hydroxylapatite[24] and suggests the possibility that T14 V–saliva

[21] N. R. Krieg, "Bergey's Manual of Systematic Bacteriology," Vol. 1. Williams & Wilkins, Baltimore, Maryland, 1984.

[22] P. H. A. Sneath, "Bergey's Manual of Systematic Bacteriology," Vol. 2. Williams & Wilkins, Baltimore, Maryland, 1986.

[23] S. M. Brecher, J. van Houte, and B. F. Hammond, *Infect. Immun.* **22,** 603 (1978).

[24] T. T. Wheeler, W. B. Clark, and D. C. Birdsell, *Infect. Immun.* **25,** 1066 (1979).

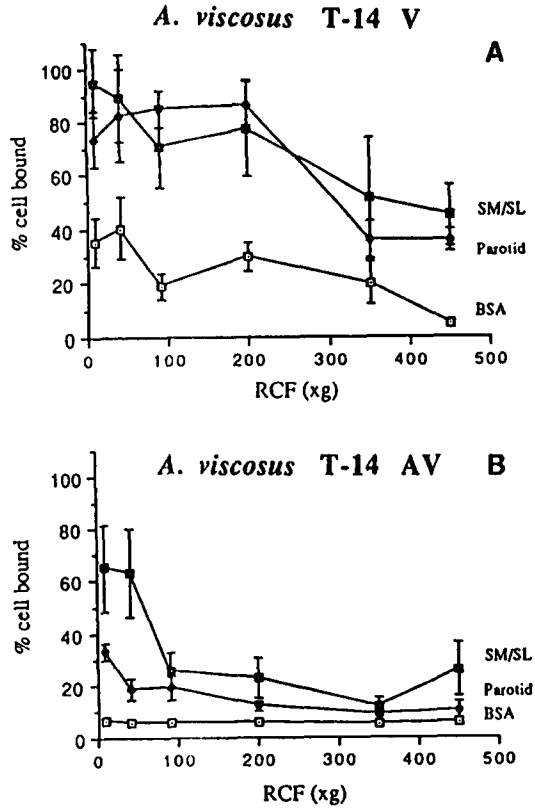

FIG. 4. Interactions of *A. viscosus* with parotid and submandibular/sublingual (SM/SL) salivas assayed by centrifugal force. RCF, Relative centrifugal force; BSA, bovine serum albumin. [Reprinted by permission from A. Prakobphol, C. Burdsal, and S. J. Fisher, Quantifying the strength of bacterial adhesive interactions with salivary glycoproteins, *J. Dent. Res.* (1995) in press.]

interactions have a greater adhesive force than those between saliva and T14 AV.

Conclusions

Two assays have been developed that can be used to gain important information concerning bacterial receptors. In the first, nitrocellulose blots are overlaid with radiolabeled bacteria, allowing the rapid identification, by autoradiography, of proteins that can act as bacterial receptors. In the second, the force by which bacteria bind to the receptors is quantified

by determining the centrifugal force required to break the interaction. Modifications to both protocols can be used to gain even more information about the interactions detected. For example, data concerning structural specificity of adhesion mechanisms can be obtained by including a variety of inhibitors in the adhesion assay.

Acknowledgments

Research was supported by a grant from the National Institutes of Health (DE07244). We thank Dr. Carol Burdsal and Dr. David McClay for assistance in applying the centrifugal adhesion assay to quantify the strength of bacteria–receptor interactions and Ms. Evangeline Leash for excellent editorial assistance.

[13] Electron Microscopical Methods in Adhesion

By STUART KNUTTON

Introduction

Pioneering studies of diarrheas and gonorrhea in the early 1970s highlighted the importance of bacterial adhesion as an essential early event in bacterial colonization of mucosal surfaces. The last decade has seen an explosion of information in our understanding of bacterial adhesion at both the molecular and genetic level, and electron microscopy has contributed significantly to this knowledge. Initially, electron microscopy, mainly of negatively stained preparations, was used to identify and characterize the proteinaceous filamentous bacterial surface structures, termed fimbriae or pili, which functions as adhesins. Indeed, bacterial fimbriae were unknown before their visualization in the electron microscope. Negative staining has been the technique extensively used to characterize adhesion fimbriae, and, in a few cases, subsequent image analysis has allowed a detailed description of the fimbrial structure. The proteinaceous nature of fimbriae makes them good antigens, a feature which has been exploited in immunological studies of bacterial fimbriae and their constituent proteins using immunonegative staining. Electron microscopical studies of bacteria–cell interactions have mainly employed scanning electron microscopy and transmission electron microscopy of thin sections together with associated immunological applications of the above methods. Other electron microscopical techniques (e.g., shadowing, freeze fracture) have been applied to studies of bacterial adhesion, but this chapter reflects the most commonly used methods.

Negative Staining

An objective of all microscopy is to attain good contrast and resolution of the specimen. In negative staining this is achieved by surrounding/embedding the specimen in a thin film of heavy metal stain such that unstained structures show up against the heavy metal stain in negative contrast.[1,2] The following procedure is one we have developed and which routinely yields good quality preparations for examining bacterial fimbriae.

[1] M. A. Hayat, *in* "Principles and Techniques of Electron Microscopy." Macmillan, 1989.
[2] A. W. Robards and A. J. Wilson (eds.), "Procedures in Electron Microscopy." Wiley, 1993.

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Negative Staining Procedure

1. Prepare a concentrated bacterial suspension in water ($\sim 10^8$–10^9 cells/ml).
2. Mix 10-μl aliquots of the bacterial suspension, the negative stain (2% ammonium molybdate, pH 6.8), and bacitracin (150 μg/ml).
3. Apply 10 μl of the mixture to carbon-coated 400-mesh copper grids for 2 min.
4. Remove excess liquid and allow to air-dry.

Although negative staining is basically a very simple technique, there are a number of points which need to be addressed in order to obtain good quality preparations routinely. Bacteria should be washed in phosphate-buffered saline, pH 7.2 (PBS), and only suspended in distilled water just prior to negative staining. The bacterial density and length of time the droplet is left on the grid need to be worked out empirically, although the figures quoted should provide a good starting point. Crucial to good negative staining, however, is good spreading of the negative stain, and the most important factor for this is to use hydrophilic grids. Glow discharge in a vacuum evaporator is probably the best method to achieve good spreading, but if this is not available the more simple and convenient UV irradiation of grids for 30 min prior to use is effective. Additionally, bacitracin is included as a "wetting" agent[3] to assist spreading of the negative stain. Carbon-coated grids give the best results, but carbon–Formvar grids can be used and do have the advantage of providing more stable supports for particles such as bacteria. Fine crossover tweezers are best for holding grids during staining; several grids are usually prepared simultaneously.

There are a number of negative stains commonly in use,[1,2] but we have found ammonium molybdate to give the best results for visualizing bacterial fimbriae. Other negative stains (e.g., phosphotungstic acid, uranyl acetate) are not very effective when mixed with bacteria and thus have to be applied secondarily to drying bacteria down on a grid. Removal of the excess liquid in the final stage of the procedure is also very important. As much as possible should be removed in order to leave as thin a film of negative stain as possible. It is easy to see when the main bulk of the droplet has been removed, but the filter paper should be kept in contact with the grid for a further 5–10 sec to ensure complete removal. Adsorption of bacteria to the grid surface is improved by partial drying of the suspension during step 3. This can be achieved by placing grids beneath and close to the bulb of an anglepoise lamp.

Factors such as the hydrophilicity of the grids are impossible to control

[3] D. W. Gregory and B. J. S. Pirie, *J. Microsc.* **99,** 251 (1973).

precisely, and so poorly stained preparations can occur. However, the quality of a preparation can be assessed prior to examination in the electron microscope using phase-contrast microscopy at low power (10× objective). Well-stained preparations will show discrete, sharply defined bacteria and the background will be clean. However, the whole of the grid should be examined carefully because unidirectional drying often results in just part of the grid being optimally stained. Poor spreading of the negative stain around bacteria results in dense, enlarged, more diffuse bacterial shapes. Only well-stained grids with relatively large numbers of bacteria should be examined in the electron microscope because (a) phase variation means that many bacteria may not be producing fimbriae and (b) delicate structures like fimbriae will not be seen if they are embedded in a thick layer of negative stain surrounding bacteria. The same protocol can be used to examine concentrated purified fimbrial suspensions.

Bacterial fimbriae have a cross-sectional diameter ranging from 2 to 7 nm and vary in length from very short to several microns. A good negatively stained preparation will have a very thin, uniform layer of negative stain right up to the bacterial surface, thus allowing the finest fibrillar fimbriae to be resolved (Figure 1). The narrow 2–2.5 nm diameter channel along

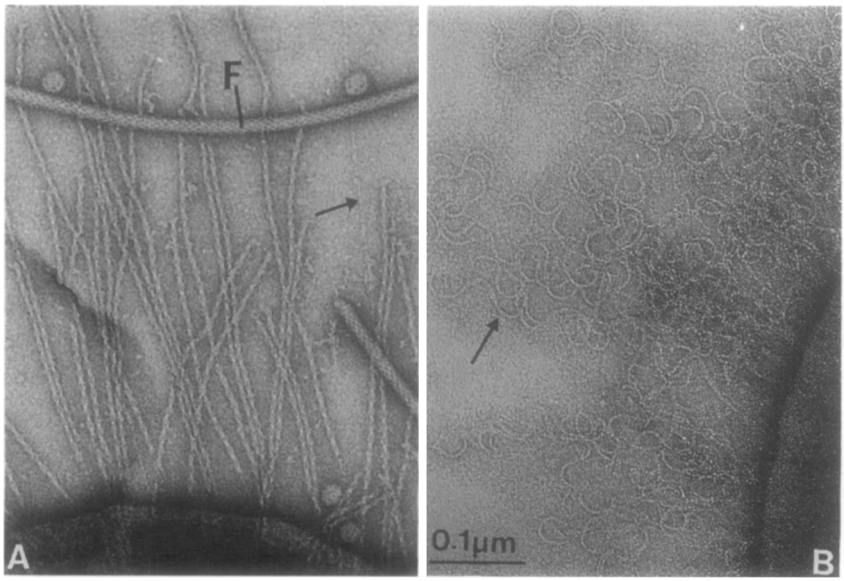

FIG. 1. Negative stain electron micrographs of bacterial fimbriae. Good negative stain preparations have a clean, uniformly thin layer of negative stain up to the bacterial surface such that fine structural detail and the finest fibrillar fimbriae are resolved (arrows). F, Flagellum.

the center of 7 nm diameter rigid rodlike fimbriae should also be resolved.[4] Care must be taken not to miss fine fibrillar fimbriae, especially when they are produced simultaneously with more prominent fimbrial structures. Some fine fibrillar fimbriae, for example, surface antigen 6 (CS6) of enterotoxigenic *Escherichia coli* are difficult to visualize routinely by negative staining even when, by immunolabeling, they are present in large amounts.[5] Such fimbriae must either be very labile or possibly prone to collapsing down onto the bacterial surface during drying. Stabilization by mild chemical fixation or antibody[6] prior to negative staining may prove useful in such cases. In a few cases, image analysis of negative stain images of fimbriae has allowed the detailed helical arrangement of fimbrial subunits to be determined.[7]

Immunonegative Staining

Immunocytochemical techniques are powerful tools for the localization of specific antigens. The development of colloidal gold probes[8] revolutionized the electron microscopical application of such techniques. Immunonegative staining was developed to label small particles which can be adsorbed onto a grid, immunolabeled *in situ,* and then contrasted by negative staining.[9] It is thus an ideal method for the immunological examination of bacterial fimbriae and nonfimbrial surface antigens. The technique is basically quite straightforward and simply incorporates an immunolabeling stage in between adsorption of bacteria onto a grid and negative staining. The aim of immunolabeling is to produce the highest specific signal with the lowest nonspecific background. Thus, indirect labeling, in which a primary unlabeled antibody is applied to the specimen to locate the antigen followed by a gold-labeled second antibody that detects the primary antibody, is now the method of choice.[2,10] It has a number of advantages over direct labeling methods: (1) it provides a universal method for detecting any primary antibody from the same primary species; (2) it leads to a significant amplification of the primary signal; and (3) a broad range of gold probes in a wide range of gold particle sizes is now widely available.

[4] S. Knutton, D. R. Lloyd, D. C. A. Candy, and A. S. McNeish, *Infect. Immun.* **44,** 519 (1984).
[5] S. Knutton, M. M. McConnell, B. Rowe, and A. S. McNeish, *Infect. Immun.* **57,** 3364 (1989).
[6] A. M. Lawn, *Nature (London)* **214,** 1151 (1967).
[7] W. Folkhard, K. R. Leonard, S. Malsey, D. A. Marvin, J. Dubochet, A. Engel, M. Achtman, and R. Elmuth, *J. Mol. Biol.* **130,** 145 (1979).
[8] W. P. Faulk and G. M. Taylor, *Immunochemistry* **8,** 1081 (1971).
[9] J. E. Beesley, *in* "Colloidal Gold, Principles, Methods and Applications" (M. A. Hayat, ed.), Vol. 2, p. 243. Academic Press, London, 1989.
[10] J. M. Polak and I. M. Varndell (eds.), "Immunolabelling for Electron Microscopy." Elsevier, Amsterdam, 1984.

Immunonegative Staining Procedure

1. Wash and prepare a concentrated bacterial suspension in PBS.
2. Apply 10-μl of the suspension to coated 400-mesh grids for 2 min and remove excess fluid.
3. Float the grid, specimen side down, on a 10- to 20-μl drop of antibody diluted with PBS containing 1% (w/v) bovine serum albumin (PBS–BSA) for 20 min.
4. Wash by transferring grids sequentially onto 5 drops of PBS–BSA for 1 min each.
5. Transfer grid to a 10- to 20-μl drop of an appropriate gold probe diluted in PBS–BSA for 20 min.
6. Wash by transferring grids onto 3 drops of PBS–BSA and 3 drops of distilled water.
7. Negatively stain with 1% ammonium molybdate, pH 6.8.

Monoclonal or polyclonal antibodies can be used; however, a high-titer primary antibody is essential for successful labeling of small structures such as bacterial fimbriae, and cross-reactivity, especially with the host tissue, must be low. Ideally polyclonal antisera will have been raised against highly purified antigen, although successful studies have been performed using antisera raised against whole bacteria which has then been absorbed with bacteria grown under conditions where surface antigens (e.g., fimbriae) are not produced (e.g., 20° instead of 37°).[11] A high-quality second antibody is essential to label the primary antibody specifically and with low background. This usually means affinity-purified conjugates which have been absorbed against serum proteins from an appropriate species to reduce cross-reactions to a minimum. Such antibodies are now widely available commercially. Goat anti-rabbit or goat anti-mouse immunoglobulin G (IgG) conjugates are the most common for use with polyclonal antibody raised in rabbits or mouse monoclonal antibodies. Smaller gold particles give higher labeling intensities, and so for higher magnification work 5- to 10-nm particles are the preferred size; 10-nm gold is probably a good size to start with for most studies. Appropriate dilutions of the antibody reagents have to be found empirically by finding the highest dilution giving good specific labeling of the antigen and a low background; a 1/50 to 1/200 dilution is typical for high-quality commercial gold conjugates.

Bovine serum albumin is included in all the immunolabeling steps to help reduce nonspecific binding of the antibodies; some protocols also include nonionic detergents such as Tween 20 in the buffer solutions for

[11] M. M. Levine, P. Ristaino, G. Marley, C. Smyth, S. Knutton, E. Boedeker, R. Black, C. Young, M. L. Clements, C. Cheney, and R. Patnaik, *Infect. Immun.* **44,** 409 (1984).

the same purpose. Tween 20 appeared to have little effect on *E. coli*,[11] although detergents may damage other unfixed organisms. Specimens must be washed thoroughly with buffer between antibody incubations. For good quality negative staining it is important not to get the underside of the grid wet during the numerous labeling and washing steps. This is most easily achieved by using a platinum loop slightly larger than the grid to transfer the grids from one droplet to another. The immunolabeling process is carried out on a hydrophobic surface such as dental wax or Parafilm. After the final wash the grid can be placed on a drop of negative stain, picked up with a pair of tweezers, inverted, and then dried as described for negative staining.

Good immunonegative stain preparations require good specific labeling of the antigen and good contrasting of the specimen by negative staining (Fig. 2). The appearance of fimbriae in negative stain preparations and after immunolabeling will be somewhat different because (a) there is cross-linking of fimbriae with divalent antibody and (b) the fine structure of fimbriae is generally lost when heavily coated with antibody (cf. Figs. 1 and 2). In addition to allowing visualization of major fimbrial subunit antigens,[5,11] immunonegative staining has now been used to localize minor

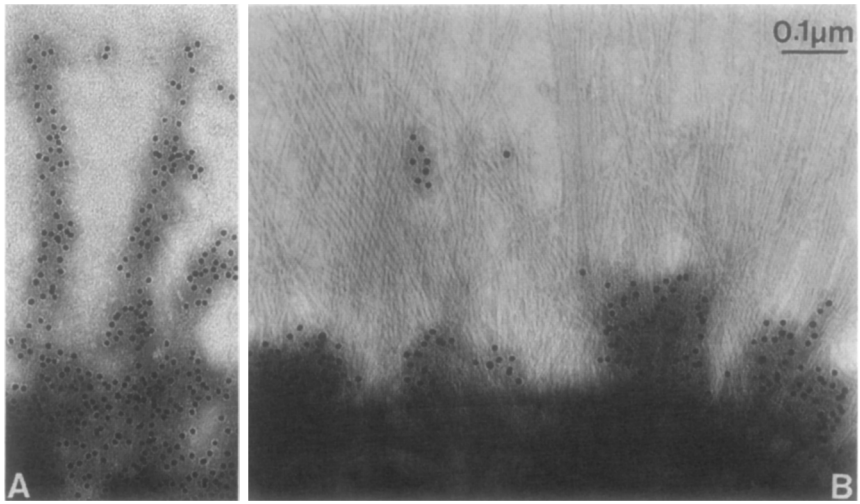

FIG. 2. Immunonegative stain electron micrographs of rodlike (A) and fibrillar bacterial fimbriae (B). Good immunonegative stain preparations show specific staining of the antigen in question (in this case with 10-nm gold), very low levels of nonspecific and background labeling, and well-contrasted unlabeled structures such as the rodlike fimbriae (B).

subunit proteins such as receptor binding adhesin subunits located at the tips of adhesion fimbriae.[12]

A major advantage of gold probes is that probes of different sizes are available and can be used for double-labeling studies. Incubating an antigen with antibody and gold probe does not usually saturate all the available antibody that is bound to the antigen, and so if the first label was followed by a second antibody and gold probe there would be cross-reaction of the second gold probe on the first antibody. Double labeling with different sized gold particles using antisera raised in the same species is possible, but it is necessary to saturate all available first antibody sites by carrying out successive incubations with the first gold probe. Appropriate control experiments must be carried out to ensure saturation of the primary antibody.[9] A better alternative would use primary antibodies raised in different hosts where there were minimal cross-reactions. Double labeling has been applied to studies of fimbrial biogenesis[13] and to demonstrate multiple antigenicity of pilus antigens.[14]

Scanning Electron Microscopy

Bacterial adhesion is a cell surface interaction which makes it ideal for examination by scanning electron microscopy (SEM). Compared to transmission electron microscopy of sectioned material, SEM has the advantage that large areas of cell surface can be rapidly examined for adherent organisms; specimen preparation and examination are also much simpler.

Specimen Preparation for Scanning Electron Microscopy

1. Washed tissue/cells are fixed in glutaraldehyde fixative (3% (v/v) in 0.1 *M* phosphate buffer, pH 7.2) for 1 hr to overnight.
2. Postfix in osmium tetroxide (1% in 0.1 *M* phosphate buffer) for 1 hr.
3. Dehydrate samples through a graded series of acetone solutions as follows:

50% acetone	15 min
70% acetone	15 min
90% acetone	15 min
100% acetone	15 min (two times)
100% dry acetone	15 min

[12] F. Lindberg, B. Lund, L. Johansson, and S. Normark, *Nature (London)* **328,** 84 (1987).
[13] M. A. Lowe, S. C. Holt, and B. I. Eisenstein, *J. Bacteriol.* **169,** 157 (1987).
[14] J. E. Beesley, S. E. J. Day, M. P. Betts, and C. M. Thorley, *J. Gen. Microbiol.* **130,** 1481 (1984).

4. Critical Point Dry samples from acetone.
5. Mount samples onto SEM stubs.
6. Sputter coat samples with gold.

As with all electron microscopy specimen preparation, fixation should be performed using fixative freshly made up from electron microscopy (EM) grade reagents; postfixation with osmium is not always necessary for SEM specimens. The duration of fixation will vary with the sample; in general, fixation for 1 hr in buffered glutaraldehyde should be adequate for most animal and plant tissue. Mucus may be a problem when looking at some mucosal surfaces by SEM. This is best removed prior to fixation by thorough washing although, since mucus is only weakly associated, it is sometimes possible to remove mucus following critical point drying using a jet duster. For most samples a buffer wash after fixation is sufficient to give a clean surface for the SEM. Critical point drying, which removes the interface between liquid and vapor before drying and thus eliminates damaging surface tension forces, is the drying method of choice[2] and is carried out using a dedicated piece of apparatus available in most routine electron microscopy laboratories.

It is frequently useful to design sample holders for individual applications because commercial instruments are usually very limited in this respect. For example, a spring is useful for holding glass coverslips when the specimen consists of cell monolayers. Double-sided cellotape, rapid Araldite, or silver paint is generally used to mount samples on SEM stubbs; some samples (e.g., pieces of tissue) will also need orienting during mounting to ensure that mucosal surfaces are uppermost. Samples placed in the SEM must be conductive enough to remove electrons deposited by the primary beam. Sputter coating a 10- to 20-nm layer of gold or other appropriate metal (e.g., gold/palladium or platinum) onto the specimen surface is the method of choice[2]; a commercial sputter coating apparatus should be available in electron microscopy laboratories with SEM facilities.

Scanning electron microscopy has been mainly used to examine mucosal surfaces from both natural and experimental infections of animals and humans and to examine tissue and tissue culture cells experimentally infected *in vitro*. Its main advantage is that large areas of mucosal and cell surfaces can be examined for adherent bacteria rapidly by scanning a specimen at low (200–300×) magnification (Fig. 3A). Adhesion can then be assessed qualitatively or quantitatively. For quantitative analysis a defined number of fields are selected at random, photographed, and bacterial adherence assessed to give an adhesion index consisting of numbers of bacteria/

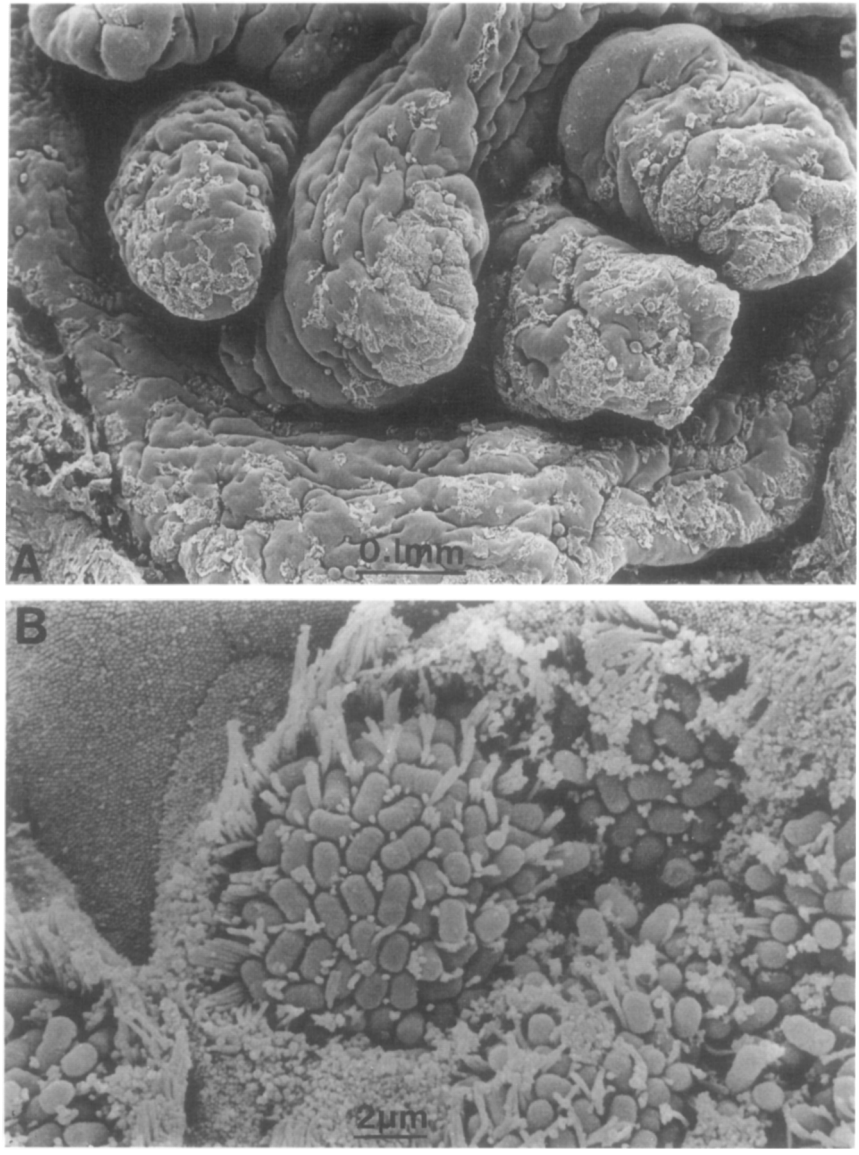

FIG. 3. Scanning electron micrographs of human small intestinal mucosa infected with enteropathogenic *Escherichia coli* (EPEC). The micrographs illustrate the ease of screening large areas of mucosal surface for adherent bacteria at low magnification (A) and the fine detail of adhesion mechanisms visible at higher magnifications (B). In this example EPEC are seen to adhere intimately in microcolonies and to cause gross alterations to the brush border surface of infected enterocytes.

unit area[15] or percentage of area colonized by bacteria.[16] Patterns of bacterial adhesion can be of diagnostic value, and this can also be assessed in tissue samples by low-power SEM,[17] although tissue culture cell adhesion assays examined by light microscopy are generally the method of choice for this application.[18] Another application of SEM is determination of the tissue and cell type specificities of adhesion.[19] The resolution of the SEM is rarely sufficient to obtain detailed information about the mechanisms of adhesion although it has proved useful in a number of cases where bacterial adhesion results in gross cell surface alterations[20] (Fig. 3B). *In vitro* SEM studies have also been performed using formalin-fixed tissue,[17] but receptors for bacterial adhesion may be altered by the fixation process. Furthermore, fixed tissue would be inappropriate for studies of organisms which cause structural alterations during the adhesive process.

Large gold particles (>20 nm) can be imaged in the SEM with good resolution, but immunolabeling for SEM does not appear to have been used to any degree in studies of bacterial adhesion. The procedure would involve a preembedding method (as described below for transmission electron microscopy) followed by the standard SEM preparative procedure.[2] Gold particles 20–30 nm in diameter need to be used; however, for higher labeling intensities, smaller (5 nm) gold conjugates could be used and subsequently enlarged by silver enhancement.[21] Yamamoto *et al.* used gold labeling methods for SEM to distinguish between indigenous and experimental bacteria in studies of mucosal adhesion.[15]

Transmission Electron Microscopy

Transmission electron microscopy (TEM) of sectioned material, with or without immunolabeling, has primarily been used to provide high-resolution information about the mechanisms of interaction of bacteria with cell surfaces and the antigens involved. The most commonly used procedure involving embedding in epoxy resin is described. Bacterial adhesion involves surface antigens of both the bacterium and target cell. Preembedding

[15] T. Yamamoto, S. Endo, T. Yokota, and P. Echeverria, *Infect. Immun.* **59,** 3722 (1991).
[16] S. Knutton, A. D. Phillips, H. R. Smith, R. J. Gross, R. Shaw, P. Watson, and E. Price, *Infect. Immun.* **59,** 365 (1991).
[17] G. Hinson, S. Knutton, M. K.-L. Lam-Po-Tang, A. S. McNeish, and P. H. Williams, *Infect. Immun.* **55,** 393 (1987).
[18] M. S. Donnenberg and J. P. Nataro, this volume [26].
[19] T. Yamamoto and T. Yokota, *Infect. Immun.* **57,** 2410 (1989).
[20] S. Knutton, D. R. Lloyd, and A. S. McNeish, *Infect. Immun.* **55,** 69 (1987).
[21] C. S. Holgate, P. Jackson, P. N. Cowen, and C. C. Bird, *J. Histochem. Cytochem.* **31,** 938 (1983).

rather than postembedding immunolabeling is therefore the method of choice for studies of bacterial adhesion.[2,10] This is achieved by carrying out the immunolabeling stage prior to fixation and subsequent tissue processing.

Schedule for Transmission Electron Microscopy

1. Fix specimen with glutaraldehyde fixative (3% (v/v) in 0.1 *M* phosphate buffer, pH 7.2) for 1 hr to overnight.
2. Postfix in osmium tetroxide (1% (w/v) in 0.1 *M* phosphate buffer) for 1 hr.
3. Stain *en bloc* with 0.5% (w/v) aqueous uranyl acetate for 2–4 hr.
4. Dehydrate samples through a graded series of ethanol and propylene oxide solutions as follows (note that specimens can be stored at 4° in 70% alcohol after the first stage):

70% (v/v) ethanol	15 min
90% (v/v) ethanol	15 min
100% ethanol	15 min (two times)
Dried 100% ethanol	15 min
Propylene oxide	15 min (two times)

5. Infiltrate specimens with epoxy embedding resin (Epon or equivalent) on a rotator as follows:

3:1 (v/v) propylene oxide/resin	1 hr
1:1 propylene oxide/resin	1 hr
1:3 propylene oxide/resin	1 hr
100% resin	overnight

6. Place specimens into an embedding mold in fresh resin, orient if necessary, and polymerize at 60° for 24 hr.
7. Select areas for ultrathin sectioning by light microscopic examination of 1-μm-thick sections stained with 1% toluidine blue.
8. Cut 60- to 100-nm-thick (gray-gold) sections.
9. Stain sections with uranyl acetate (25% solution in methanol) for 10 min followed by three washes in methanol.
10. Stain sections with Reynold's lead citrate[22] for 5 min followed by two distilled water washes, 10 sec, in 0.001 *N* sodium hydroxide and two further distilled water washes.

Note. An automatic section stainer should be used if available.

For tissue samples, small approximately 2 mm^2 pieces should be processed; tissue culture cells grown on plastic coverslips or membrane filters can be processed directly. *En bloc* uranyl acetate staining gives significantly

[22] E. S. Reynolds, *J. Cell Biol.* **17,** 208 (1963).

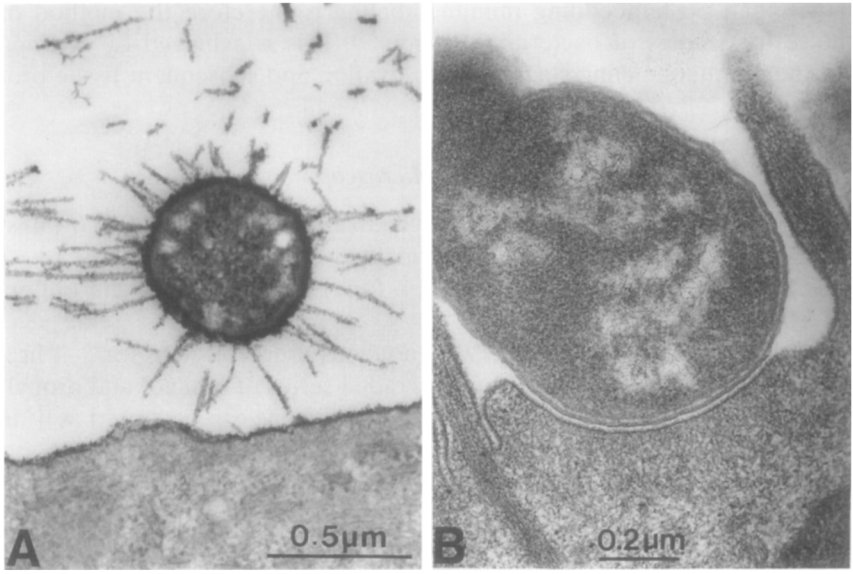

FIG. 4. Transmission electron micrographs of bacterial adhesion illustrating nonintimate adhesion mediated by rodlike fimbriae visualized by ruthenium red staining (A) and intimate adhesion mediated by nonfimbrial outer membrane proteins (B). Good TEM preparations show fine detail of bacterial and cell structure and the interaction between them.

improved contrast.[23] To save time on resin infiltration and embedding, vacuum embedding is a preferred method.[24] The procedure, which replaces step 5 in the above schedule, is as follows: (1) Transfer samples to a 1:1 mixture of propylene oxide/epoxy resin on a rotator for 45 min. (2) Outgas 100% resin for 30 min (while specimens are being dehydrated) and place samples just below the surface of the resin in a Teflon (not silicone rubber) embedding mold. (3) Place the mold in a vacuum embedding oven, switch the oven heater on (set to 60°) and pull a vacuum of 760 torr. Leave under vacuum for 20 min only and return very slowly (5–10 min) to atmospheric pressure. (4) Orient specimens if necessary and polymerize the resin at 60° for 24 hr. Several stages of the protocol involve toxic chemicals, and so the procedure needs to be carried out in a fume hood and the appropriate safety measures adhered to.

The routine production of ultrathin sections requires a high level of skill, and the technique is time-consuming; it should therefore only be used

[23] D. P. Knight, *in* "Practical Methods in Electron Microscopy, Volume 5: Staining Methods for Sectioned Material" (A. M. Glauert, ed.), p. 25. North-Holland, Amsterdam, 1977.

[24] M. P. Osborne, *in* "Neuroanatomical Techniques" (N. J. Straussfield and T. A. Miller, eds.), p. 205–239. Springer-Verlag, New York, 1980.

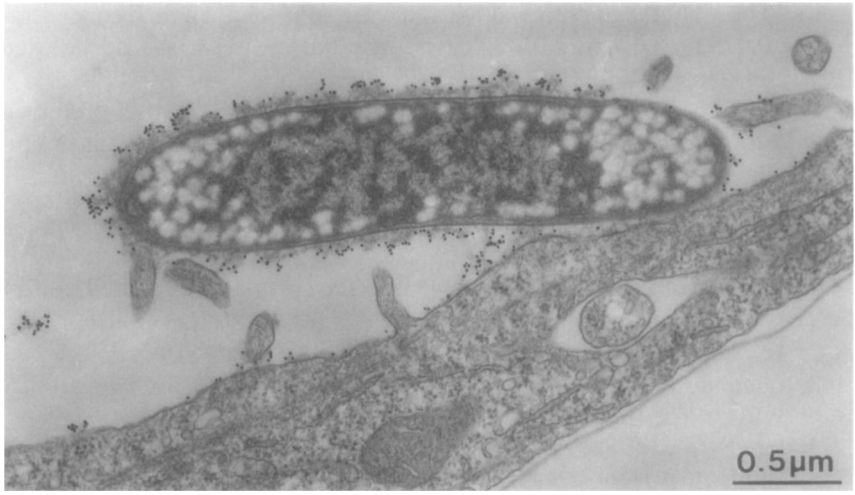

FIG. 5. Preembedding immunolabeling of fine fibrillar fimbriae which mediate adhesion of the bacterium to tissue culture cells. Good preparations show specific gold labeling of the fimbrial antigen (in this case with 10-nm gold), very low or no nonspecific or background labeling, and good resolution and contrasting of the bacterial and cell structure.

for studies where alternative simpler techniques are inappropriate. Thin sectioning will reveal both the bacterial and cell surface structure at high resolution, the nature of the interaction, plus any associated alterations in cell surface architecture. In general, bacterial adhesion will be fimbrially or nonfimbrially mediated and thus show nonintimate or intimate association of the bacterial and cell surfaces (Fig. 4). One problem in studying fimbrially mediated adhesion is a general lack of fimbrial staining with traditional heavy metal stains, probably owing to the hydrophobic nature of many fimbrial proteins. Thus, micrographs frequently show poorly defined fimbriae or no fimbriae at all and simply a translucent gap between the bacterium and the cell surface. Nonspecific staining of fimbriae has been achieved using stains such as ruthenium red[25] (Fig. 4A) although the explanation for this is not clear. Ruthenium red stains acidic polysaccharides[26] which are present at cell surfaces as glycocalyx but which are not a component of bacterial fimbriae. A possible explanation is that fimbriae become coated with glycocalyx polymers and thus show up after ruthenium red staining. If this is the case, care needs to be taken with interpretation since positively stained fibrils could either be glycocalyx fibers or fimbrial structures coated with glycocalyx. If ruthenium red staining is performed,

[25] S. Knutton, D. R. Lloyd, D. C. A. Candy, and A. S. McNeish, *Infect. Immun.* **44,** 514 (1984).
[26] J. H. Luft, *Anat. Rec.* **171,** 347 (1971).

it should be included in both fixative and wash solutions at a final concentration of 0.075% (w/v), and 0.1 *M* cacodylate buffer, pH 4, should be used instead of phosphate buffer.[26]

A preembedding immunogold labeling technique is the preferred method for demonstrating involvement of specific fimbrial antigens in mucosal adhesion.[2,10]

Preembedding Immunogold Staining Procedure

1. Prefix samples for 10 min at 4° in 0.1% (v/v) glutaraldehyde or 2–4% (w/v) formaldehyde in PBS, pH 7.4.
2. Wash three times in PBS.
3. Incubate tissue with antibody diluted in PBS–BSA for 1 hr at 4°.
4. Wash three times with PBS–BSA.
5. Incubate with gold probe diluted in PBS–BSA for 1–4 hr at 4°.
6. Wash three times in PBS and then follow the above schedule for TEM.

The choice of glutaraldehyde or formaldehyde depends on the sensitivity of the antigen. Prefixation with glutaraldehyde results in better preservation of ultrastructure but sometimes destroys the antigenicity of proteins; formaldehyde preserves antigenicity better but preserves ultrastructure less satisfactorily. Fifty millimolar glycine can be added to the subsequent wash solutions to quench free aldehyde groups. As with all immunolabeling procedures, optimal dilutions of antibodies and incubation times have to be determined empirically. In our experience quite a long incubation (several hours) with the gold conjugate is required for good labeling of infected intestinal mucosa using this procedure. The specificity of the gold labeling can be assessed by omitting the primary antibody or by using a heterologous antibody, although, in the case of bacterial surface antigens, it is usually apparent if labeling is specific to the bacterium. As described for immunonegative staining, double-antigen labeling can be performed, but the technique requires that still available antigen binding sites of the first antibody are saturated before incubation with the second antibody. Preembedding immunogold labeling has been used to localize specific bacterial surface antigens and demonstrate their role in mediating cell adhesion[27] (Fig. 5).

Acknowledgment

This work was supported by the Wellcome Trust.

[27] S. Knutton, D. R. Lloyd, D. C. A. Candy, and A. S. McNeish, *Infect. Immun.* **48,** 824 (1985).

[14] Use of Confocal Microscopy in Studying Bacterial Adhesion and Invasion

By PAUL A. MANNING

Introduction

Light microscopy has undergone a revolution with the advent and commercial availability of the laser-scanning confocal microscope. This instrument is the product of developments in a number of areas including high numerical aperture plan-apochromatic objectives, confocal imaging, laser-illuminated optics for the generation of fluorescent and transmitted light images, image scanning, and high-speed computing and video recording. The resultant technology provides a means of optically generating discrete thin sections which can be viewed individually, in a desired sequence, or subjected to image analysis to produce high-resolution three-dimensional images of the specimen. The history, theoretical and practical aspects of confocal microscopy, and numerous applications are presented in a treatise by Pawley.[1]

In the confocal system, the illumination and detection are confined to the same spot on the specimen at any one time, and only that part which is in focus is able to be detected, that is, out-of-focus regions appear black. Thus, by focusing on various planes through a specimen and storing the individual images, it is possible to analyze the relative three-dimensional locations of different components of the sample either in the individual optical sections or in a composite image comprising the sections, or, by means of suitable computational software packages, it is possible to perform a three-dimensional reconstruction of the specimen.

In an epilumination confocal format, the same lens functions as both the condenser and the objective lens. Thus, light from an aperture is reflected into the rear of the objective lens and is focused on the specimen. The light returning from the specimen, arising from either reflection or emission produced by fluorescence, passes back through the lens and is focused on a second aperture, and it is the size of that aperture which determines the amount of light detected. The confocal effect is enhanced by decreasing the size of the aperture and also increasing the numerical aperture of the lens. For optimal results, the objective lens must give a flat field because even a small degree of curvature to the field will reduce the

[1] J. B. Pawley, "Handbook of Biological Confocal Microscopy." Plenum, New York and London, 1990.

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ability to produce good optical sections. A plan-apochromatic objective with a numerical aperture (N.A.) of 1.4 and about 60× magnification is recommended, and further magnification usually can be applied digitally by means of the software.

The use of a transmission detector, to detect the light that has passed through the specimen, also permits the generation of a pseudo-phase-contrast or Nomarski-type image even though it is effectively using bright-field illumination. This is due to the nature of the scanning beam. This image will also be perfectly superimposable on the confocal image. Because of the capacity for electronic enhancement, these images may offer better resolution than conventional light microscopy.

The computer is an important component of the confocal system as it stores the individual scans being made by the laser, and it later brings the scans together to generate the complete optical image of the specimen. The resultant image may be a superimposition of a contiguous series of scans through the whole specimen or of a particular region, or, with the aid of suitable image analysis software packages, the data obtained from each of the two-dimensional scans can be assembled with the third-dimension sectional data to produce a three-dimensional image which may be looked at from various directions by rotation on the computer screen. The moving images may also be stored on videotape. All of the information can be obtained without physically sectioning the specimen.

Studies of Bacterial Adhesion and Invasion

Studies in microbial pathogenesis have proceeded at a rapid rate owing to the combination of molecular genetic and cell biological technologies to examine the interaction of the bacterium with its future host. Such studies are facilitating the definition of the various stages of the invasion process in a number of bacterial systems. Two well-studied examples from the laboratory of Philippe Sansonetti are the pathogenic bacteria *Listeria monocytogenes*[2,3] and *Shigella flexneri*.[4,5] In conjunction with cloning of the relevant genes and isolation of specific mutants, the analyses of the adhesion and invading process have involved the use of fluorescence light microscopy and scanning and transmission electron microscopy. Studies using confocal microscopy with fluorescent markers have now added new information to

[2] J.-L. Gaillard, P. Berche, J. Mounier, S. Richard, and P. J. Sansonetti, *Infect. Immun.* **55,** 2822 (1987).

[3] J. Mounier, A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti, *Infect. Immun.* **58,** 1048 (1990).

[4] P. Clerc and P. J. Sansonetti, *Infect. Immun.* **55,** 2681 (1987).

[5] M. L. Bernardini, J. Mounier, H. d'Hauteville, M. Coquis-Rondon, and P. J. Sansonetti, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 3867 (1989).

these analyses, because this type of confocal microscopy permits not only the analysis of the individual markers but also the precise superimposition of the different fluorescent images. In addition, it provides data in the third dimension.[6] Thus, confocal analyses such as those shown in Figs. 1 and 2 have confirmed and are extending and adding molecular precision to the model shown in Fig. 3.

Preparation of Samples for Immunofluorescence Staining

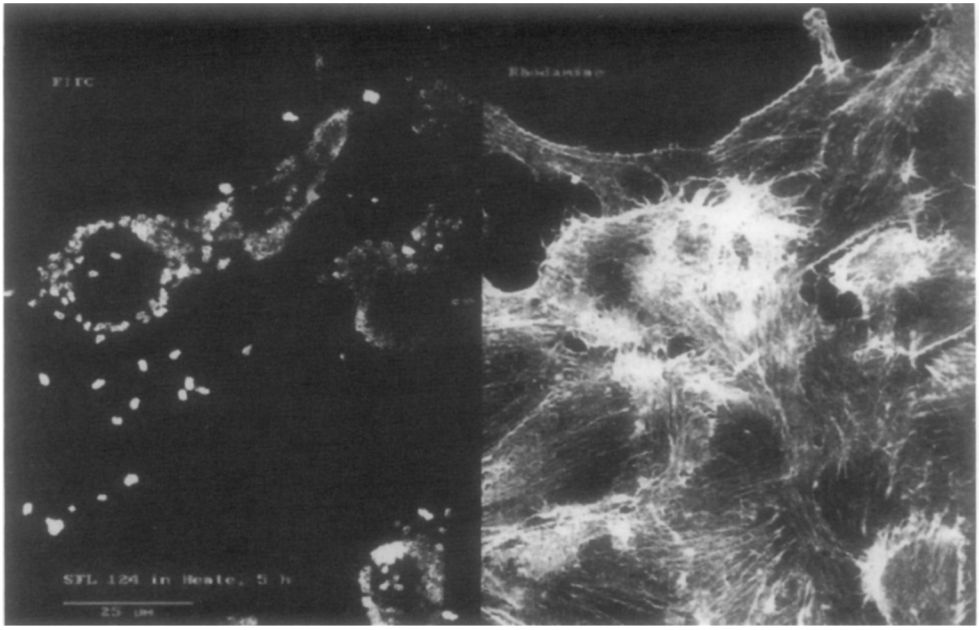
In studying bacterial invasion of either cultured cells or tissues, the bacteria must first be introduced to the cells or tissue of interest. In the case of invasion of cultured cells by bacteria this may be achieved by the simple addition of the bacteria to the cell layer in medium deficient in antibiotics (often present to eliminate potential bacterial contamination) to which the bacteria may be sensitive. In the case of *Listeria monocytogenes*, this leads to an efficient invasion given the appropriate cell lines. However, with nonmotile bacteria such as *Shigella flexneri* and other *Shigella* spp., the process is relatively inefficient, and it is best to gently centrifuge the bacteria onto the cell layer. A typical experiment is as follows.

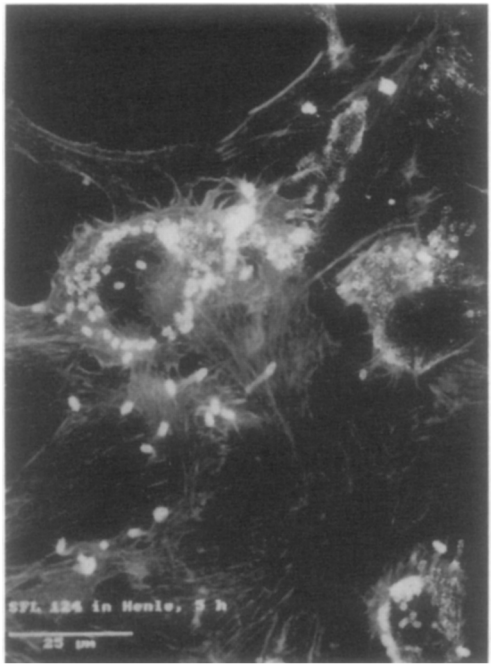
Invasion and Fixation of Cells

1. Incubate the bacteria under suitable conditions to the desired phase of growth, and then harvest the bacteria (500 μl in a microcentrifuge tube) by centrifugation for 5 min at 9000 rpm in a microcentrifuge. The bacteria are washed with antibiotic-free tissue culture medium (e.g., RPMI), recentrifuged, and suspended in 500 μl of fresh medium.

2. At the same time the cultured cells are washed with fresh medium to remove any traces of antibiotics which may be inhibitory to the bacteria. (Six-well microtiter trays are usually used for the growth of the cells as several coverslips can be placed in each well. The monolayers grow across the coverslips so that duplicates are readily generated. The larger wells also make it easier to remove the coverslips.) Washing of cells can usually be done by simply tilting the microtiter tray to about 45° and gently removing the overlying medium by suction, replacing with fresh, prewarmed medium, and repeating the process. It is preferable to grow the cultured cells on coverslips to facilitate the later fixation, permeabilization and immunostaining, and observation. (Most microtiter trays are too thick to be used with other than long working distance objectives that greatly reduce the ability to generate a good confocal image.)

[6] P. A. Manning, R. Morona, and M. Rohde, *Today's Life Science* in press (1995).


FITC
Rhodamine
SFL 124 in Henle, 5 h
25 µm


SFL 124 in Henle, 5 h
25 µm

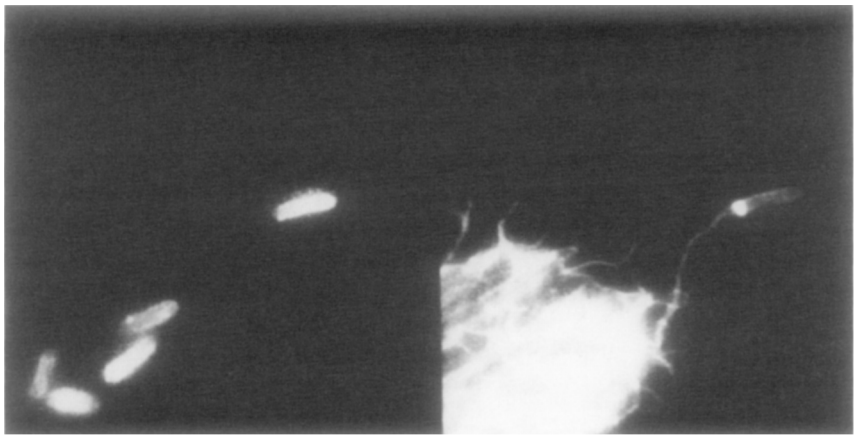


FIG. 2. Protrusion formation by *Shigella flexneri* strain M90T following invasion of Henle 407 cells. *Left:* Staining of the bacteria with FITC. *Right:* Staining of the cell cytoskeleton and polymerized actin using rhodamine. The dense polar accumulation of actin corresponds to the trailing edge of the bacterium within the protrusion.

3. The bacteria are then added at the desired multiplicity, and the microtiter trays are centrifuged for 10 min at about 2000 *g* in a swinging-bucket rotor adapted for such trays. This brings the bacteria into gentle contact with the cells and has the added benefit of synchronizing the invasion process. The 2000 *g* force is close to the limits that the microtiter trays can withstand without cracking, and so care should be taken not to exceed this limit. It may be necessary to do test runs with trays from different manufacturers.

4. The trays are then returned to the CO_2 incubator and incubated until required. With low multiplicities of *Shigella* and cell lines such as HeLa, HEp-2, and Henle 407, incubation may be up to 4–5 hr to produce the whole range of changes, whereas macrophage lines such as J774 are readily killed and lyse within 1–2 hr.

5. At the appropriate time points the cells are fixed for about 30 min using 3.7% formalin (v/v) in phosphate-buffered saline (PBS; containing 8.0 g NaCl, 2.0 g KCl, 2.0 g $Na_2HPO_4 \cdot 2H_2O$, and 2.0 g K_2HPO_4 per liter

FIG. 1. Multiplication of *Shigella flexneri* strain Sfl124 after invasion of cultured Henle 407 epithelial cells. *Top left:* Localization of bacteria as visualized using FITC-labeled goat anti-rabbit Ig bound to primary antiserum (rabbit anti-*Shigella flexneri* LPS). *Top right:* Cell cytoskeleton revealed using rhodamine-labeled phalloidin to depict the actin filaments. The resultant image from superimposition of the two views is shown at the bottom.

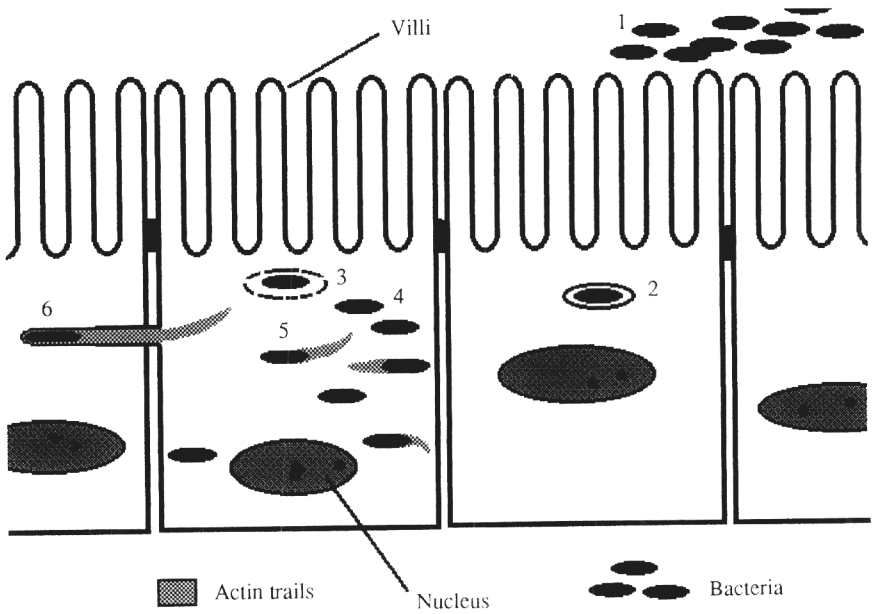


FIG. 3. Model for the invasion of epithelial cells by *Shigella flexneri* [based on that of M. Goldberg and P. J. Sansonetti, *Infect. Immun.* **61,** 4941 (1993)]. Free bacteria (1) are induced to be taken up and enter the epithelial cells within a phagocytic vacuole (2). As a result of lysis of the vacuole (3) the bacteria are released into the cytoplasm (4). Here they begin to interact with the cytoskeleton, leading to polarized accumulation of actin (5). Because the distal ends of the actin molecules are fixed to the cytoskeleton, this facilitates intracellular movement of the bacteria and formation of protrusions into adjacent cells (6). This results in the bacteria being contained within a double-membrane-bounded vacuole from which they become freed, starting the cycle at (3) again.

water, pH 7.4). At this stage the coverslips can be stored at 4° for at least several days.

Fluorescent Staining

To visualize the bacteria within the cell and to put them in a three-dimensional perspective, both the bacteria and the cell need to be visualized, and this can be readily done using different fluorochromes. Generally we use rhodamine for visualizing the cell cytoskeleton and FITC (fluorescein isothiocyanate) for the bacteria. This makes it easier, than the converse combination, to find the bacteria against the background of the cell cytoskeleton as the fluorescein isothiocyanate is a brighter stain and may mask the rhodamine.

A humid chamber is first prepared using a layer of Whatman (Clifton, NJ) No. 1 filter paper in the bottom of a plastic petri dish, saturating the filter with PBS, and then draining off the excess buffer. The humid chamber is required to prevent the samples from drying out during the staining process as only small volumes of reagents are used. Three small beakers each containing about 50 ml of PBS are also set up for use as a washing series. The procedure continues from step 5 (see above).

6. The cells are permeabilized by transferring the coverslips, cell side up, into a clean microtiter tray and overlaying with 0.2% Triton X-100 (v/v) in PBS for about 1–2 min. The coverslips are then dipped sequentially into the three beakers to wash away the Triton X-100 and transferred cell side up to the humid chamber.

7. The bacteria are stained first. This is most readily accomplished using a rabbit antibody [e.g., an antibody directed against the lipopolysaccharide (LPS) and induced by immunizing rabbits with heat-killed bacteria], which is then visualized using an FITC-conjugated goat immunoglobulin anti-rabbit (Ig). A typical rabbit antiserum can be used at a 1 : 1000 or greater dilution. The coverslips are incubated with 10 μl of diluted anti-LPS for 30 min followed by the usual washing procedure.

8. The cell cytoskeleton and polymerized actin can then be visualized by staining with rhodamine-labeled phalloidin. Phalloidin is a fungal toxin from *Amanita phalloides* which binds specifically to F-actin. A 10-μl portion of the FITC-labeled goat anti-rabbit Ig (Sigma, St. Louis, MO; used at a concentration of 0.01 μg/ml in PBS containing 10% fetal calf serum) is then added, and plates are again incubated for 30 min. At this time 10 μl of rhodamine-labeled phalloidin (Sigma); used at a concentration of 0.01 μg/ml in PBS containing 10% fetal calf serum) is added and incubation continued for a further 30 min, after which the coverslips are washed and allowed to dry briefly in air prior to mounting.

Adhesion Assays

The above procedure for studying the intracellular behavior of the bacteria with respect to actin and the cell cytoskeleton; however, the protocol can be easily varied for studying adhesion to cell layers or biopsy material. In the case of adhesion assays, the centrifugation in step 3 may not be required, and the incubation in step 4 is likely to be considerably shorter. However, it is advisable to include several washings prior to the fixation in step 5, and the permeabilization in step 6 should be omitted. Because the cells are still intact the phalloidin conjugates cannot be used, but polyclonal antibodies and monoclonal antibodies (MAbs) to various

tissue cell-surface markers or to general components such as fibronectin[7] can be employed. Their binding can be detected using a rhodamine-labeled goat anti-mouse Ig conjugate, assuming that the MAbs are mouse derived. This has the potential to be able to identify adhesion to particular cell populations within a tissue sample, or to specific regions of a structure such as the tips versus the crypts of villi on the gut epithelium. Alternatively, instead of using a double-staining procedure, the tissue culture cells or biopsy material could be visualized using the transmission detector, and only the bacteria are stained.

Mounting Specimens for Observation

Fluorochromes such as fluorescein are readily bleached by exposure to light, and this necessitates the use of an antibleaching agent because of the high energy of the laser beam being used to stimulate them. Even though this reduces the problem, bleaching is not eliminated, and often the antibleaching agents will lead to a coloration of the sample with prolonged storage. Several alternative agents are used: 0.1% NaN_3, 1,4-diazabicyclo[2.2.2]octane (DABCO) (Sigma) at 100 mg/ml, or *p*-phenylenediamine (quickly made up as a speck on the tip of a spatula, then dissolved in ethanol, and used at 1:50 with the mounting medium). All appear to work well.

The coverslips are mounted specimen side down on microscope slides using about 5 μl of mounting medium (e.g., 50% glycerol in PBS) containing the antibleaching agent. It is best to use a micropipettor to deliver the medium to the slide and place the coverslip onto it. The coverslips are then ringed with clear acrylic nail polish to prevent dehydration and stored in a dark container until ready to view.

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[7] M. C. Plotkowski, M. Chevillard, D. Pierrot, D. Altenmayer, J. M. Zahm, G. Colliot, and E. Puchelle, *J. Clin. Invest.* **87,** 2018 (1991).

[15] Epidemiological Considerations in Studies of Microbial Adhesion

By JAMES R. JOHNSON

Introduction

The host, an obligate participant in all host–pathogen interactions, is a necessary component of microbial adhesion and thus is explicitly or implicitly included in studies of adhesion. It is the host component of microbial adhesion and specifically its epidemiological aspects (i.e., characteristics of human or microbial populations related to disease causation by adhering microorganisms) that this chapter addresses. Also discussed are principles of study design and analysis fundamental to (but not exclusive to) sound epidemiological investigation. Examples are drawn primarily from the literature regarding adhesins of uropathogenic *Escherichia coli,* but the underlying principles apply equally well to other organisms and other clinical settings. It is to be hoped that attention to these principles (Table I) will allow the laboratory methods detailed elsewhere in this volume to be used with greater clinical relevance.

Type II Errors

Statistical tests to confirm that observed differences are "significant," that is, unlikely to be due to chance alone, are essential for all scientific studies involving comparisons between groups, including studies of microbial adhesion. Failure to test differences for statistical significance (as in Ref. 1) can lead to a "type I" error, namely, the conclusion that a difference exists when there is no true difference.[2] Type I errors are uncommon in the contemporary scientific literature, because most investigators are aware of this pitfall and use appropriate statistical tests for significance.

In contrast, type II errors, which result from wrongly concluding that no difference exists when there is a true difference, are comparatively more common.[2] This probably is because the inability to demonstrate a significant difference is easily misinterpreted as the demonstration of the absence of a difference. For example, the authors of one study[3] concluded that host

[1] T. Tambic, V. Oberiter, J. Delmis, and A. Tambic, *Clin. Ther.* **14,** 667 (1992).

[2] J. A. Freiman, T. C. Chalmers, H. Smith, Jr., and R. R. Kuebler, *N. Engl. J. Med.* **299,** 690 (1978).

[3] U. Kärkkäinen, R. Ikäheimo, M.-L. Katila, and R. Mäntyjärvi, *J. Clin. Microbiol.* **29,** 221 (1991).

TABLE I
EPIDEMIOLOGICAL CONSIDERATIONS OF MICROBIAL ADHESION IN CLINICAL PRACTICE

1. Data analysis
   a. Type II errors (power calculations)
   b. Confounding variables (stratification; multivariate analysis)
   c. *Post hoc* hypotheses
2. Internal controls
3. Uniform (comprehensive) evaluation of subjects
4. Clinical characterization of subjects
5. *In vivo* relevance of assays and receptors
6. Population relevance of adhesin
7. *In vivo* expression of adhesin
8. Clinical applications of adhesin characterization

compromise was not associated with a decreased prevalence of P fimbrial expression among urosepsis isolates, because the observed difference of 29/54 (54%: compromised hosts) versus 17/24 (71%: noncompromised hosts) was not statistically significant. However, had the study been larger, a difference of this magnitude would have been statistically significant,[4] leading to the opposite conclusion.

Because of limited sample size, many studies lack the power to exclude the presence of a difference. The sample size required to exclude with confidence intergroup differences of various magnitudes can be estimated based on standard nomograms.[4] Sample size should be considered when studies are designed and also before concluding that negative results demonstrate the absence of a significant difference between comparison groups.[2]

Confounding Variables: Stratification and Multivariate Analysis

Studies of adhesion and other virulence properties often include several measures of adhesion (e.g., adhesion to epithelial cells, mannose-resistant hemagglutination, and expression of P fimbriae as demonstrated by latex bead agglutination) and may also include multiple host characteristics (e.g., different categories of host compromise). It is important to determine whether associations noted between microbial and host factors are primary or can be accounted for by other more fundamental associations within the same study. When two variables both exhibit an association with a particular property, the primary association can be identified by stratifying according to the first variable before analyzing for the second variable. For

[4] M. J. Young, E. A. Bresnitz, and B. L. Strom, *Ann. Intern. Med.* **99,** 248 (1983).

example, both P fimbrial expression and uroepithelial cell adhesion have been associated with development of bacteremia during pyelonephritis in adult women.[5] However, stratification according to P fimbrial expression eliminated the association of adhesion with bacteremia, demonstrating that adhesion was in essence a surrogate marker for P fimbrial expression.

For more complex combinations of variables, multivariate analysis can be useful. For example, in a data set where univariate analysis had shown that multiple bacterial properties significantly differentiated pyelonephritic from fecal *E. coli* isolates, multivariate analysis demonstrated that only P fimbriae, hemolysin, and mannose-resistant hemagglutination (but not hydrophobicity, cytotoxic necrotizing factor, and aerobactin) were independently associated with pyelonephritic strains.[6] With respect to host characteristcs, in another study[7] multivariate analysis showed that each of the three defined categories of host compromise (urological abnormalities, urinary tract instrumentation, and medical illness) was independently associated with a decreased likelihood of P fimbriae among *E. coli* isolates causing urosepsis, confirming the results of univariate analysis.

Post Hoc Hypotheses

Conclusions based on the results of studies specifically designed to address a clearly stated prior hypothesis can be very powerful. In contrast, conclusions based on after-the-fact reviews of data collected in the absence of a clearly stated prior hypothesis are suspect, even when substantiated with "significant" P values. The problem is that in reviewing data after the fact, it is difficult for investigators to insulate themselves from the tendency to focus on apparent differences and to formulate *post hoc* hypotheses that are supported by the observed findings. This bias toward positive findings negates the assumptions of standard statistical tests, rendering P values meaningless.

Post hoc hypotheses generated in this fashion must be considered unproved until substantiated in subsequent studies. For example, in a study evaluating whether the increased incidence of urinary tract infection (UTI) following renal transplantation might be due to an increased bacterial adhesion capacity of uroepithelial cells among transplant recipients,[8] subjects receiving azathioprine as part of an immunosuppressive regimen were incidentally noted to have higher levels of uroepithelial cell adhesion than

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[6] K. Tullus, S. H. Jacobson, M. Katouli, and A. Brauner, *J. Urol.* **146,** 1153 (1991).

[7] J. R. Johnson, P. L. Roberts, and W. E. Stamm, *J. Infect. Dis.* **156,** 225 (1987).

[8] H. Ruder, C. Thurn, and J. P. Guggenbichler, *Transplant. Proc.* **24,** 2576 (1992).

subjects receiving other regimens. The authors appropriately did not test this association for statistical significance, and they tempered their conclusions by observing that "these data suggest that adherence ... is increased in patients receiving azathioprine," without proposing a causal relationship.[8]

Relevant Internal Controls

Inclusion of control groups in studies of adhesion allows for the strongest possible conclusions, because results for control groups and study groups can be compared directly. For example, it is common in studies of uropathogenic *E. coli* to compare the adhesion properties of urinary isolates from patients with UTI (study group) with those of fecal isolates from healthy individuals (control group).[9] Selection of an appropriate control group from among the many possible alternatives is critical, and it can be challenging. For example, if the goal is to characterize the adhesion characteristics of *E. coli* strains causing nosocomial UTI, possible comparison groups would include strains causing community-acquired UTI; fecal isolates from healthy community dwellers without UTI; fecal isolates from hospitalized patients without UTI; and isolates from extraurinary nosocomial infections. Which control group is used will determine to some extent the conclusions that can be drawn from findings in the study group.

In selected instances, studies lacking internal controls can lead to meaningful conclusions.[10] However, in the realm of microbial adhesion such studies generally can do little more than describe the characteristics of a population, such as *E. coli* urinary isolates from patients with spinal cord injury[11] or nosocomial UTI.[12] Explicit or implicit reference to historical controls, or to an ill-defined "common knowledge,"[10] is required for meaningful interpretation of such reports. Nonetheless, even in such studies stratification according to secondary variables such as clinical UTI syndrome[11,12] can provide the basis for informative internal comparisons.

Uniform, Comprehensive Evaluation of Subjects

Unless all study subjects (whether humans, bacteria, purified adhesins, or host cells) are evaluated identically, unrecognized bias may be introduced and lead to spurious conclusions. For example, the unavailability of certain bacterial isolates for testing may be due to bacterial characteristics that affect viability during storage ("hardiness," auxotrophy) or to patient-

[9] J. R. Johnson, *Clin. Microbiol. Rev.* **4,** 80 (1991).
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[12] R. Ikäheimo, A. Siitonen, U. Kärkkäinen, and P. Helena Mäkelä, *Clin. Infect. Dis.* **16,** 785 (1993).

related factors that influence the likelihood that a strain would be saved (clinic or institution of origin, clinical syndrome, presence of other organisms in original specimen). These factors may in turn be related to the microbial or host characteristics under investigation. Because of this, selective losses from certain subsets of the study population may occur that would distort the apparent distribution of the properties of interest within the population.

The assumption that the subjects available for testing (if less than the total population) are representative of the study population as a whole may therefore be incorrect and could result in false conclusions. For example, in a study of P fimbrial expression among *E. coli* from children with febrile UTI,[13] only 70 of the 83 initial isolates were available for phenotype testing. Given the small numbers in some of the subgroups analyzed, inclusion of the missing strains could have substantially altered the results of the study, depending on the (unknown) phenotype of the missing strains. The potential problems associated with incomplete sampling can best be avoided by ensuring that all subjects are tested fully; alternatively, that any subset of subjects selected for special testing is chosen randomly or systematically by the investigator (rather than by happenstance); or, if neither of these is possible, that analysis is limited to those subjects available for uniform testing, and that the possibility of inadvertently biased selection is acknowledged.

Clinical Characterization of Study Population

Many studies of microbial adhesion involve comparisons of microorganisms (or host cells) from one clinical setting (e.g., infected individuals) with those from a different setting (e.g., infection-free individuals), to permit conclusions about the association of particular microbial or host traits with disease. It follows that the precision of such conclusions depends on how precisely the comparison groups are defined. Early studies of *E. coli* virulence commonly compared commensal strains with "pathogenic" strains, without regard for the site or severity of infection, or host status; this is still sometimes done today.[14–17]

[13] M. Majd, H. G. Rushton, B. Jantausch, and B. L. Wiedermann, *J. Pediatr.* **119,** 578 (1991).

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[16] J. Blanco, M. Blanco, M. P. Alonso, J. E. Blanco, E. A. González, and J. I. Garabal, *Res. Microbiol.* **143,** 869 (1992).

[17] A. Cherifi, M. Contreposis, B. Picard, Ph. Goullet, J. de Rycke, J. Fairbrother, and J. Barnouin, *FEMS Microbiol. Lett.* **70,** 279 (1990).

For studies of *E. coli* adhesion, stratification according to "UTI" versus "other"[18] is to be preferred over grouping all infection isolates together, because of the unique adhesion characteristics of uropathogenic organisms.[9] However, numerous studies demonstrate the importance of even more detailed characterization (and narrower stratification) of the clinical material. In the arena of UTI, subjects and associated strains are commonly stratified according to specific clinical syndrome (e.g., urosepsis, versus pyelonephritis or febrile UTI, versus cystitis, versus asymptomatic bacteriuria) because of the dramatic implications of the clinical syndrome for adhesion and other virulence properties.[9]

Other clinical variables important to consider in studies involving adhesion in UTI include gender,[19] age, site of infection (e.g., upper versus lower urinary tract),[13,20–23] inflammatory manifestations,[24,25] presence of compromising host conditions,[5,7,9,26–29] blood group and secretor status,[30–33] and past UTI history.[28,34] Unless these characteristics are known to be similarly distributed among different comparison groups, they should be suspected of possibly confounding the analysis, giving rise to spurious associations (or lack of associations) between adhesion properties and clinical features.

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[34] A. Stapleton, S. Moseley, and W. E. Stamm, *J. Infect. Dis.* **163,** 773 (1991).

In Vivo Relevance of Adhesion Assays and Receptors

Because the goal of studies of microbial adhesion is to advance understandings of how adhesins function in the "real world," it is important that experimental conditions reflect the natural conditions present *in vivo* during disease pathogenesis, and that assay systems detect the actual property of interest. This is sometimes problematic, because natural conditions frequently are undefined, or are difficult to reproduce in an experimental situation, and because it may not be clear which alternative assay system is most relevant to the hypothesis under study.

Different assay systems for adhesin expression can give different results for the same strains, depending on the property being detected. For example, immunofluorescent detection of P fimbriae is more sensitive than latex agglutination,[35] but it relies on expression of pilus antigen rather than of a functional adhesin and so may be less relevant to pathogenesis. Latex agglutination gives variable results for detection of P fimbriae, depending on the particular oligosaccharide attached to the latex beads.[36] Hemagglutination is commonly used as a surrogate for cellular adhesion in studies of uropathogenic *E. coli.* However, despite their ability to bind to various renal tissues,[37] F1C fimbriae do not mediate hemagglutination and are not detected by hemagglutination assays.

Definition of the binding specificity of P fimbrial variants has been pursued through the use of thin-layer chromatography (TLC), with radiolabeled bacteria allowed to adhere to glycolipids that are separated and immobilized on TLC plates.[38–40] It has become apparent, however, that this artificial model does not faithfully reproduce the receptor–ligand interactions that occur *in vivo* (or on natural membranes), possibly because steric constraints are different on artificial as opposed to natural membranes.[41] These considerations have clear implications for the clinical rele-

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vance of results derived from artificial adhesion systems and argue for the importance of using natural adhesion targets.

However, natural targets for adhesion pose their own quandries regarding *in vivo* relevance. When epithelial cell adhesion is used to assay adhesin expression or to evaluate receptor specificity, the source and nature of the cells must be considered carefully. The P fimbrial variant from strain J96 termed Prs and Pap-2 mediated adhesion to monolayers of a canine kidney cell line (Madin–Darby: MDCKI), but not of a human bladder carcinoma cell line (T24), contributing to the conclusion that this adhesin may be important for canine but not human UTI.[38] Paradoxically, no adhesion was seen to a second canine kidney cell line (MDCKII).[38] In contrast, in a subsequent study a strain expressing an adhesin of similar receptor specificity bound to glycolipids extracted from voided human uroepithelial cells, and to the uroepithelial cells themselves, of an A_1 secretor individual (but not those of an A_1 nonsecretor or of an A_2 secretor). This was interpreted as evidence that the Pap-2 and similar adhesins may function in uropathogenesis in humans after all, but only in secretors and those of blood type A_1[39]. However, in yet another study[42] the Pap-2 adhesin adhered to specific structures in tissue sections from the kidney of a man of blood type B, evidence suggesting the possibility of a broader role for this adhesin in human UTI than suggested by cellular adhesion assays.

These seemingly contradictory observations indicate that transformed cell lines may not be representative of normal cells from the same organ; that different primary cell lines from the same organ and species may differ with respect to adhesive capacity; that voided epithelial cells may differ from individual to individual, depending on blood type and secretor status; and that voided epithelial cells may not accurately reflect the adhesive capacity of renal tissues. Which natural target is most representative of the *in vivo* substrate for adhesion during infection must be considered in selecting an assay system to study adhesion.

Population Relevance of Adhesins

In evaluating the relevance to pathogenesis of results from studies of microbial adhesion, one must consider not only the assay system and the availability of receptors in relevant host tissues, but also the adhesin itself. Key questions include whether the adhesin is sufficiently prevalent among organisms causing a particular clinical syndrome to possibly be important in pathogenesis, and whether the adhesin is expressed *in vivo* during infection.

[42] J. F. Karr, B. J. Nowicki, L. D. Truong, R. A. Hull, J. J. Moulds, and S. I. Hull, *Infect. Immun.* **58,** 4055 (1990).

The P fimbriae from strain J96 have been regarded as prototypic P fimbriae[43] and have formed the basis for elegant investigations of P fimbrial structure and assembly.[44–46] Ironically, the PapG adhesin molecule from strain J96 appears not to be representative of the PapG molecules of most human uropathogenic strains and may be unique.[47,48] Whether this applies to other aspects of J96 P fimbriae is unknown, but evidence is available to suggest that P fimbrial structure (and presumably also assembly) does differ substantially between different P fimbrial serotypes.[49] Thus, the validity of generalizing results derived from one specific adhesin to other presumably similar adhesins may be questioned.

A variety of mannose-resistant adhesins other than P fimbriae have been identified among urinary strains (reviewed in Ref. 9), and in some cases clever molecular studies have been done to determine their genetic basis and receptor specificity. However, the apparent rarity among clinical isolates of such adhesins as the M adhesin, G fimbriae, and the NFA (non-fimbrial) adhesins[50–55] casts doubt on their clinical significance. The prevalence of S fimbriated strains in clinical collections is difficult to assess because of the variable expression of S fimbriae[56] and the cross-hybridization of DNA probes for S and F1C fimbriae.[57,58] Adhesins of the Dr family

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[52] J. Grünberg, R. Perry, H. Hoschützky, B. Jann, K. Jann, and J. Goldhar, *FEMS Microbiol. Lett.* **56,** 241 (1988).

[53] H. Hoschützky, W. Nimmich, F. Lottspeich, and K. Jann, *Microb. Pathog.* **6,** 351 (1989).

[54] M. Archambaud, P. Courcoux, V. Ouin, G. Chabanon, and A. Labigne-Roussel, *Ann. Inst. Pasteur (Paris)* **139,** 557 (1988).

[55] J. N. Maslow, M. E. Mulligan, K. S. Adams, and R. D. Arbeit, *Clin. Infect. Dis.* **17,** 89 (1993).

[56] G. Schönian, W. Sokolowska-Köhler, R. Rollmann, A. Schubert, Y. Gräser, and W. Presber, *Zentralbl. Bakteriol.* **276,** 273 (1992).

[57] N. Riegman, R. Kusters, H. van Veggel, H. Bergmans, P. van Bergen en Henegouwen, J. Hacker, and I. van Die, *J. Bacteriol.* **172,** 1114 (1990).

[58] G. Zingler, G. Blum, U. Falkenhagen, I. Orskov, F. Orskov, J. Hacker, and M. Ott, *Med. Microbiol. Immunol.* **182,** 13 (1993).

appear to be more prevalent among urinary isolates than the above adhesins.[59–63] Here too, however, the nonspecificity of the DNA probes used in several molecular epidemiologic studies precludes assessment of the relative prevalences of the different members of the Dr family, namely, the Dr hemagglutinin, AFAI, AFAIII, and F1845 fimbriae,[60,61,64] leaving in question the clinical importance of each adhesin subtype.

Adhesin Expression *in Vivo*

Even an adhesin that is expressed by all isolates causing a particular type of infection, and for which receptors are available at the appropriate site within the host, may not be active in pathogenesis if it is not expressed *in vivo* during infection. UTI-associated adhesins are notorious for their variable expression in response to temperature, other environmental conditions, and subinhibitory concentrations of antibiotics.[9] Consequently, adhesin expression in the laboratory by passaged isolates is no guarantee that the same adhesins are expressed in the host. Some of the variability between different epidemiological studies of adhesin expression in UTI doubtless is due to laboratory artifact.[9] Direct testing of urinary organisms[35,65–68] brings the investigation closer to the actual site of infection than does working with passaged organisms but still fails to provide a definitive answer, as the bacterial population shed in the urine may differ from that adherent to the mucosa.[69] Indeed, different sites within the host actually may harbor distinct subpopulations of the same bacterial strain, with differing adhesin phenotypes.[65] Direct determination of adhesin expression in animal model systems or in biopsy material from human subjects would be needed to clarify when, where, and to what degree specific adhesins are expressed in the host during infection.

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[62] B. Nowicki, J. Moulds, R. Hull, and S. Hull, *Infect. Immun.* **56,** 1057 (1988).
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[67] I. Ofek, A. Mosek, and N. Sharon, *Infect. Immun.* **34,** 708 (1981).
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[69] J. R. Johnson, *J. Infect. Dis.* **158,** 495 (1988).

Clinical Utility of Adhesion Testing

A final epidemiological consideration relates to proposed potential clinical uses of adhesion testing. To date, adhesion testing has been primarily a research tool, used to advance understandings of microbial function, disease pathogenesis, and host–parasite interactions, without any direct impact on patient care. However, some investigators have proposed that testing for expression of P fimbriae could be clinically useful in a variety of settings in individual patients with UTI, and perhaps should be included as part of the laboratory evaluation of selected UTI episodes. Critical to the evaluation of such proposals are the following questions: Will the test provide clinically useful information? Will the patient be better off for having the test done? Is the potential benefit worth the cost and risks resulting from the test itself, and from any other tests or treatments that would be done because of the testing?[70]

For many of the proposed clinical applications of P fimbrial testing, the information needed to answer these questions is unavailable. For example, the possibility of horizontal transmission of P fimbriated uropathogenic strains within households and hospital wards[71,72] prompted the suggestion that family members of a patient with pyelonephritis should be screened for fecal carriage of the P fimbriated strain of the patient, and that asymptomatic carriers should be treated with antibiotics to prevent subsequent UTI.[73] However, the prevalence of asymptomatic carriage among family members and the risk of progression to symptomatic UTI are unknown; clearly, the lower these values, the lower the yield of a screening program. Furthermore, the efficacy of preventive antibiotic treatment in this context has not been studied. Even if such treatment should prove efficacious, it might be that adverse effects associated with antibiotic use would outweigh the benefits of treatment. Finally, the (undetermined) cost of testing and treatment must be considered, as must the (undetermined) cost of evaluating and treating any adverse effects of antibiotic therapy.

As another example, the association of non-P fimbriated *E. coli* strains with subsequent development of renal scarring in boys with UTI[74] and with underlying urological or medical compromising conditions in patients with pyelonephritis or urosepsis[9] has led to the suggestion that P fimbrial testing

[70] Department of Clinical Epidemiology and Biostatistics, McMaster University Health Sciences Centre, *Can. Med. Assoc. J.* **124,** 703 (1981).

[71] K. Tullus, B. Fryklund, B. Berglund, G. Kallenius, and L. G. Burman, *J. Hosp. Infect.* **11,** 349 (1988).

[72] K. Tullus, K. Horlin, S. B. Svenson, and G. Kallenius, *J. Infect. Dis.* **150,** 728 (1984).

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[74] P. de Man, I. Claeson, I. M. Johanson, U. Jodal, and C. Svanborg Eden, *J. Pediatr.* **115,** 915 (1989).

of UTI isolates could be used to predict the development of renal scarring in boys[74] or the presence of compromising host conditions in adults.[75] How identifying boys with UTI who are at risk for subsequently renal scarring would alter their medical management is unclear. Perhaps more frequent screening for and rapid treatment of asymptomatic bacteriuria could be undertaken in such individuals.[74] However, whether this would prevent renal scarring is unknown, as are the costs and side effects associated with such a program. It is similarly unclear whether the information regarding host compromise provided by the isolation of a non-P fimbriated strain from an adult with pyelonephritis or urosepsis adds to what can be learned by reviewing the medical record and from tests that are commonly done even in the absence of adhesin characterization.[76] Furthermore, the benefit to the patient of any added information that adhesin characterization might provide remains to be demonstrated.

Thus, several crucial epidemiological steps are commonly lacking in extrapolations from research observations regarding adhesion to recommendations for clinical practice. Other proposed clinical applications for P fimbrial testing that are as yet incompletely substantiated epidemiologically include the following: determining length of therapy for children with cystitis[1]; identifying pregnant women with asymptomatic bacteriuria who are at high risk for developing pyelonephritis[77]; and identifying fecal carriers who could then be screened for an increased uroepithelial cell adhesion capacity for P fimbriated *E. coli*, to permit targeted preventive antibiotic therapy.[78]

Conclusion

Epidemiological considerations regarding study design, data collection and analysis, and interpretation of results are fundamental to studies of microbial adhesion, if relevance to the pathogenesis of clinical infection is a goal. Pitfalls to be avoided include type II statistical errors, inattention to confounding variables, and *post hoc* hypotheses; absence of internal controls; nonuniform evaluation of subjects; failure to characterize subjects adequately, or to stratify analyses according to important clinical characteristics; inattention to the *in vivo* relevance of the adhesin or receptor studied, or of the assay system used; and overconcluding from research findings to recommendations for clinical practice. Attention to these principles can

[75] K. Dowling, J. A. Roberts, and M. B. Kaack, *South. Med. J.* **80,** 1533 (1987).
[76] J. R. Johnson, *South Med. J.* **81,** 1070 (1988).
[77] K. Stenqvist, G. Lidin-Janson, T. Sandberg, and C. Svanborg Eden, *Scand. J. Infect. Dis.* **21,** 193 (1989).
[78] G. Kallenius, S. H. Jacobson, K. Tullus, and S. B. Svenson, *Infection* **13,** 159 (1985).

enhance the scientific quality and clinical relevance of studies of microbial adhesion.

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[16] Kinetic Analysis of Microbial Adhesion

By MARJORIE MURPHY COWAN

Introduction

The adhesion of bacteria to other cells and to surfaces has many biological and mechanical implications. However, bacterial adhesion processes are not of an all-or-nothing nature. Some attachments are loose and easily reversible, whereas others are firm and essentially irreversible, and even others are somewhere in between. In fact, most bacteria possess multiple strategies for attachment to different surfaces or ligands. Many adhesion interactions which are considered irreversible only become firmly bound after having "survived" the reversible, loosely bound state. Unraveling the steps in the adhesion process is the goal of microbial adhesion studies.

The strength, or affinity, of attachment can be estimated from data obtained in two types of adsorption experiments: (1) equilibrium binding experiments, in which varying concentrations or densities of ligand (bacteria) are allowed to reach a steady-state of interaction with a constant concentration of receptors; and (2) kinetic binding experiments, in which a single concentration of ligand is allowed to bind to a single concentration of receptors. In the first experiment, data points obtained after the attainment of steady-state conditions are graphed so that K_A, the equilibrium or affinity constant, can be determined. The use of this approach assumes a single-step binding reaction which is completely reversible. The kinetic method collects data throughout the course of the binding reaction and yields graphic estimations of k_a and k_d, rate constants for association and dissociation, which provide $K_A(k_a/k_d)$, but, more importantly, also provide insight to the progress of the binding reaction. Other advantages to the kinetic approach include (1) there is no assumption about single-step mechanisms, and (2) there is no requirement for reversibility.[1]

[1] M. T. Boughey, R. M. Duckworth, A. Lips, and A. L. Smith, *J. Chem. Soc. Faraday Trans. 1* **74,** 220 (1978).

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[1] M. T. Boughey, R. M. Duckworth, A. Lips, and A. L. Smith, *J. Chem. Soc. Faraday Trans. 1* **74,** 220 (1978).

The Langmuir adsorption isotherm yielding the mathematical expressions for affinity was developed to describe the adsorption of gases to interfaces,[2] and it has proved useful in the analysis of colloidal particles adsorbing to surfaces and other systems consisting of larger components. Applying the theories to the adsorption behavior of biological cells requires careful attention to their structural and physiological complexities. Bacteria have highly heterogeneous surfaces, made up of many different types of macromolecules which contribute to their binding behavior. These molecules contribute both to "average" properties of the cell surface and to localized forces. The degree of hydrophobicity (sometimes called the interaction force) and/or cell surface charge can influence long-range interactions and may result in a cell being bound in the secondary energy minimum as described by DLVO theory.[3,4] Localized forces, found in the form of stereospecific groups or patches of hydrophobicity, are likely to bring about short-range interactions and result in adhesion in the primary energy minimum. The secondary and primary minima are separated by a substantial energy barrier, and cells which traverse the barrier to effect close adsorption (in the primary minimum) are considered to be irreversibly attached. The energy barrier between the primary and secondary minima exists at a distance of 1–10 nm from the substratum surface, under most conditions. Irreversible adhesion can be achieved when a cell is farther away from the substratum, however, as slim appendages (i.e., fimbriae) experience a much smaller repulsive force and are often able to cross the barrier.[5]

Each bacterial cell may use multiple strategies for a single attachment. In addition, in most biological environments, the receptor and ligand surfaces are coated with a mixture of adsorbed macromolecules, adding to the complexity. The kinetic approach allows investigators to identify any departure from ideal pseudo-first-order attachment kinetics which can help identify the cell structures responsible for attachment. Kinetic analysis also allows the separate investigation of low-affinity (long-range) association and stereospecific binding occurring at a short range. Systems in which the energy barrier between the long-range attachment and closer, stronger binding is large benefit greatly from an analysis of the first, reversible stage of adhesion, because this is the most influential determinant of eventual adhesion. This stage is best studied kinetically.

Advances in computer-assisted microscopy allow the simultaneous calculation of adsorption and desorption rates of bacteria flowing over smooth

[2] I. Langmuir, *J. Am. Chem. Soc.* **40,** 1361 (1918).
[3] B. V. Derjaguin and L. Landau, *Acta Physicochim. URSS* **14,** 633 (1941).
[4] E. J. W. Verwey and J. T. G. Overbeek, *in* "Theory of the Stability of Lyophobic Colloids; the Interaction of Sol Particles Having an Electric Double Layer." Elsevier, New York, 1948.
[5] B. A. Pethica, *Exp. Cell. Res. Suppl.* **8,** 123 (1961).

transparent surfaces in flow chambers.[6,7] This design has many advantages, including well-defined fluid dynamic conditions, but so far is impractical for studying bacterial adsorption to nonideal surfaces, such as fibers, tissues, other cells, or even transparent surfaces with biological coatings. Other assays utilizing continuous-flow conditions for studying adhesion to nontransparent surfaces have been described. For example, Elimelech[8] studied the deposition of positively charged colloids in packed beds of negatively charged spheres. The setups generally require a level of instrumentation and expertise in fluid dynamic theory that many microbiologists do not possess and which is not necessary for the simple investigation of biological adhesion. For many investigations, adhesion assays in which bacteria and substratum are mixed together in a batch vessel and then harvested separately from suspension remain the most useful method for studying microbial adhesion.

A variety of sources provide detailed descriptions of microbial adhesion experiments[9–11]; this chapter identifies the major experimental pitfalls in modeling bacterial adhesion to surfaces using a mixed or stirred system and describes the graphical and mathematical analysis of the adsorption kinetics.

Adhesion Assay Conditions

Bacteria

Quantification of both substratum-associated and suspended bacteria is usually accomplished by labeling; often radioisotopes are employed, though fluorescent tags may be used if adhesion is to be assessed microscopically. The label itself must be demonstrated to have no effect on surface charge or composition; in this respect [^{3}H]thymidine labeling of nucleic acids is useful. Indium-III labeling has been used with good results in adhesion experiments.[12]

Metabolizing cells have the capacity to change their surfaces by manufacturing new polymers or change the physicochemical makeup of their external environment via the extrusion of ions or migration of particular

[6] J. M. Meinders, J. Noordmans, and H. J. Busscher, *J. Coll. Interface Sci.* **152,** 265 (1992).
[7] H. J. Busscher and H. C. van der Mei, this volume [37].
[8] M. Elimelech, *J. Colloid Interface Sci.* **146,** 337 (1991).
[9] W. B. Clark, M. D. Lane, J. E. Beam, S. L. Bragg, and T. T. Wheeler, *Infect. Immun.* **4,** 730 (1985).
[10] M. E. Konkel, M. D. Corwin, L. A. Joens, and W. Cieplak, *J. Med. Microbiol.* **37,** 30 (1992).
[11] W. E. Nesbitt, R. J. Doyle, and K. G. Taylor, *Infect. Immun.* **38,** 637 (1982).
[12] R. Ardehali and S. F. Mohammad, *J. Biomed. Mater. Res.* **27,** 269 (1993).

chemical groups within their envelopes. We discuss here the passive adhesion of metabolically inert bacteria in order to simplify the analysis.

Care should be taken that bacteria in the adhesion experiment occur as single cells. Bacteria which occur as clusters, or which clump during growth, can often be separated by gentle sonication. Bacteria which grow in chains can usually be readily reduced to a mixture of singlets and doublets through sonication without noticeable damage to the bacterial surface, but reducing the remaining doublets to singlets is very difficult and probably too traumatic to the bacterial surface. The proper balance must be found between the degree of damage to the cell surface and inaccuracy in the calculations caused by the presence of doublets,[13] especially in the early portion of the experiment where overall numbers of bound bacteria are lowest.

Bacterial Density

Conventional enzyme-binding experiments call for a density of bacteria which greatly exceeds the number of possible binding sites on the surface under study to ensure that lack of saturability (owing to complete lack of specificity) not be confused with insufficient amounts of ligand.[14] When preliminary equilibrium experiments are performed to determine saturation in advance, as advocated here (see below), this problem would already be detected. Experiments conducted with relatively fewer cells in suspension proceed more slowly,[15] and the slower kinetics often reveal subtle aberrations in the binding mechanism. It is therefore advisable to perform the kinetic experiments at several cell densities, especially if the adsorption is thought to be of low affinity.

Buffer

Both bacteria and surface must be equilibrated in the same buffer to be used in the assay. Attention must be paid to the ionic strength of the buffer and valency of buffer ions, because both can greatly affect long-range interaction forces. In general, the higher the ionic strength of the suspending buffer, the greater adhesion, as the presence of negative ions can counteract the cations comprising the "electric cloud" surrounding the

[13] J. Sjollema, H. J. Busscher, and A. H. Weerkamp, *J. Microbiol. Methods* **9,** 73 (1989).

[14] J. C. Matthews, "Fundamentals of Receptor, Enzyme, and Transport Kinetics." CRC Press, Boca Raton, Florida, 1993.

[15] H. J. Busscher, M. H. W. J. C. Uyen, A. W. J. van Pelt, A. H. Weerkamp, and J. Arends, *Appl. Environ. Microbiol.* **51,** 910 (1986).

negatively charged bacterium and substratum (the electric double layer).[16] At high electrolyte concentrations this effect can be tempered by possible alterations in the three-dimensional architecture of cell surface extensions such as fibrils and fimbriae, which may collapse owing to the inability of repulsive forces to compensate for increased attractive van der Waals forces between appendages.[17]

Reaction Conditions

In practice, the attachment of microorganisms to surfaces comprises two processes: transport to the vicinity of a surface followed by attachment to it.[16] Often the transport portion of the process is ignored in bacterial adhesion assays, with varying degrees of success. It is this step in adsorption which depends heavily on the hydrodynamic conditions. When performing adhesion assays in mixed or stirred conditions with relatively uncontrolled fluid dynamics, steps must be taken to ensure the measured rates are due mainly to colloidal interaction phenomena. First, rates and extent of deposition must be determined from separate kinetic experiments conducted at varying speeds of mixing. Plots of K_A against mixing speed which are linear indicate that Brownian motion is not controlling. Equilibrium levels of binding should be independent of mixing speed, however, if the contribution of the hydrodynamic potential is to be excluded in the kinetic analysis.[1]

When using noncontinuous flow setups, every effort should be made to prevent the passage of substrata with adherent cells through air–liquid interfaces. Rather than removing the substratum from the reacting solution and placing it in the rinse buffer, the incubating suspension should be displaced with the fixative and/or rinse solution. Busscher and van der Mei[18] described very clearly the damaging effects of passing cells adsorbed to a substratum through an interface. They found not only that much desorption occurred, but also that shifting and clumping of bound bacteria took place, especially on hydrophobic substrata.

Rinsing in general is a very risky endeavor in adhesion experiments. Less firmly bound bacteria (i.e., those bound in the secondary minimum by virtue of long-range forces) are susceptible in varying degrees to displacement by the shear forces involved. The susceptibility differs markedly depending on the type of adhesive interactions, that is, whether both the

[16] K. Marshall, *in* "Bacterial Adhesion: Mechanisms and Physiological Significance" (D. C. Savage and M. Fletcher, eds.), Plenum, New York, 1985.

[17] H. M. Uyen, H. C. van der Mei, A. H. Weerkamp, and H. J. Busscher, *Appl. Environ. Microbiol.* **54,** 837 (1988).

[18] H. J. Busscher and H. C. van der Mei, personal communication (1989).

bacterium and/or substratum are relatively hydrophobic or hydophilic.[19] It follows that if rinsing must be done it should be well controlled and reproducible, and the flow rates of the rinsing solution should be determined and documented.

Another practical consideration in the performance of adhesion experiments is the avoidance of adventitious binding of bacteria to spurious surfaces such as vessel walls or membrane filters. Especially when bacteria are labeled with radioisotopes for detection, large errors may be introduced by undetected bacterial trapping or adsorption to nonsubstratum surfaces. This binding can be minimized by careful planning of experiments. For example, Cowan, *et al.*[20] separated the reactants (substratum and ^{3}H-labeled bacteria) from the reaction vessel and from the collecting filter before scintillation counting.

Adsorption Experiments

In most adhesion assays, bacterial cells are mixed with some quantity of "surface" such as other bacterial cells, hydroxylapatite powders, or glass beads. Kinetic experiments require that samples of the substratum and/or the supernatant be removed at specified time intervals for quantification of bound and unbound bacteria. Often, multiple reaction vessels are prepared for each time point to be studied so that the reaction may be stopped in those vessels and both substratum and supernatant analyzed in duplicate or triplicate for the numbers of bacteria present. Numbers of bound (or unbound) cells can be plotted against time (Fig. 1). The plots depict the time required for the achievement of equilibrium.

Desorption Experiments

Reaction mixtures which have reached the end point in adsorption experiments can be transferred to desorbing conditions, usually by replacing the bacterial suspension with plain buffer. Samples are then harvested and analyzed during the course of the desorption experiment and numbers of unbound cells plotted against time, as above.

Determination of Saturation

The kinetic analysis described below requires knowledge of θ, the fractional saturation, or coverage, which is defined as the percentage of potential binding sites which are actually occupied. It is often impossible to determine the number of "sites" which are available for a bacterium to bind on a biological or irregular substratum. Instead the maximum possible number

[19] H. J. Busscher and A. H. Weerkamp, *FEMS Microbiol. Rev.* **46,** 165 (1987).
[20] M. M. Cowan, K. G. Taylor, and R. J. Doyle, *J. Dent. Res.* **65,** 1278 (1986).

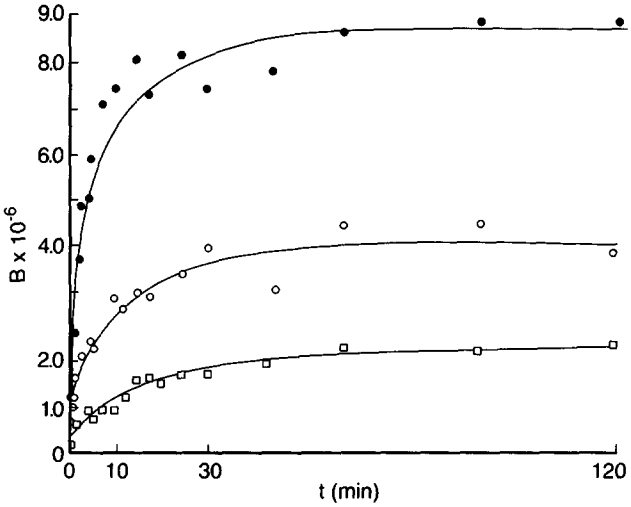

FIG. 1. Adsorption of *Streptococcus sanguis* 10556 to saliva-coated hydroxylapatite (SHA). *B*, Bound cells. Curves represent a constant number of total bacteria (1.66×10^8) and increasing amounts of SHA: □, 10 mg; ○, 20 mg; and ●, 60 mg. [Reprinted from M. M. Cowan, K. G. Taylor, and R. J. Doyle, *J. Dent. Res.* **65,** 1278 (1986).]

of cells bound when using a saturating density of bacteria can be used as an approximation of number of sites. This is determined in equilibrium experiments using a range of bacterial densities and a single concentration of substratum. The two are allowed to react until no further binding occurs. Then substratum and supernatant are separated and numbers of bacteria associated with each are counted. Numbers of bound cells for each density are plotted against numbers of unbound cells (Fig. 2), and the maximum number of bound cells is taken as the denominator in the above percentage.

The equation used to analyze adsorption kinetics includes a term for desorption, so that both k_a, the rate constant for adsorption, and k_d, the rate constant for desorption, can be determind from a single adsorption experiment. It is prudent, however, to conduct separate desorption experiments to confirm the calculated k_d. More than one complex adhesion mechanism has been unraveled by studying carefully the desorption process.[15,20]

Calculations

Adsorption Rate Constant

Kinetic experiments yield numbers of bound (B) and unbound (U) cells at time t. A kinetic form of the Langmuir adsorption model describes the net rate of deposition of bacteria onto substratum[1]:

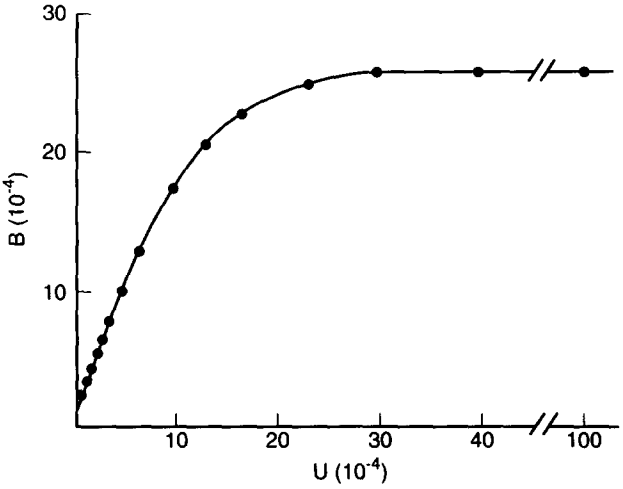

FIG. 2. Binding isotherm for determining saturation of bacterial adhesion to substrata. B, Bound cells; U, unbound cells.

$$\frac{dB_t}{dt} = k_a(U_0 - B_t)(1 - \theta_t) - k_d B_t \tag{1}$$

where U_0 is total number of bacteria added to the mixture, B_t is the number of bound cells at time t, and θ_t is the fractional saturation at time t and is equal to B_t/B_{max}, where B_{max} is the number of cells bound at saturation coverage. Applying the equilibrium condition so that $dB_t/dt = 0$ yields

$$k_d = k_a(U_0 - B_f)(1 - \theta)/B_f \tag{2}$$

where B_f is the number of cells bound at equilibrium. Substituting for k_d and integrating Eq. (1) yields

$$-\ln \frac{(U_t - U_f)/(U_0 - U_f)}{1 - [1 - (U_t/U_0)]\theta} = k_a N t\,[U_0/(U_0 - U_f) - \theta] \tag{3}$$

where U_f is the number of unbound cells at infinity. Plotting the left-hand side of Eq. (3) against t yields a strength line (Fig. 3), the slope of which (m) is related to k_a by

$$m = k_a N\,[U_0/(U_0 - U_f) - \theta] \tag{4}$$

The plot is linear when the adsorption rate is independent of substratum coverage. When linearity is not found, it may help to decrease initial bacterial density to levels corresponding to $\theta \leq 0.20$.

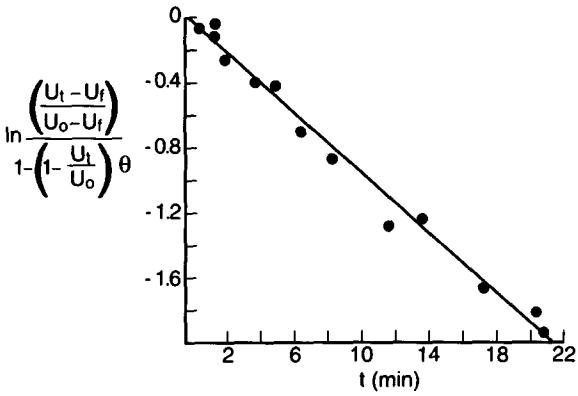

FIG. 3. Kinetics of *Streptococcus sanguis* 10556 adhesion to saliva-coated hydroxylapatite (SHA). The left-hand side of Eq. (3) is plotted against time. U_t, Unbound cells at time t; U_f, unbound cells at infinity; U_0, total cells; and θ, fractional saturation. The standard error of estimate for the left-hand side of Eq. (3) is 0.13. [Reprinted from M. M. Cowan, K. G. Taylor, and R. J. Doyle, *J. Dent. Res.* **65,** 1278 (1986).]

If the straight line does not pass through the origin, cell–cell interactions (aggregation) may be influencing the adsorption process, and rate constants for adsorption and desorption cannot be calculated.

Desorption Rate Constant

The desorption rate constant k_d can be calculated from

$$k_d = k_a(U_0 - B_f)(1 - \theta)/B_f \tag{5}$$

where B_f is the number of cells bound at equilibrium. When desorption experiments are performed to determine k_d independently, the time required for 50% of the adsorbed cells to desorb is estimated from the plot of unbound cells (U) against time t and related to k_d by

$$1/2U_0 = U_0 e^{-k_d t_{1/2}} \tag{6}$$

or

$$t_{1/2} = \frac{0.693}{k_d} \tag{7}$$

Complex Adsorption Processes

If the k_d obtained from desorption experiments does not correspond with the calculated k_d from Langmuir theory [Eq. (1)] and if all the condi-

tions noted above have been met, then some binding anomaly may be predicted. Often a slow two-step attachment mechanism is indicated in which the initial equilibrium (sampled in the kinetic adsorption experiments) gives way at some rate to a new (final) equilibrium which is sampled in the desorption experiments and which is governed by an additional set of rate constants for adsorption and desorption. This situation may be represented

$$A \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} C$$

in which A $\rightleftharpoons$ B represents the "equilibrium" between unbound cells and cells bound in a reversible state (probably in the secondary minimum), and B $\rightleftharpoons$ C represents the process of loosely bound cells traversing the energy barrier to be bound firmly in the primary minimum. The affinity constant K_A can be calculated from k_a/k_d for any given step in the adsorption process. K_A is also available from experiments which sample only the equilibrium condition. In these experiments numbers of bound and unbound cells at equilibrium are analyzed using models, such as the (nonkinetic) Langmuir equation or the Scatchard equation,[21] to yield graphical estimations of K_A. It is vital to note that K_A values obtained in equilibrium-type binding experiments describe only the last step in the adhesion process, and when adhesion proceeds in a multistep fashion they reveal nothing about the initial interaction of bacteria with substratum.[20]

In the two-step process described above k_{d2} may be nonexistent or negligible since adhesion in the primary minimum is often (but not always) irreversible. Also, the model predicts that k_{a2} is slower than k_{a1} (but not k_{d1}) and for this reason is detectable in experiments.

Summarizing, k_a can be determined experimentally using the kinetic form of the Langmuir equation described here, and k_d can be calculated once k_a is known. The value of k_d can also be independently determined in desorption experiments and, if found to differ from k_{d1}, may in actuality represent a k_{d2} or even k_{d3}, indicating multistep adsorption kinetics. In this case k_{d1} can also be experimentally verified by conducting experiments in which desorption conditions are instituted very early in the adsorption process, that is, while B $\rightleftharpoons$ C (or possible later steps) are still exerting a negligible influence on the initial binding.

Conclusion

Kinetic adsorption and desorption experiments provide a fundamental method for examining mechanisms of bacterial adhesion. Violations of

[21] G. Scatchard, *Ann. N.Y. Acad. Sci.* **51,** 660 (1949).

assumptions (about cell–cell interactions, for example) are readily apparent and themselves yield information about the nature of the binding. Combining initial kinetic data with data gained from "equilibrium" experiments can also help to unravel complex adhesion mechanisms. Additional experiments assessing the effects of temperature and buffer changes on the kinetic constants can provide information about the particular long-range and short-range forces and bacterial surface constituents which are responsible for attachment.

[17] Observation and Measurement of Bacterial Adhesion to Plants

By ANN G. MATTHYSSE

Introduction

The measurement of bacterial adhesion to plants and plant cells can be carried out using two fundamentally different approaches. In one, the focus of interest is the determination of the number of adherent bacteria and the changes in number with time and exposure to various treatments. A variety of methods are used in this approach, including viable cell counts of free and attached bacteria and measurement of adhesion of radioactively labeled bacteria. In the second approach, the focus is on the site of bacterial adhesion and the appearance of the bacteria and surrounding plant cells. The methods used here are largely microscopic. This chapter discusses the use of each of these approaches in the study of bacterial adhesion to exterior leaf surfaces, roots and root cap cells, the interior of the plant, wounded tissue, and tissue culture cells. This is followed by a general discussion of the interpretation and limitations of each approach. Considerations of limitations and interpretation of data which apply to adhesion to only one type of plant surface are considered with the description of the methods for that surface.

In general, any study of bacterial adhesion to plant surfaces should make use of both approaches because they provide complementary information. The first approach provides quantitative numbers, but the meaning of the numbers can only be assessed if the spatial distribution of the bacteria is also known. In any experiment involving bacterial adhesion to plants live bacteria should be used, and it should be remembered that there are

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assumptions (about cell–cell interactions, for example) are readily apparent and themselves yield information about the nature of the binding. Combining initial kinetic data with data gained from "equilibrium" experiments can also help to unravel complex adhesion mechanisms. Additional experiments assessing the effects of temperature and buffer changes on the kinetic constants can provide information about the particular long-range and short-range forces and bacterial surface constituents which are responsible for attachment.

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numerous studies showing the induction and repression of many bacterial genes by substances coming from the plant.

General Considerations

Before measuring bacterial adhesion to a plant surface, the type of tissue useful for measuring adhesion must be determined; roots or leaves, intact tissues or wounded tissues, or artificial systems, such as tissue culture cells. The physiology of the plant will inevitably determine the nutrients available for bacterial growth and the composition of the surface to which the bacteria can adhere. Thus, consideration must be given to the method of growth and note taken of the state of the tissue with respect to light–dark cycles, flowering or stressed plants, mineral nutrition, and whether the plants are grown axenically. Similar considerations apply to the prior growth of the bacteria. If they are grown in complex medium, then the plant surface is likely to represent a considerable nutritional shift down. Bacteria in exponential or in stationary phase can show different adhesion properties. All such variables should be noted and controlled.

It is also important to determine the conditions used for measuring adhesion. If excess bacteria are present, then the ability of the plant surface to bind any of them can be examined, but the percentage of the bacteria capable of adhesion cannot be examined. Excess bacteria are generally present in methods which use radioactively labeled bacteria as a method of determining the extent of bacterial adhesion. Excess plant cells can be used if one wishes to allow all bacteria which are capable of binding to do so. Methods which use viable cell counts of bacteria are often used in this situation. Microscopic methods must be used if one wishes to examine the spatial localization of the bacteria. Microscopic methods can be used under conditions of either excess bacteria or excess plant cells.

Observation and Measurement of Bacterial Adhesion to Leaf Surfaces

Methods for Enumerating Adherent Bacteria

Procedure. Leaves or strips of leaves from plants which were grown axenically are submerged in a suspension of bacteria. The bacteria are labeled with radioactivity by growth in ^{14}C-labeled amino acids. The bacteria are collected by centrifugation and washed once with nonradioactive growth medium. Typical specific activities obtained are 10^{-3} counts/min (cpm)/bacterium. Romantschuk and Bamford[1] used leaf strips 15 × 7 mm

[1] M. Romantschuk and D. H. Bamford, *Microb. Pathog.* **1,** 139 (1986).

in size and 1 ml of 2×10^7 bacteria/ml in M9 medium. The leaf strips are incubated with the bacteria at room temperature with agitation for varying periods of time up to 120 min. The leaves or leaf strips are removed from the incubation mixture and washed four times with 100 ml of water. The radioactivity adhering to the leaves is then determined.

Variations. Because many leaf surfaces are hydrophobic and do not wet with water, the leaf can be pretreated with 0.1% Triton X-100 for 2 min followed by extensive washing with water to remove the detergent.[2] The procedure is then carried out as described above.

If the numbers of adhering bacteria to be measured are less than 10^5 cells/leaf then viable cell counts of the adhering bacteria or bacterial colonies may be used. Incubations with unlabeled bacteria can be carried out as described above and the washed leaves ground in a blender. The numbers of adhering bacteria released can then be determined by plating appropriate dilutions on suitable media. Alternatively, the washed or unwashed leaves can be shaken dry and simply pressed against a petri dish containing appropriate medium, the leaves are removed and the numbers of colonies formed after incubation of the dish determined. This technique is referred to as making a leaf print.

Leaves from plants grown under ordinary greenhouse or field conditions can be used and the adhesion of radioactive bacteria or of bacteria marked with antibiotic resistance can be determined. If the adhesion of more than one type of bacterium is to be measured, the types can be labeled with different isotopes or marked with resistance to different antibiotics.

Useful Circumstances and Limitations. One of the major advantages of the method is its simplicity. When radioactive bacteria are used the method requires that large numbers of bacteria adhere to the leaves in order for them to be detected. All methods of examining adhesion to leaves encounter the difficulty that the surface of many leaves is markedly hydrophobic. If the leaf is washed with Triton X-100 in order to wet it, then the surface to which the bacteria are adhering is not the same as the surface exposed in natural leaves.

When cut leaf strips are used it is advisable to combine the determination of the number of adherent bacteria with microscope observations because many bacteria may adhere to the cut edge of the strips and not to the leaf surface per se. The use of leaf prints has the advantage that some information about the spatial distribution of the adherent bacteria can be obtained. However, adherent colonies of bacteria and closely spaced adjacent bacteria cannot be distinguished from single adherent bacteria using this method.

[2] E.-L. Numaiho-Lassila, E. Pantala, and M. Romantschuk, *Micron Microsc. Acta* **22,** 71 (1991).

Microscopic Methods

Procedure. Identical samples to those used in the enumeration of adherent bacteria as described above can be prepared and used for studies in the scanning electron microscope. After incubation of the leaves with bacteria and the removal of nonadherent bacteria by washing, the leaves are fixed in 3–4% glutaraldehyde in 10–100 m*M* phosphate buffer, pH 7.2, containing 1 m*M* $MgCl_2$ for 1–3 hr at room temperature. The exact buffers and times depend on the bacterial and plant species to be examined. Excess glutaraldehyde is removed by washing in buffer three times, and the sample is postfixed in 1% OsO_4 in 100 m*M* phosphate buffer for 2 hr at room temperature. The tissue is then dehydrated in an ethanol series (30, 70, 80, 90, 95, 95, 100, 100, 100%) and dried in a critical point dryer. The sample is coated with gold and/or platinum and examined in the scanning electron microscope.[1]

Variations. As an alternative to using the methods described for incubating the bacteria with the leaf surface, bacteria can be applied directly from a culture as a small spot on the leaf (e.g., 50 μl of a bacterial culture). The leaf is then incubated at room temperature for 20 to 120 min, washed four times in water or phosphate buffer, and processed as above for scanning electron microscopy (SEM).[1] This method has the advantage of concentrating the adherent bacteria in a known location of limited size.

Observation and Measurement of Bacterial Adhesion to Root Surfaces

Methods for Enumerating Adherent Bacteria

Procedure. Bacteria are grown in complex or defined media. The medium and phase of bacterial growth are often observed to affect the ability of the bacteria to bind to roots. If it is desired to measure the number of bacteria bound using radioactive bacteria, radioactive amino acids, for example, [^{3}H]leucine, can be included in the medium. If the bacteria are grown without radioactive isotopes and are to be used at a considerable dilution, they can be directly diluted into the medium containing the roots. If the bacteria are labeled radioactively or if high densities of bacteria are to be used (e.g., 10^6 cells/ml or more), the bacteria are collected by centrifugation and washed with buffer or the medium to be used in the incubation.

Plants are grown axenically and roots or root segments of a defined length (e.g., 1 cm) are cut from a defined region of the root. The roots are placed in water or buffer in a glass dish. Bacteria are diluted and added

to the roots, and the mixture is incubated on a shaker at 2–50 rpm at room temperature for 1 to 120 min. The roots are then removed, drained, and washed by shaking or soaking them for 10–60 sec in water or buffer. The washing can be repeated as many as 10 times. The roots are recovered on a Büchner funnel. The number of adherent bacteria is measured by determining the radioactivity remaining with the roots after incubating them with a solubilizing agent such as Lumisolve.[3] Alternatively, the bacteria are removed from the roots and plated for viable cell counts. To remove adherent bacteria the roots are placed in a 10-ml tube with 2 ml of water containing Tween 40 (1 drop/100 ml). The tube is placed in the cup horn of a Heat Systems model 370 sonicator which is run at 50% power for 5 min. The samples are then plated on an appropriate medium.[4]

Variations. As an alternative to the use of a sonicator to remove the bacteria, a blender or homogenizer can be used. Plants can be grown in sterile dishes or in sterile pouches. There are several methods for exposing the roots to the bacteria. Pueppke[5] hung plants from bent paper clips in tubes with the roots dipping into the solution containing the bacteria. There are also several variations in the washing procedure. Some investigators wash the roots in flowing water. Others wash the roots by vortexing them in water or buffer.[6]

The procedure of Anollés and Favelukes[7] combines the enumeration of adherent bacteria with the use of microscopic observations. Plants are grown aseptically from seed, and 15 5-day-old seedlings are placed in 22.5 ml of nitrogen-free Fåhraeus solution in a wide-mouth 250-ml flask. Bacteria are added, and the mixture is incubated for 4 hr on a shaker at 28° and 50 rpm. The fluid is discarded, and the plants are washed four times in 25 ml of the same solution for 1 min at 120 rpm. The seedlings are then laid flat in a petri dish and embedded in 45° agar medium. The number of colonies formed and their location are determined after 2 days of incubation at 30° by observation in a dissecting microscope.

Useful Circumstances and Limitations. The root is a complex organ, and the average adhesion over the entire surface may miss significant localizations of the bacteria. In the case of rhizobia adhering to legume roots, it is the specific adhesion to root hairs which might be expected to play a role in the formation of root nodules. This localized adhesion can only be documented in the microscope.

[3] A. J. Anderson, P. Habibzadegah-Tari, and C. S. Tepper, *Appl. Environ. Microbiol.* **54,** 375 (1988).
[4] S. J. Vesper and W. D. Bauer, *Symbiosis* **1,** 139 (1985).
[5] S. G. Pueppke, *Plant Physiol.* **75,** 924 (1984).
[6] D. W. James, Jr., T. V. Suslow, and K. E. Steinback, *Appl. Environ. Microbiol.* **50,** 392 (1985).
[7] G. C. Anollés and G. Favelukes, *Appl. Environ. Microbiol.* **52,** 371 (1986).

When root segments are used it is important to distinguish between adhesion to the surface of the intact root and adhesion at the cut surface. The distinction can be made in the microscope or by making a fresh cut to remove the original cut surface and bacteria bound to it before the numbers of adherent bacteria on the remaining root surface are determined.

The ability to obtain quantitative numbers of adherent bacteria over time is an advantage over the more subjective microscopic scoring of adhesion. However, these numbers appear to be markedly affected by the method of washing chosen. Thus the data of Anderson[3] suggest that in analyzing the attachment of pseudomonads to roots using sequential washes the results depend on how many washes in a particular medium are necessary to remove nonadherent bacteria. More than 90% of the bacteria which could be removed by water washing were removed by the first water wash, but significant numbers of bacteria were still removed by the tenth wash. A subsequent wash with EDTA removed an equivalent number of bacteria to those still found to be associated with the root and recovered by grinding. In the absence of microscopic data on where the bacteria are located and of chemical data on the nature of the mechanism of adhesion, it is difficult to know how to make distinctions between adherent bacteria and bacteria which are simply trapped by the anatomy of the root. Probably the best procedure is to measure the number of bacteria released by washing and those remaining associated with the root after various washing protocols and to continue washing until the number removed is much less than those which remain. Microscopic observation of the location of the adherent bacteria after various washing procedures should also aid in deciding on a washing protocol.

Mathematical analysis of bacterial adhesion is severely limited by the complex nature of the surface of the root to which the bacteria adhere. Any mathematical model which assumes the presence of only one type of binding site on the root surface is open to question.

Microscopic Methods

Procedure. Bacteria can be incubated with roots as described above and the material then prepared for microscopic observation. Living material can be observed directly after washing using phase-contrast microscopy. To observe bound bacteria using fluorescent antibodies, the roots are fixed for 10 min in 3.5% paraformaldehyde, washed twice in phosphate-buffered saline (PBS), stained with the fluorescent antibody in PBS containing 1% bovine serum albumin for 30 min at room temperature, washed twice in PBS, and mounted in 50% glycerol.[8]

[8] K. Haahtela, T. Laakso, and T. K. Korhonen, *Appl. Environ. Microbiol.* **52,** 1074 (1986).

For scanning electron microscopy the roots are washed and fixed in 3% glutaraldehyde in 50 m*M* potassium phosphate buffer, pH 7, for 4 hr. They are rinsed three times in the phosphate buffer followed by three rinses in water (each rinse is 30 min at room temperature). The roots are then dehydrated in a 10-step acetone series (from 10 to 100% acetone), dried in a critical point dryer, coated with gold, and examined in the SEM.[9]

Variations. Bacteria and roots can be grown together in hydroponic slide cultures (Fåhraeus slide cultures). Seeds are surface sterilized and germinated on water agar. After 36 hr of germination the roots are placed between two slides in a medium containing salts and no agar. For the study of interactions of rhizobia with roots, N-free Fåhraeus solution is often used. Bacteria which have been collected and washed in the same medium are added. The mixture is incubated for varying lengths of time up to several days. The slide is then gently disassembled, and the roots are washed while still on the slide with the medium used during the incubation. The slide is covered with a glass coverslip and examined.[10] Obviously this method is only feasible with plants which have small, thin roots.

Use of Root Cap Cells

An innovative variation for the study of bacterial adhesion to roots is the use of root cap cells. Root cap cells are produced in the root meristem and are shed into the rhizosphere as the root grows through the soil.

Procedure. Seeds are surface sterilized and germinated on water–agar overlaid with Whatman (Clifton, NJ) filter paper. The root tip is excised and immersed in 50 μl sterile water in the well of a microtiter plate. The plate is agitated for 30 to 60 sec to release the cells. If desired the cell density can be adjusted. In some cases the number of binding sites per root cap cell was so large that excess plant cells were obtained with experiments involving 10^2 cells/ml and 10^7 bacteria/ml. Viability of the root cap cells can be verified by observing cytoplasmic streaming in the light microscope. Bacteria (50 μl of 10^7 bacteria/ml) are added to the plant cells, and the mixture is incubated for 2 hr at 25°. A sample (e.g., 20 μl) of the mixture is collected on a 10-μm mesh screen and washed with 1 ml of water. The plant cells are then suspended in water and examined in the light microscope using interference contrast optics. Bacterial binding is determined in one focal plane and is expressed as the number of bacteria bound on the cell perimeter.[11]

[9] K. Haahtela and K. Kari, *Plant Soil* **90,** 245 (1986).

[10] F. B. Dazzo, *in* "Experimental Microbial Ecology" (R. G. Burns and J. H. Salter, eds.), p. 431. Blackwell, Oxford, 1982.

[11] M. C. Hawes and S. G. Pueppke, *Plant Physiol.* **91,** 113 (1989).

Variations. Numbers of bound bacteria could be determined by disrupting the washed root cap cells in a blender or homogenizer and determining the number of bacteria released by viable cell counts. Radioactively labeled bacteria could be used and the amount of radioactivity remaining with the washed root cap cells determined. Root cap cells and adherent bacteria could be prepared for examination in the SEM using the techniques described for the microscopic study of bacteria adhering to suspension culture cells described below.

Useful Circumstances and Limitations. Root cap cells are easy to obtain and to manipulate. They represent a uniform cell population. However, their surface may differ from the surface of other root epidermal cells. Thus, if results with root cap cells are to be extrapolated to other cell types, some comparisons of the major characteristics of the adhesion being studied should be made using both cell types.

Observation and Measurement of Bacterial Adhesion to Plant Interior

Enumeration of Adherent Bacteria

Few, if any, attempts have been made to enumerate bacteria which have attached to interior surfaces of the plant. Such sites include the surfaces of the cells which line the air spaces in the mesophyll. Bacteria would presumably gain access to this area through the stomata. In addition, there are the surfaces of the xylem conducting vessels and tracheids. Several species of plant pathogenic bacteria which cause wilting diseases grow in the xylem. However, no information is available as to whether these bacteria adhere to the surface of the vessels. A major obstacle in such experiments is that in the case of air spaces the bacteria will be found on the plant cell surfaces unless the air space has become water soaked. In the case of xylem vessels there seems to be no obvious reason for the bacteria to adhere to the surface of the vessels.

Measurements of the adhesion of bacteria to such surfaces could be made using the techniques described for roots, wounded tissues, and tissue culture cells.

Microscopic Methods

The majority of studies of bacterial adhesion to the interior surfaces of leaves have been microscopic studies. These studies have implicated bacterial adhesion in the development of the hypersensitive response (HR) and the resistance of plants to disease.

In the examination of plants for internal adherent bacteria thin sections are usually used. Specimens of this type have also been examined for the presence of structures which attach the bacteria to the plant cell surface. Such structures have frequently been seen. However, it is difficult to determine if they are made by the bacteria or the host cells.

Because the tissue is fixed with the internal bacteria in place, it is not possible to distinguish bacteria which are simply lying in a particular location from bacteria which are bound to that surface. Numbers of adherent versus nonadherent bacteria cannot be determined. In addition, the absence of the opportunity to wash the tissues means that the relative strength of the adhesion cannot be examined.

Procedure. Bacteria are introduced into fully expanded leaves by injection or by infiltration under vacuum or increased pressure. Some researchers have even used an air brush on the abaxial side of the leaf. High densities of bacteria are generally used (10^7 to 10^9 cells/ml) in order to find the introduced bacteria easily and to induce a visible HR. After varying times of incubation (generally 2–72 hr), pieces of the leaf are excised and fixed in 3–5% glutaraldehyde in 80 m*M* cacodylate buffer, pH 7.4, in vacuum container for 2 hr. The tissue is rinsed in the buffer and then postfixed in 1% osmium tetroxide. The tissue is dehydrated in an ethanol series and then embedded in Epon epoxy resin, and thin sections are cut with an ultramicrotome. The sections are stained with 0.5% uranyl acetate for 45 min and then with lead citrate for 7 min. The sections are examined in the transmission electron microscope.[12]

Variations. There are many variations in the details of the fixation and staining of the samples. Phosphate buffer (0.2 *M*, pH 7.3) can be substituted for the cacodylate buffer. Fixation is sometimes carried out at 4°. Some researchers extend the fixation time to overnight.[13]

One variation allows the examination of the specimen in the scanning electron microscope. Bacteria are introduced into the leaves as described above. Leaf disks are cut with a cork borer and fixed overnight in 2% glutaraldehyde buffered with 0.1 *M* sodium cacodylate, pH 7.2. The disks are then rinsed twice in the buffer and postfixed in 1% OsO_4 in the cacodylate buffer for 4 hr. After two buffer rinses the disks are dehydrated in an acetone series and dried in a critical point dryer. Dried leaf disks are placed on a stub covered with double-stick tape. Another stub covered with the same tape is pressed onto the leaf disk and then removed. In this way the epidermal surfaces of the leaf become attached to the tape and the interior

[12] W. F. Fett and S. B. Jones, *Phytopathology* **72,** 488 (1982).
[13] D. J. Politis and R. N. Goodman, *Phytopathology* **68,** 309 (1978).

of the mesophyll is revealed. The sample is coated with gold–palladium and examined in the SEM.[14]

Interpretation and Limitations. The inability to wash the tissue results in difficulties in interpretation of results. Bacteria in liquid suspension which are infiltrated into the air spaces in a leaf must as the infiltrated liquid dries be pulled to the surface of the plant cells surrounding the cavity. In the absence of washing these bacteria cannot be distinguished from adherent bacteria. The comparison of bacterial mutants altered in known properties with an isogeneic wild-type strain would allow the determination of the role of various bacterial genes in the interaction seen in the microscope. Thus, before concluding that pili or fibrils play a major role in the interaction of the bacteria with the plant cell surface, one should examine the appearance of bacterial mutants which are unable to make these structures.

Observation and Measurement of Bacterial Adhesion to Wounded Tissue

Many phytopathogenic bacteria interact with and infect wounded plant tissues. Thus the use of disks of wounded tissues is a useful model for interactions which might be expected to occur under natural conditions.

Enumeration of Adherent Bacteria

Procedure. Bacteria are radioactively labeled by growth in a medium containing a radioactive amino acid such as [^{35}S]methionine. Because the radioactivity will later be measured using solubilized tissue pieces, the use of an isotope which emits high-energy particles and thus is less subject to quenching by the sample is advised. The bacteria are harvested by centrifugation and washed in phosphate-buffered saline. The specific activities obtained are usually between 20 and 300 bacteria/cpm.

Plant material is surface sterilized and disks are cut axenically from the tissue to be used such as potato tubers, carrot roots, or leaves. Care should be taken to make the disks as uniform as possible. Pueppke has used potato tuber disks 9 mm in diameter and 4 mm thick. The disks are placed in a petri dish on water agar or wet filter paper. The disks are all oriented in the same way (e.g., apical end up). Radioactive bacteria are suspended in buffer at 10^8 to 10^9 bacteria/ml. A small measured volume of the suspension, for example, 25 μl, is placed on each disk. The dish is covered, and the disks are incubated at room temperature for 1 to 90 min. The disks are then washed three times in a tube with 5 ml of PBS and transferred to

[14] M. C. Deasey and A. G. Matthysse, *Physiol. Mol. Plant Pathol.* **33,** 443 (1988).

scintillation vials, where the tissue is solubilized by using a commercial tissue solubilizer or by drying the disks at 80° overnight followed by digestion with perchloric acid. The radioactivity remaining with the disk is determined in a scintillation counter. As an internal standard to measure quenching, the same volume of bacteria is applied to a disk and the disk placed directly into a scintillation vial and processed in the same way as the experimental disks. Results are expressed as the cpm in the experimental disks divided by the cpm in the standard.[15]

Variations. If the tissue to be tested is very thin, such as leaf disks, or easy to homogenize, then nonradioactive bacteria can be used and the number of adherent bacteria determined by viable cell counts after washing and homogenizing the plant disks.

Although bacterial adhesion to wounded tissues usually has not been measured using disks of tissue submerged in liquid medium to which the bacteria are added, there is no reason why that method should not be used. The only difficulties would be the tendency of wounded tissues to fragment when submerged and the necessity to keep the tissue aerobic. Such materials could be processed as described for tissue culture cells below.

Microscopic Methods

Procedure. Wounded plant tissues generally have irregular surfaces and are too thick for easy observation of living material in the light microscope. Thus bacterial adhesion to such samples is usually examined in the SEM. The tissue is washed with the incubation medium and fixed in 3% glutaraldehyde for 5 to 18 hr. Long fixation times may aid in holding the tissue together. The sample is then washed in the buffer used in the glutaraldehyde fixative or water and postfixed in 1% OsO_4 for 1 to 2 hr. The tissue becomes brittle with overly long exposure to osmium. The sample is again washed with buffer and dehydrated in a series of increasing concentrations of ethanol or acetone (30, 50, 70, 80, 90, 95, 95, 100, and 100%). The sample is dried in a critical point dryer, coated with gold or platinum, and examined in the SEM.[16]

Variations. The exact protocol used in preparing the tissue for microscopy should be monitored in the light microscope to determine if pieces of the wounded surface are sloughing off during the preparation. Wounded tissue is difficult to work with because of the propensity for the wounded surface to be further damaged during processing. If it is possible to observe the plant material and adherent bacteria in the light microscope, that pro-

[15] S. G. Pueppke and U. K. Benny, *Can. J. Microbiol.* **30,** 1030 (1984).

[16] A. E. Graves, S. L. Goldman, S. W. Banks, and A. C. F. Graves, *J. Bacteriol.* **170,** 2395 (1988).

vides a useful control for artifacts which may be introduced during preparation for SEM.

Samples may also be examined in the transmission electron microscope using the protocols described above for the examination of adherent bacteria in the interior of leaves. However, the tendency of the wounded tissue to tear during sectioning may limit the use of thin sections.

Observation and Measurement of Bacterial Adhesion to Tissue Culture Cells

Although tissue culture cells are not a naturally occurring cell population, several investigators have considered that the growth of cells in callus or suspension cultures may represent a prolonged wound response. The use of tissue culture cells in studies of bacterial adhesion has several advantages. Measurement of bacterial adhesion is generally relatively easy. Time courses of adhesion can be determined. In suspension cultures usually only one cell type is present. Changes in the surrounding medium including timed additions and removal of substances by washing are possible.

The major limitation of the use of tissue culture cells is that the cell type may not represent the cell type involved in adhesion to intact or wounded plants. In particular, adhesion to surfaces and surface structures such as the cuticle or root hairs cannot be observed in this system. However, in cases where adhesion to the cells present at a wound site appears to have occurred, tissue culture cells can be a useful model. Major conclusions reached with studies using tissue culture cells should be checked with whole plants in so far as practicable.

Enumeration of Adherent Bacteria

Procedure. If it is desired to have a large number of bacteria present, then the bacteria can be grown with radioactive substances such as amino acids, collected by centrifugation, washed, and suspended in the medium to be used in the incubation. If an excess of plant cells is wanted, then the bacteria can be grown in medium and diluted to an appropriate density. Plant suspension culture cells are diluted to the desired density in tissue culture medium such as Murashige and Skoog medium (MS). It is convenient to use plant cells at between 1 and 5×10^5 cells/ml freshly diluted 1/10 in MS. When an excess of plant cells is desired, bacteria are added to a final density of $3–6 \times 10^3$ cells/ml. When an excess of bacteria is desired, bacteria are added to a final density of $3–6 \times 10^8$ cells/ml. The mixture of plant cells and bacteria is incubated for varying times and the plant cells and adherent bacteria separated from free bacteria by filtration through a

Miracloth or Whatman number 1 filter. The plant cells and adherent bacteria are homogenized in the incubation medium or in PBS in a blender to break the plant cells and separate bacteria which have attached to the same plant cell. Numbers of both free and adherent bacteria can be determined by plating on an appropriate medium for viable cell counts. If the bacteria were radioactively labeled, the free bacteria can be collected on 0.2-μm filters, dried, and counted. The adherent bacteria and plant cells can be dried and dissolved in a solubilizer and radioactivity determined. A quench control consisting of a known amount of radioactively labeled bacteria mixed with plant cells should be included.[17,18]

Variations. Plant cells and adherent bacteria can be separated from free bacteria using filtration through wire mesh screens, nylon sieves, Mitex 60-μm-pore filters (Millipore Corp., Bedford, MA), or by low-speed centrifugation. Depending on the plant cells used, cells will often pellet at forces as low as 500 *g* for 4 min. If the plant cell culture contains some large clumps of cells, it is often advisable to remove these before the start of the experiment by filtration through large mesh size wire screens (e.g., 400-μm wire mesh).

Callus tissues can be used as well as suspension culture cells. Callus tissues provide a much more varied surface. The lumps of callus can be suspended in liquid medium and bacterial adhesion measured as described for suspension culture cells. Alternatively, bacteria can be applied directly to the surface of callus cultures on agar and incubated and then the callus washed in water, buffer, or tissue culture medium and the numbers of free and adherent bacteria determined using the methods described for suspension culture cells.

Microscopic Methods

Although the plant cell type is uniform in suspension cultures, all such cultures will contain a small number of broken or dead cells. As with all methods of measuring numbers of adherent bacteria, it is helpful to observe the bacteria and plant cells in the microscope. Such observations will reveal bacterial aggregate formation, specific sites of adhesion if they exist, whether the bacteria are bound to live or dead cells, and whether the bacteria adhere individually or in clusters. Live plant cells can be recognized in the light microscope by the presence of cytoplasmic streaming. This can be a slow and stately process, so that patient observation is required to determine whether particular cells show cytoplasmic streaming.

Procedure. Bacteria are incubated with tissue culture cells as described above. Cells are fixed for microscopy by adding glutaraldehyde to a final

[17] A. G. Matthysse, P. M. Wyman, and K. V. Holmes, *Infect. Immun.* **22,** 516 (1978).
[18] A. G. Matthysse, K. V. Holmes, and R. H. G. Gurlitz, *J. Bacteriol.* **145,** 583 (1981).

concentration of 1% directly to the mixture of bacteria and plant cells. The mixture is fixed for 1 to 5 days. The cells are washed with water and postfixed in 1% osmium tetroxide for 4 to 16 hr. The cells are dehydrated in aqueous solutions of 30, 50, 70, 90, 95, and 100% acetone and dried in a critical point drying point apparatus with liquid CO_2. The cells are attached to aluminum specimen holders with Microstick (Ted Pella Co., Tustin, CA), coated in a rotary evaporator with about 10 nm of 60% gold–40% palladium, and examined in the SEM at 25 kV.[18] The observations made in the SEM are compared with observations of living specimens in the light microscope using Nomarski optics.

Limitations and Interpretation of Experiments Involving Enumeration of Adherent Bacteria

Methods of counting adherent bacteria usually provide only limited information about the spatial distribution of the bacteria on a surface. Therefore, it is important that the methods be combined, if at all possible, with microscopic examination of the adherent bacteria. Enumeration of adherent bacteria is useful for screening different types of bacteria and incubation conditions for their effects on adhesion and for obtaining quantitative data.

The physiology of the plant cells and in particular of leaves may influence bacterial adhesion. Thus, leaf age, growth conditions, and time of day (relative to light–dark cycles and the start of photosynthesis in the morning) may affect the structure of the leaf surface and the nutrients available to bacteria in various locations. The surface of the leaves of axenically grown plants may not be the same as the leaf surface of field-grown plants.

In analyzing the quantitative data one should be careful to remember that the attachment may be to several different types of sites exposed on the surface. Most mathematical formulas dealing with adhesion assume that adhesion is to only one type of site, and thus such models must be used with considerable caution. Even with suspension cultures or root cap cells which are relatively uniform, one must still be cautious in the use of mathematical models of attachment. More than one type of attachment may still be present. The bacteria may alter the surface of the plant cell in their vicinity. The bacteria may produce structures or materials which entrap free bacteria, resulting in two classes of adherent bacteria: those attached to the plant cell and those bound to the attached bacteria. This is the case for *Agrobacterium tumefaciens* incubated with carrot suspension cells. These bacteria adhere directly to the plant cell surface. They also produce cellulose fibrils which bind the bacteria tightly to the plant cell surface and which entrap free bacteria.[18]

In analyzing the kinetics of bacterial adhesion, one must also remember that the bacteria may grow during the incubation. In general, the doubling time of adherent bacteria is not the same as that of free bacteria. When the bacteria are grown in rich medium such as Luria broth and diluted into plant tissue culture medium such as MS, the change of medium constitutes a nutritional shift down, and the bacteria enter a lag phase which may last for 20 to 200 min depending on the bacterial species and media used.

Some species of bacteria tend to form aggregates which may confuse the enumeration of adherent bacteria. Even with studies of adhesion to suspension culture cells, the aggregates can cause problems. Bacterial aggregates may be retained by the filters used to separate free bacteria from plant cells or organs with adherent bacteria. In that case the aggregated bacteria will be counted as adherent bacteria. It is best to check for the formation of bacterial aggregates both in the bacterial growth medium and during the incubation with plant cells. Plant tissue culture media or substances released from the plant cells may cause or increase the formation of bacterial aggregates.

The use of radioactive bacteria to measure numbers of adherent bacteria carries the problems of measuring radioactivity in the presence of large amounts of plant tissue causing quenching. In the case of discs of colored tissue such as leaf discs or carrot root discs, this can be a very difficult problem.

With the use of tissue discs or wounded tissues the problem of removing nonadherent bacteria becomes significant. If the bacteria are motile and chemotactic to substances released by the plant tissue, many bacteria will swim into the tissue and be trapped and not released on washing. An upper limit on the magnitude of this problem can be obtained by the comparison of the adhesion of wild-type motile bacteria with that of nonmotile bacterial mutants which would not move far into the tissue. However, motility may also play a role in attachment to the surface of the wounded tissue. Microscopic examination of the tissue may aid in distinguishing trapped from adherent bacteria if one remembers to ask whether a bacterium in any particular location would be likely to be removed by washing.

It is important to remember that some species of bacteria do not grow in plant tissue culture media and may find the media toxic. For example, the growth of *Agrobacterium rhizogenes* is inhibited by high salt. The amount of salt found in MS medium is sufficient to inhibit the growth of the bacteria. In addition, some plant cells may release substances into the medium which are toxic or inhibitory to some bacteria. Thus, it is useful to incubate known numbers of viable bacteria with the medium and plant material intended for use in adhesion studies to determine the effect of the incubation on bacterial viability. To prevent the bacteria from adhering

to the plant material during these incubations, the plant organs or cells may be separated from the bacteria by a membrane filter which will allow the passage of large molecules or dialysis tubing which will not allow the passage of toxic proteins. The interaction between the bacteria and the plant cells is by nature a sort of conversation so that the plant cells may release toxic materials in response to molecules coming from the bacteria. This is the case in at least some examples of the hypersensitive response.

Useful Circumstances and Limitations of Microscopic Observations of Bacterial Adhesion

Microscopic examination is the only technique which will allow determination of the spatial distribution of the adherent bacteria. For example, clustering of bacteria around stomata or at the junctions between adjacent cells on a leaf or adhesion localized at the root hair tip can only be determined in the microscope. The direct observation of bacterial adhesion in the microscope is important in measuring attachment to a complex organ such as the root. Different bacteria may adhere to different structures, for example, root hairs, root tips, or the zone of elongation. In the case of rhizobia, because the penetration of the root by the bacteria proceeds through infection threads which enter the root through the root hairs, attachment to the root hairs is thought to be the only meaningful adhesion leading to nodule formation.

Observations of living material in the light microscope are the most reliable and subject to the fewest artifacts. However, it may be difficult to distinguish adherent bacteria on the surface of the plant at the magnification possible in the light microscope. This is particularly true if the bacteria are small or if they are bound lying on their side along the plant cell surface or if the plant material has an uneven surface.

These problems can be overcome by the use of scanning and transmission electron microscopy (SEM and TEM). Nevertheless, those techniques may introduce significant artifacts into the material. The live material must undergo extensive processing before it can be examined in the electron microscope. In preparing samples for SEM care must be taken that the sample is never exposed to an air–liquid interface until after it is dried. All of the steps in the preparation need to be carried out slowly so that no sudden shrinkage or swelling of the sample results. Drying artifacts in which various parts of the sample have pulled away from one another, leaving strands of dried material behind, are frequently encountered. The artifacts are often due to too rapid dehydration or inadequate fixation of the sample. Examination of locations in the specimen in which structures are known to be close together without such strands provides information

as to whether there is a problem in the particular sample. Bacteria can often move during sample preparation. In general, it is wise to be certain that longer fixation times do not change the appearance of the sample.

If at all possible living samples similar to those to be prepared for SEM should be examined in the light microscope. Any discrepancy between the living sample and the SEM sample suggests the presence of artifacts generated during the preparation of the SEM sample. Buffer concentrations and fixation and dehydration times can be adjusted to attempt to correct the problem.

The use of the light microscope and SEM allow the survey of a large area of the plant surface. Adherent bacteria which do not protrude far beyond the surface of the plant cells are best seen in the SEM. Estimates of the numbers and frequency of adherent bacteria as well as their general location are best made in the SEM. However, the resolution of the SEM is not usually high enough for many bacterial structures potentially involved in adhesion to be seen. Flagella can sometimes be seen, but fimbriae are not visible in the SEM. Fibrillar material can only be seen if it has a large enough diameter or if it forms aggregates with a large diameter. The metal coating obscures some surface details.

In the transmission electron microscope fine details of the adherent bacteria can be distinguished, but it is not possible to survey many samples or regions of samples. In addition, if the bacteria are widely dispersed on the plant cell surface, one may have to examine a great many sections just to find one which shows an adherent bacterium. Using TEM, small structures involved in adhesion such as pili and fibrils can be seen. However, caution should be used in the interpretation of such structures. It is easy to confuse proteinaceous fimbriae and carbohydrate-containing fibrils in the TEM.

One of the major limitations of microscopic measurement of adhesion is the difficulty in quantitating the number of adherent bacteria observed. Some investigators have tried to surmount this obstacle by using various scales to report degrees of adhesion. For example, in the case of rhizobia adhering to roots, both Dazzo *et al.*[19] and Smit *et al.*[20] use scales in which 1 indicates little adhesion, 2 indicates a few bacteria spread over the surface of the root hair, 3 indicates adhesion mainly to the tip of the root hair, and 4 indicates that the tip of the root hair is covered with a cap of bacteria, in this case with some bacteria also being found along the side of the root hair. When one uses a scale such as that, the steps on the scale are unlikely

[19] F. B. Dazzo, G. L. Truchet, J. E. Sherwood, E. M. Hrabak, M. Abe, and S. H. Pankratz, *Appl. Environ. Microbiol.* **48,** 1140 (1984).

[20] G. Smith, T. J. J. Logman, M. E. T. I. Boerrigter, J. W. Kijne, and B. J. J. Lugtenberg, *J. Bacteriol.* **171,** 4054 (1989).

to be evenly spaced. Thus, the distance and number of bacteria and plant functions required to go from 1 to 2 may be very different from the distance and number of steps required to go from 2 to 3 on the scale. Although the use of such scales does aid in describing various types and degrees of attachment, the measurement of the magnitude of bacterial adhesion in the microscope still remains largely subjective.

Conclusion

Although microbial adhesion to plant surfaces must play a significant role in the ecology and incidence of disease of plants growing in the field, relatively little attention has been given to the topic. The methods described above are not particularly complicated or difficult to apply. It is hoped that more information about the adhesion of bacteria to plants will be obtained in the near future, allowing a more accurate assessment of the role of adhesion in the life of those bacteria which grow in association with plants.

[18] Strategies for Studying Bacterial Adhesion *in Vivo*

By HAKON LEFFLER, WILLIAM AGACE, SPENCER HEDGES, RAGNAR LINDSTEDT, MAJLIS SVENSSON, and CATHARINA SVANBORG

Introduction

The ultimate goal of studies on microbial adhesion is to understand what molecular interactions between host and microbe occur *in vivo* and the impact of these interactions on disease processes. With this goal in mind the problem can be approached at four levels. At the biochemical level the host receptors at the relevant colonization site are identified; at the cell biology level, consequences of bacterial binding to host epithelial cells are studied in cell culture; at the physiological level the consequences of bacterial binding are studied in experimental animals or humans; and at the population level the consequences of receptor binding for colonization are studied by epidemiological methods. In this chapter, we give an example of studies at each level and discuss the implication for what might occur *in vivo*.

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Biochemical Level: Sialoglycoprotein Receptor for Enteropathogenic *Escherichia coli*

Host Cell Surface Glycoconjugate Microarchitecture

Many bacteria carry adhesins that bind specific carbohydrate structures. This binding has functional and medical consequences at different levels depending on the receptor-bearing molecule. The carbohydrate chain structure recognized by the bacteria targets the bacteria to the site of infection and also determines species specificity and tissue tropisms. Within a given cell, the carbohydrate structure targets the bacteria to specific glycoconjugates; it determines which of the many glycoproteins (or glycolipids) will interact with the bacteria. However, the nature of the noncarbohydrate part, whether a protein with a specific function or a lipid, is likely to be most important for determining the effects bacteria have on the cell.

Identification of the carbohydrate specificity of bacterial binding and mapping of carbohydrate structures in relevant tissue are most important in understanding the spread and tropism of infection, and for development of inhibitory receptor analogs. On the other hand, the identification of the noncarbohydrate part of receptors is most important in understanding the dynamic functional interaction with the target cell. Because the same carbohydrate structural motifs can be found on many different proteins in different cell types, it is essential to identify the whole receptor structure, carbohydrate and noncarbohydrate, in the relevant target cell.

The availability of glycoconjugate receptors and their function depends on the molecular architecture at the cell surface. Two layers of structural variation influence the cell surface architecture; one is the variability of the carbohydrate chains; the other is the variation of the protein part of glycoproteins and the lipid part of glycolipids. The protein and lipid moieties are usually inserted in the membrane and are often directly involved in specific physiological functions (at least in the case of glycoproteins).

The carbohydrate chains are not inserted in the membrane, and for the most part they do not directly influence the function of the protein or lipid. Instead they act as markers decorating the proteins. A limited number of structural motifs occur in the carbohydrate chains of a large number of glycoconjugates. Their structures do not correlate with tissue type or function in a simple way, and frequently the saccharides of a given glycoconjugate vary extensively among species.

The erythrocyte membrane is the best studied natural membrane, and demonstrates distinct cell surface microarchitecture of glycoconjugates.[1]

[1] J. Viitala and J. Jarnefelt, *Trends Biochem. Sci.* **10,** 392 (1985).

For example, 90% of the sialic acid (recognized by some strains of *Escherichia coli*) is found on glycophorins, whereas other glycoproteins have preferentially other types of saccharides such as polylactosaminoglycans.[1] Galα1,4Galβ sequences (recognized by other *E. coli*) are found mainly in glycolipids. The same carbohydrate structures as found in erythrocytes occur in many other cell types, but they may be linked to different proteins or to glycolipids. Some cell types have mainly other carbohydrate structures on their cell surface glycoconjugates. In the intestinal epithelium, glycolipids[2,3] and glycoproteins[4–6] carry different types of saccharides, compared to erythrocytes.

Below, we describe the identification of a specific receptor for enteropathogenic *E. coli* in the rabbit intestine. In this example, sialic acid appears to be part of a carbohydrate "flag" that targets bacteria to a specific type of protein. Sialic acid is recognized by many bacterial strains[7] and viruses[8] that cause disease in the gastrointestinal tract. In most cases, binding activities have been detected by microbe-induced hemagglutination or by interaction with various model sialoglycoconjugates. Sialic acid has been demonstrated in the intestine, but the sialylated glycoconjugates in the intestinal epithelium have not been identified at the biochemical level, except for a few mucins[9,10] and glycolipids (e.g., Ref. 3). In fact, most of the N-linked protein-bound saccharides in rat and human intestine do not contain sialic acid[4–6] nevertheless, sialic acid does appear to be carried by a limited subset of glycoproteins in the normal intestinal epithelium.[9,11]

Isolation of Sialoglycoprotein Receptor from Rabbit Intestinal Brush Borders

Strain RDEC-1 is an enteropathogenic *E. coli* (EPEC) strain isolated from rabbits with spontaneous enteritis. Like other EPEC, it induces effac-

[2] S. Björk, M. E. Breimer, G. C. Hansson, K.-A. Karlsson, and H. Leffler, *J. Biol. Chem.* **262,** 6758 (1987).
[3] M. E. Breimer, G. C. Hansson, K.-A. Karlsson, and H. Leffler, *J. Biol. Chem.* **257,** 557 (1982).
[4] J. Finne, M. E. Breimer, G. C. Hansson, K.-A. Karlsson, H. Leffler, J. F. G. Vliegenthart, and H. van Halbeek, *J. Biol. Chem.* **264,** 5720 (1989).
[5] S. Takasaki, R. H. Erickson, Y. S. Kim, N. Kochible, and A. Kobata, *Biochemistry* **30,** 9102 (1991).
[6] H. Norrsell, J. Bengtson, P. A. Jovall, and G. C. Hansson, *Eur. J. Biochem.* **230,** 285 (1992).
[7] P. O. Sjöberg, M. Lindahl, J. Porath, and T. Wadström, *Biochem. J.* **255,** 105 (1988).
[8] R. E. Willoughby, *Glycobiology* **3,** 437 (1993).
[9] S. V. Litvinov and J. Hilkens, *J. Biol. Chem.* **268,** 21364 (1993).
[10] I. Carlstedt, A. Herrmann, H. Karlsson, J. Sheehan, L. A. Fransson, and G. C. Hansson, *J. Biol. Chem.* **268,** 18771 (1993).
[11] P. Raffie, H. Leffler, J. Byrd, F. J. Cassels, E. C. Boedeker, and Y. S. Kim, *J. Cell Biol.* **115,** 1021 (1991).

ing–attaching lesions of the brush borders.[12] A bacterial chromosome encoded protein (e.g., Eae) is required for such lesions,[13] but plasmid-encoded pili (AF/R1 in RDEC-1) may facilitate the effacing lesion and mediate *in vivo* the tissue tropism and species specificity of infection.[14,15] Therefore, it was of interest to identify the receptor for AF/R1 pili-mediated adhesion.

Earlier work has shown that adhesion of *E. coli* RDEC-1 to isolated rabbit brush borders is correlated with experimental infection of live rabbits.[14] To identify the receptor of the adhesion, intact brush borders are used as a starting material. First, an assay to measure binding of RDEC-1 to intact brush borders or fractions of brush borders had to be developed. The assay is used to monitor the subsequent fractionation of brush borders into different classes of components and purification of a receptor active glycoprotein complex. Details of the methods are given in Ref. 11.

Binding Assay. Intact brush borders, microvilli, or insoluble fractions (pellets) are sonicated (10 sec on ice with a Branson sonifier) to generate a fine particulate suspension before immobilization in wells. Soluble extracts, with or without detergents, are immobilized without prior sonication. For immobilization, 0.3-ml/well portions of the sonicated or soluble fractions, undiluted or diluted with phosphate-buffered saline (PBS), are incubated in 24-well tissue culture plates for 14 hr at 4°. To detect binding activity, *E. coli* RDEC-1 cells labeled by culture in the presence of [^{14}C]glucose are added to the wells. After washing to remove unbound bacteria, bound bacteria are quantitated by lysing in 0.5 ml of 1% (v/v) SDS and determining the radioactivity of the solubilized material in a scintillation counter.

The binding assay is suitable for monitoring receptor activity in extracts and fractions. Activity can be detected whether the sample is an insoluble and sonicated pellet or is extracted in the presence of detergent. In contrast to that of other bacterial adhesion receptors,[16] this receptor activity can not be detected after blotting onto nitrocellulose after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Preliminary Characterization of Receptor. To gain preliminary insight into the nature of the receptor, the intact brush borders are treated with different reagents before or after immobilization, and bacterial binding is analyzed. Periodate and sialidase abolish binding activity, suggesting that sialic acid is an important component of the receptor. This suggests that the receptor is a glycoprotein or a glycolipid.

[12] R. M. Robins-Browne, *Rev. Infect. Dis.* **9,** 28 (1987).
[13] A. E. Jerse, K. G. Gicquelais, and J. B. Kaper, *Infect. Immun.* **59,** 3869 (1991).
[14] M. K. Wolf, G. P. Andrews, D. L. Fritz, R. W. Sjögren, and E. C. Boedeker, *Infect. Immun.* **56,** 1846 (1988).
[15] J. R. Cantey, L. R. Inman, and R. K. Blake, *J. Infect. Dis.* **160,** 136 (1989).
[16] B. Gillece-Castro, A. Prakobphol, A. L. Burlingame, H. Leffler, and S. J. Fisher, *J. Biol. Chem.* **266,** 17358 (1991).

Extraction of Brush Borders with Different Solvents. Because most glycoproteins can be extracted with nonionic detergents, brush borders are first extracted with buffers containing Triton X-100 or Nonidet P-40 (NP-40), and aliquots of the supernatant and sonicated pellet are immobilized and assayed for binding of *E. coli* RDEC-1. In all cases, over 80% of the activity remains with the pellet. Therefore, it appears that the receptor is linked to the cytoskeleton.

Based on these findings, the receptor could be a membrane glycoprotein linked to the cytoskeleton via its cytosolic domain. Alternatively, it could be a phospholipid-anchored glycoprotein or a glycolipid, as these classes of compounds have also been bound in detergent-insoluble complexes in certain cells.[17] In separate experiments, the latter possibilities have been ruled out by treatment of brush borders with phosphatidylinositol-specific phospholipase C (PI-PLC) or by analysis of lipid extracts.[11]

Association of Receptor Activity with Microvilli. An efficient technique, based on further homogenization followed by differential centrifugation, permits fractionation of brush borders into microvilli and terminal web material.[18] When the fractions are immobilized and assayed as described above, most binding activity is found in the microvillar fraction.

Extraction of Binding Activity with ATP. As for intact brush borders, most of the receptor activity could not be extracted from microvilli with nonionic detergents. This supported the hypothesis that the receptor is a microvillar glycoprotein linked to the cytoskeleton, which in microvilli consists of an actin core linked to the membrane via myosin I side arms.[19] Because the bond between actin and myosin I is broken in the presence of ATP, we have tried to extract the receptor activity in ATP-containing buffer. Buffer containing ATP and detergent extracts most of the receptor activity. The receptor activity can also be extracted from intact brush borders in the presence of detergent plus either ATP or high salt concentrations. This is consistent with the hypothesis that the receptor is linked to the microvillus cytoskeleton via myosin I.

Fractionation of Solubilized Receptor Complex. Once the receptor is solubilized, it can be purified further by biochemical techniques. Size-exclusion chromatography, ion-exchange chromatography, and lectin affinity chromatography are used. With each method a sharp peak can be eluted [eluting as 450 kDa on Superose-12, with 200 m*M* NaCl from DEAE-Sepharose, and with 0.5 *M* GlcNAc from wheat germ agglutinin (WGA)-

[17] W. Rodgers, B. Crise, and J. K. Rose. Signals determining protein tyrosine kinase and glycosyl–phosphatidylinositol-anchored protein targeting to a glycolipid–enriched membrane fraction Molecular and Cellular Biology, **14,** 5384 (1994).

[18] M. S. Mooseker, K. A. Conzelman, T. R. Coleman, J. E. Heuser, and M. P. Sheetz, *J. Cell Biol.* **109,** 1153 (1989).

[19] M. B. Heintzelman and M. S. Mooseker, *Curr. Top. Dev. Biol.* **26,** 93 (1992).

Sepharose]. On analysis by SDS–PAGE and Western blotting, each purified fraction reveals two glycoprotein bands and myosin I. Apparently, the receptor consists of a tight complex of either one or two glycoproteins with myosin I. The binding to wheat germ agglutinin further supports the hypothesis that the receptor is sialylated.

Biological Implications. The role of the sialoglycoprotein in the effacing lesion is unclear. The finding of a distinct glycoprotein that is linked to the cytoskeleton and sialylated is interesting. Most glycoproteins of the brush border are not linked to the cytoskeleton, but are easily extracted with nonionic detergents. Most of the N-linked saccharides of intestinal epithelial glycoproteins do not contain sialic acid.[4–6] Sialic acid is found in certain mucins,[9] although other intestinal mucins are not highly sialylated. Little is known about sialylated glycoconjugates on epithelial cells. Sialylated glycolipids (gangliosides) are sparse in rabbit intestine compared to neutral glycolipids.[20] In glycoproteins, most of the sialic acid is probably O-linked as it is scarce in N-linked chains.[4,6] Therefore, the receptor probably represents a specific glycoprotein enriched in sialic acid, found mostly in O-linked saccharides. Apparently, binding to such a receptor has been favored among pathogenic *E. coli* in the intestinal tract as many enterotoxigenic *E. coli* (ETEC) also bind to sialic acid in model systems.[7] This raises the possibility that the bacteria bind the same receptor as RDEC-1, because it is linked to the cytoskeleton, and thereby may facilitate a more stable adhesion and/or other effects.

Cell Biology Level: Cytokine Response in Epithelial Cells Elicted by P Fimbriated *Escherichia coli*

Cytokines include interleukins (IL), colony-stimulating factors, tumor necrosis factor (TNF), interferons (IFN), and chemokines. They are produced by a variety of cells and participate in normal tissue homeostasis and in immune reactions. Cytokine production is increased during a variety of pathological conditions including infections and trauma.

Epithelial cells at mucosal surfaces are often the first to encounter microorganisms and may produce certain cytokines in response to stimulation by bacteria. For example, cultured cell lines from the human urinary tract were found to secrete IL-6 when exposed to uropathogenic *E. coli* bacteria.[21–24] Subsequently, a variety of epithelial cells have been found to

[20] M. E. Breimer, G. C. Hansson, K.-A. Karlsson, and H. Leffler, *J. Biochem.* **93,** 1473 (1983).
[21] S. Hedges, P. de Man, H. Linder, C. van Kooten, and C. Svanborg-Edén, *in* "Advances in Mucosal Immunology." Kluwer, London, 1990.
[22] S. Hedges, M. Svensson, and C. Svanborg, *Infect. Immun.* **60,** 1295 (1991).
[23] W. Agace, S. Hedges, U. Andersson, J. Andersson, M. Ceska, and C. Svanborg, *Infect. Immun.* **61,** 602 (1993).
[24] W. Agace, S. Hedges, M. Ceska, and C. Svanborg, *J. Clin. Invest.* **92,** 780 (1993).

secrete cytokines in response to bacteria. Bacteria increase the mRNA levels for IL-1, IL-6, and IL-8 in epithelial cells.[22]

P fimbriae are a specific class of fimbriae common to *E. coli* from cases of pyelonephritis. The fimbriae were first thought to contribute to the virulence of uropathogenic *E. coli* mainly by mediating bacterial adhesion to uroepithelial cells.[25–27] Subsequent epidemiological studies[28] and animal experiments[29] suggested that P fimbriae also directly enhance the inflammatory response in the epithelial cells (e.g., release of cytokines). Because P fimbriae are specific for the saccharide sequence Galα1,4Gal, which in uroepithelial cells is found exclusively in glycolipids, it is suggested that P fimbriae may induce specific responses in the epithelial cells by interacting with the glycolipid receptors. This premise prompted studies at the cell biological level, described below.

Mucosal Inflammatory Response Induced by P Fimbriated Escherichia coli

P fimbriated *E. coli* attach to epithelial cells through specific interactions with Galα1,4Galβ-containing glycoconjugates.[25–27] The receptor function of Galα1,4Galβ-containing oligosaccharides has been documented in several ways. Presence of receptor on the cell is a prerequisite for binding in that P fimbriated *E. coli* attach to cells expressing Galα1,4Galβ-containing oligosaccharides but not to cells lacking these receptors.[25,26] Inert surfaces or particles that lack the receptors can be induced to bind bacteria by coating them with the Galα1,4Galβ-containing glycosphingolipids.[26] The attachment to receptor-positive cells or particles can be competitively inhibited by pretreatment of the bacteria with soluble Galα1-4Galβ-containing oligosaccharides or glycosphingolipids.[27]

P fimbriae are encoded by the *pap* DNA sequences. The receptor specificity of the fimbriae is determined by the G adhesins encoded by the *papG* DNA sequences.[30,31] Three classes of *papG* adhesins have been defined[31] ($papG_{J96}$, $papG_{IA2}$, and $prsG_{J96}$). The pap_{J96} sequences were the first to

[25] G. Källenius, R. Möllby, S. B. Svenson, J. Winberg, A. Lundblad, S. Svensson, and B. Cedergren, *FEMS Microbiol. Lett.* **7,** 297 (1980).
[26] H. Leffler and C. Svanborg-Edén, *FEMS Microbiol. Lett.* **8,** 127 (1980).
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[28] P. de Man, U. Jodal, K. Lincoln, and C. Svanborg, *J. Infect. Dis.* **158,** 29 (1988).
[29] H. Linder, I. Engberg, H. Hoschützky, I. Mattsby-Baltzer, and C. Svanborg, *Infect. Immun.* **59,** 4357 (1991).
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[31] B. Lund, F. Lindberg, B. Marklund, and S. Normark, *Mol. Microbiol.* **84,** 5898 (1987).

be cloned.[32] These sequences were later shown to be unique to *E. coli* J96. Most clinical isolates of *E. coli* carry either $papG_{IA2}$, $prsG_{J96}$, or additional and as yet undefined *papG* sequences. The $papG_{IA2}$-encoded adhesins bind to most Galα1,4Galβ-containing glycosphingolipids. Consequently, strains expressing these adhesins adhere to cells from all donors except to those of blood group P who lack the globo series of glucosphingolipids. The *prsG* adhesins recognize as a receptor epitope a terminal GalNAcα linked to a Galα1,4Galβ containing oligosaccharide core.[33,35] These fimbriae bind to cells of blood group A_1P secretor individuals (see later section on epidemiological methods).

P fimbriated *E. coli* cause disease by activating a local and a systemic inflammatory response.[28] Granulocytes migrate to the site of infection and into the urine.[29] The systemic response includes fever, elevated acute phase reactants such as C-reactive protein (CRP), and elevated erythrocyte sedimentation rate. Studies have suggested that this response may be explained in part by the ability of P fimbriated *E. coli* to induce the production of cytokines, which, in turn, activate the host response.

The existence of a mucosal cytokine response in the urinary tract was first recognized in deliberately infected patients and in mice.[36,37] We found interleukin-6 (IL-6) in the urine of mice within minutes after intravesical instillation of *E. coli* bacteria or isolated P fimbriae. We also found IL-6 and IL-8 in the urine after deliberate colonization with *E. coli* bacteria,[24,37] and in patients with urinary tract infection (UTI).[38–41] There was no concomitant elevation of the serum levels of these cytokines, suggesting that the cytokines were locally produced. It was concluded that bacteria elicit

[32] R. Hull, R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow, *Infect. Immun.* **33,** 933 (1981).
[33] N. Strömberg, B.-I. Marklund, D. Ilver, A. Hamers, and W. Gaastra, *EMBO J.* **9,** 2001 (1990).
[34] G. Otto, T. Sandberg, B.-I. Marklund, P. Ulleryd, and C. Svanborg, *Clin. Inf. Dis.* **17,** 448 (1993).
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[36] P. de Man, L. Aarden, I. Engberg, H. Linder, C. Svanborg-Edén, and C. van Kooten, *Infect. Immun.* **57,** 3383 (1989).
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a mucosal cytokine response and that attachment enhances this response. The role of P fimbriae in the response was examined at the cellular level.

P Fimbriae–Glycolipid Interactions and Cytokine Response in Epithelial Cells

The role of fimbriae–glycolipid interactions for the induction of an epithelial cytokine response may be analyzed in two ways.

Isolated P Fimbriae Elicit Epithelial Cytokine Responses. Epithelial cell lines were exposed to P fimbriae of the $papG_{IA2}$ adhesion type. The secretion of cytokines was determined after 2, 6, and 24 hr. Isolated P fimbriae with intact receptor binding capacity were found to trigger an IL-6 response, whereas P fimbriae lacking the receptor-binding domain triggered a lower IL-6 response.[22] This suggested that the binding of P fimbriae to Galα1, 4Galβ-containing glycolipids elicits a cytokine response in the cells. The interpretation of these experiments is complicated by the fact that the P fimbrial proteins used for cell stimulation were contaminated by lipopolysaccharide (LPS). Binding of P fimbriae to the glycolipid receptor would therefore cause a nonspecific increase of the LPS concentration at the cell surface and possibly enhance the LPS-induced cytokine responses. This nonspecific effect is difficult to separate from a direct activation of the cells by the P fimbrial interaction with the GSL receptor.

Inhibition of Glycolipid Expression. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is a structural analog of ceramide and Glc-Cer that inhibits the glucosylation of this molecule.[42,43] Treatment with PDMP has previously been used to decrease the ganglioside content of cells or tissues and to study the resulting changes in biological properties, such as growth, morphology, and activation by mitogens. We have used PDMP treatment to reduce the concentration of Galα1,4Galβ–containing glycolipids in epithelial cell lines and to study the effects on attachment and cytokine activation. The PDMP treatment reduced the globo series of glycolipids in the cell lines. As a consequence, the attachment of P fimbriated *E. coli* to these cells was markedly reduced. Furthermore, the IL-6 response to bacterial stimulation is impaired.[43a]

Type 1 fimbriated *E. coli* were used as a control. Type 1 fimbriae recognize mannose-containing glycoprotein receptors. The expression of these receptors is not influenced by PDMP. There was no change in adhesion of type 1 fimbriated *E. coli* after PDMP treatment of the cells. These results suggest that the Galα1,4Galβ-containing glycosphingolipids are cru-

[42] J. Inokuchi and N. S. Radin, *J. Lipid Res.* **28,** 565 (1987).
[43] J. Inokuchi, I. Mason, and N. S. Radin, *Cancer Lett.* **38,** 23 (1987).
[43a] M. Svensson, R. Lindstedt, N. S. Radin, and C. Svanborg, *Infect. Immun.* **62,** 4404 (1994).

cial not only as receptors for P fimbriated *E. coli* attachment to epithelial cells but also as transducers of the cytokine response elicited by the interaction. The recent discovery that glycolipids may occur in specific membrane domains that also contain specific glycoproteins and protein kinases[17] may be relevant for signal transduction. Evidence from our laboratory suggests that the ceramide signalling pathway is activated by P fimbriated binding to the globo series of glycolipids. (Hedlund *et al.* in manuscript.)

These studies explored the consequences of bacterial adhesion at the cellular level. Other consequences include invasion of the epithelial cell or through the cell layer, induction of cell desquamation, and activation of nonepithelial mucosal cells. These effects are not further commented on here.

Physiological Level: Consequences of Bacterial Adhesion *in Vivo*

Type 1 or mannose-sensitive (MS) fimbrial adhesins recognize mannose-containing receptors.[44] Their binding is blocked by D-mannose or α-methyl-D-mannoside.[45,46] Receptors for type 1 fimbriae are present on epithelial and nonepithelial cells from many species,[44–46] and type 1 fimbriae are widely distributed among virulent and nonvirulent *E. coli* strains.[47,48] In the human urinary tract, type 1 fimbriae bind mannose epitopes on secreted glycoproteins such as the Tamm–Horsfall protein and secretory immunoglobulin A (IgA).[49–51] When these substances coat uroepithelial cells, they may provide receptor epitopes for bacterial surface colonization. When secreted, they may eliminate type 1 fimbriated *E. coli* strains and prevent colonization or infection. Furthermore, type 1 fimbriae play a complex role in the interaction with human polymorphonuclear leukocytes (PMNL). The adhesion of type 1 fimbriated *E. coli* strains to PMLN may promote bacterial uptake and killing.

fim Gene Cluster

The type 1 fimbriae are encoded by the (*pil*) *fim* gene cluster.[31,52,53] The *pil/fim* sequences from different *E. coli* strains show a high degree of

[44] J. P. Duguid and R. R. Gillies, *J. Pathol. Bacteriol.* **74,** 397 (1957).
[45] I. Ofek, D. Mirelman, and N. Sharon, *Nature (London)* **265,** 623 (1977).
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[48] J. L. Duncan, *J. Infect. Dis.* **158,** 1379 (1988).
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[52] P. E. Orndorff and S. Falkow, *J. Bacteriol.* **159,** 736 (1984).
[53] P. Klemm and G. Christiansen, *Mol. Gen. Genet.* **208,** 439 (1987).

homology. The three genes encoding the minor fimbrial subunits (*fimF, fimG,* and *fimH*) have been sequenced.[54] The *fimH* gene encodes the specific adhesin,[55,56] which is located at the tip as well as along the fimbriae[57,58]; *fimA* encodes the fimbrial subunit protein and can be expressed independently from the *fimH*-encoded adhesin protein. The *fimA* gene product has to be present on the cells to confer the adhesive phenotype. The *fimB* and *fimE* genes encode the proteins involved in regulation of transcription of the *fim* gene cluster.[56]

Role of Type 1 Fimbriae in Virulence of Escherichia coli O1 : K1 : H7 in Urinary Tract

The study[58a] was prompted by a clinical observation in children[59] with febrile UTI. Of 88 children with acute pyelonephritis, 14 carried *E. coli* of serotype O1 : K1 : H7. These children had higher fever, longer fever duration, and higher white blood cell counts than children infected with other *E. coli* strains.

Properties of the O1 : K1 : H7 isolates were examined in an attempt to explain the increased virulence associated with this serotype. The O1 : K1 : H7 *E. coli* strains were shown to be members of the same clone by several criteria. First, they belonged to the same electrophoretic type, having identical electromorphs for the 11 enzymes tested. They expressed P fimbriae of the F11 antigen type. They were hemolysin negative. They all contained the *fim* DNA sequences but differed in their expression of type 1 fimbriae; eight isolates expressed type 1 fimbriae, whereas six did not.

Children infected with the O1 : K1 : H7 strains were grouped into those infected with type 1-positive and those infected with type 1-negative isolates. Their inflammatory responses were then compared to children infected with other P fimbriated *E. coli.* Children infected with the type 1-positive O1 : K1 : H7 strains showed a shorter duration of symptoms prior to antibiotic treatment (quicker onset of symptoms), higher fever, longer fever duration, and higher leukocyte counts than individuals infected

[54] K. A. Krogfeld and P. Klemm, *Microb. Pathog.* **4,** 231 (1988).
[55] F. C. Minion, S. N. Abraham, E. H. Beachy, and J. D. Goguen, *J. Bacteriol.* **165,** 1033 (1986).
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[58a] W. Agace, *et al.,* in preparation.
[59] S. Mårild, B. Wettergren, M. Hellström, U. Jodal, K. Lincoln, I. Orskov, F. Orskov, and C. Svanborg, *J. Pediatr.* **112,** 348 (1988).

with other *E. coli* strains. The severity of the inflammatory response to the type 1-negative O1:K1:H7 isolates did not differ from that of children infected with the other P fimbriated *E. coli* strains. The results suggested that it was the expression of type 1 fimbriae by the *E. coli* O1:K1:H7 isolates that led to the increased severity of infection in the children.

This hypothesis was tested in an experimental model for UTI in the mouse. Three O1:K1:H7 isolates were selected for *in vivo* experiments in the mouse UTI model: one expressing type 1 fimbriae, a second with a weak type 1 expression, and a third that did not express type 1 fimbriae. Mice were inoculated intravesically with each of the strains. The fimbrial expression was tested prior to infection.

The persistence of the three *E. coli* strains in the mouse urinary tract differed in relation to the expression of type 1 fimbriae. Furthermore, the inflammatory response to infection was greater in animals infected by type 1 fimbriae-expressing O1:K1:H7 isolates. Neutrophil numbers and IL-6 levels in the urine were higher at 2, 6, and 24 hr after infection with a type 1-positive *E. coli* strain than after infection with the phenotypically negative isolate.

To attribute this difference to the type 1 fimbriae, a *fimH* deletion mutant was constructed from the O1:K1:H7 type 1-positive strain. A first deletion was achieved by P1 transduction and homologous recombination. The *E. coli* Δ*fimH* mutant did not induce mannose-sensitive agglutination of guinea pig erythrocytes. In the mouse UTI model, *E. coli fimH*$^+$ survived in higher numbers in both kidney and bladders compared to *E. coli* Δ*fimH*. Urinary neutrophil numbers and IL-6 levels were higher at 2 and 6 hr after infection with *E. coli* Δ*fimH* than after infection with the *E. coli fimH* strain.

The contribution of a single bacterial virulence factor to virulence may be discussed in analogy to the Koch postulates.[60] (1) O1:K1:H7 strains were isolated as the cause of pyelonephritis in 14 children. The isolates were shown to be members of the same clone but to differ in the expression of type 1 fimbriae. Type 1-positive O1:K1:H7 isolates were found to induce higher inflammatory responses in the children than type 1-negative isolates. (2) The type 1-positive isolate, when inoculated intravesically into the urinary tract of mice, induced a higher inflammatory response and survived in higher numbers than the type 1-negative isolate. (3) Inactivation of the *fimH* sequences caused a measureable loss in virulence. (4) Ongoing experiments with allelic replacement of *fimH* are required for final conclusions about the role of type 1 fimbriae in these phenomena.

[60] S. Falkow, *Rev. Infect. Dis.* **10,** 5274 (1988).

Population Level: Consequences of Receptor Binding as Studied by Epidemiological Methods

The susceptibility of the individual to colonization and infection with attaching bacteria can vary depending on the availability of receptors in the target tissue.[61,62] Molecules with receptor function for a given bacterial adhesin can be present or absent.[25–27] Furthermore, the availability of receptor molecules for bacterial binding may be influenced by other constituents of the epithelial cell membrane.

The classic example is piglet diarrhea caused by *E. coli* expressing the K88 adhesin. Adhesion mediated via the K88 adhesin was shown to enhance bacterial colonization of the pig small intestine. Adhesion acted in synergy with the bacterial exotoxin in that both factors were required to cause disease. The susceptibility to disease was further controlled by the presence or absence of small intestinal receptors for the K88 adhesin. Piglets lacking receptors were resistant to colonization and infection.[61,62] The spectrum of individuals who become infected by a certain pathogen can thus be influenced by the repertoire of receptors for attaching bacteria expressed by each individual. We examined how the fit between the receptor expression of the host and the adhesion factors of infecting bacteria influenced infection with P fimbriated *E. coli.*

Receptor Repertoire Defining Host Range for P Fimbriated Escherichia coli

The Galα1-4Galβ-containing glycolipids mediate the attachment of *E. coli* to human uroepithelial cells.[26] Their expression is determined by the P histo blood group system, whereas the elongation of the core structure depends on the ABH blood group and secretor state.[63–65] We have described a group of uropathogenic *E. coli* strains which bind with high affinity to globotetraosylceramide (the P antigen) elongated with the blood group A determinant (globo-A), but with low affinity to globotetraosylceramide.[35,66,67] These strains require the presence of globo-A or the Forssman

[61] R. Sellwood, R. A. Gibbons, G. W. Jones, and J. M. Rutter, *J. Med. Microbiol.* **8,** 405 (1975).
[62] H. W. Smith and M. A. Lingood, *J. Med. Microbiol.* **4,** 467 (1971).
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[64] H. Clausen and S. Hakomori, *Vox Sang.* **56,** 1 (1989).
[65] H. Clausen, E. Holmes, and S. Hakomori, *J. Biol. Chem.* **261,** 1388 (1986).
[66] D. Senior, N. Baker, B. Cedergren, P. Falk, G. Larson, R. Lindstedt, and C. Svanborg-Edén, *FEBS Lett.* **237,** 123 (1988).
[67] R. Lindstedt, N. Baker, R. Hull, S. Hull, J. Karr, H. Leffler, C. Svanborg, and G. Larsson, *Infect. Immun.* **57,** 3389 (1989).
[68] H. Lomberg, L. Å. Hanson, B. J. Jacobsson, U. Jodal, H. Leffler, and C. Svanborg, *N. Engl. J. Med.* **308,** 1189 (1983).

glycolipid hapten to bind to target cells.[66,67] Globo-A is present on uroepithelial cells from blood group A_1, P_1 secretor individuals. In contrast, the receptor for most attaching uropathogenic *E. coli,* globotetraosylceramide, is present on epithelial cells from all individuals except those of blood group P.[68]

In theory, it should be possible to evaluate the role of epithelial receptor expression in the susceptibility to UTI by analysis of blood group P individuals, who lack Galα1,4Galβ-containing glycolipids and consequently the receptor for P fimbriated *E. coli.* The group of individuals with this metabolic error is, unfortunately, too small for epidemiological analysis. Other P blood group variables, however, show epidemiological association with disease. Children of blood group P_1 have an increased relative risk for recurrent pyelonephritis compared with those of the P_2 phenotype.[69]

The blood group A-dependent expression of additional receptors provided another opportunity to analyze the role of the receptor repertoire in the selection of the bacteria which successfully colonize and infect the human urinary tract.[35] The receptor specificity was compared among strains that bind globotetraosylceramide and strains recognizing globo-A. Glycolipids were extracted from uroepithelial cells of donors of the following blood groups: A_1P_1 nonsecretor, A_2P_2 secretor, and A_1P_1 secretor. The extracts were fractionated by thin-layer chromatography (TLC), and specific glycolipids were detected by overlay with radiolabeled bacteria or monoclonal antibodies of defined specificity. The classic P fimbriated *E. coli* with high affinity for globotetraosylceramide bound multiple glycosphingolipids that occurred in all the urinary sediment samples (A_1P_1 secretor, A_1P_1 nonsecretor, and A_2P_1 secretor). The *E. coli* with high affinity for globo-A and the Forssmann antigen described above stained only one band (corresponding to globo-A haptoglycosylceramide) in glycolipid samples only from an A_1 secretor individual.

The receptor function of the glycolipids in the intact cell membrane and the role of blood group of this function were analyzed by bacterial adhesion. The Galα1,4Galβ-specific strain attached in similar numbers to cells from the three donors. In contrast, the globo-A-binding strain attached only to the epithelial cells from the blood group A donor with a positive secretor state.

Adhesion is proposed to determine the establishment of bacteria in the urinary tract of the human host. Because individuals of blood group A express the receptor, the globo-A binding strains should infect them more readily than other individuals. We analyzed the P fimbriae expressed by

[69] R. Lindstedt, G. Larson, P. Falk, U. Jodal, H. Leffler, and C. Svanborg, *Infect. Immun.* **59,** 1086 (1991).

2504 *E. coli* strains from 1473 children in relation to the blood group of the children. Nine infections in eight individuals were caused by *E. coli* strains expressing P fimbriae that recognized globo-A but no other detectable adhesins. Six individuals infected with seven of the strains were available for ABH blood group determination. All were blood group A positive, compared with 45% in the population at large.

The affinity of the globo-A-specific adhesins for the A blood group-positive hosts is likely to be of greater quantitative importance than suggested by this low frequency of exclusively globo-A-binding strains. About 10 to 30% of UTI strains contain more than one copy of the *pap* homologous DNA sequences, which encode Galα1,4Galβ-specific adhesins and which cross-hybridize with *prs*, the sequence encoding the globo-A-specific adhesin. Such strains have been shown to coexpress the Galα1,4Galβ- and globo-A-specific adhesins. The affinity for globo-A may thus also contribute to the selection of hosts for such strains. In this case, it was predicted that the A blood group frequency would be increased among patients infected with *E. coli* binding to globo-A.

The study was made possible by the dual function of Galα1,4Galβ-containing glycolipids as bacterial receptors and blood group antigens. Once the combined requirement of the P and A blood group for globo-A expression was shown on erythrocytes and the selective epithelial globo-A expression by secretors was demonstrated in this study, we could deduce the individual variation in receptor expression from the blood group. This is an example how the receptor repertoire can influence the susceptibility to infection at the population level. We predict that similar associations will be identified for other microbes that bind receptor epitopes which vary in expression between individuals in a given population.

[19] Assay for Adhesion of Host Cells to Immobilized Bacteria

By EVGENI V. SOKURENKO and DAVID L. HASTY

Introduction

Most methods for detecting adhesion of microorganisms are based on combining a microbial suspension with suspended or immobilized target cells obtained from the host, followed by washing away the nonadherent

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bacteria.[1-3] However, it is possible to utilize the opposite approach where target cells are allowed to bind to immobilized bacteria. At least one such assay was described previously in a study of the interaction of *Yersinia pseudotuberculosis* with HEp-2 (epidermoid carcinoma, human larynx) cells via the surface protein called invasin.[4] Bacterial cells were immobilized on a filter membrane followed by incubation for 1 hr with dispersed HEp-2 cells. The bound cells were detected by the alkaline phosphatase activity of the HEp-2 cells. The binding of mammalian cells to adhesive proteins of the extracellular matrix, such as fibronectin and collagen, has also been studied by an essentially similar approach.[5]

Here we describe a new method for detection of bacterial adhesion to target cells which we named the inverted adhesion assay (IAA). Similar to the previous assays mentioned, the IAA is based on adhesion of suspended target cells to bacteria immobilized on the bottom of microtiter plate wells, or other suitable substratum. The suspension of cells is rotated inside sealed wells so that only cells with an affinity to the bacterial layer will attach. The number of adherent cells is then counted, visualizing cells using an inverted microscope. The IAA is simpler and faster than many other adhesion assays and gives reliable results. Results can be checked within 7–10 min after combining target cells with bacteria-coated assay wells. Other advantages and disadvantages are mentioned below.

Assay Method

Microorganisms. Staphylococcus aureus Cowan 1 and *Escherichia coli* CSH-50 are grown in brain–heart infusion (BHI) broth for 18 hr at 37°. *Streptococcus pyogenes* M5 is grown in Todd–Hewitt broth (THB) under the same conditions.

Buccal Epithelial Cells. Epithelial cells are obtained from donors by gentle swabbing of the buccal mucosa with a sterile, cotton-tipped applicator stick and are pooled.

Reagents

10 m*M* phosphate, 0.15 *M* NaCl, pH 7.2 (phosphate-buffered saline, PBS)

[1] C. Svanborg-Eden, B. Erickson, and L. A. Hanson, *Infect. Immun.* **18,** 767 (1977).
[2] E. H. Beachey and I. Ofek, *J. Exp. Med.* **143,** 759 (1976).
[3] S. K. Tylewska and R. J. Gibbons, *Curr. Microbiol.* **16,** 129 (1987).
[4] J. M. Leong, L. M. de Vargas, and R. R. Isberg, *Infect. Immun.* **60,** 683 (1992).
[5] E. Ruoslahti, E. G. Hayman, M. Pierschbacher, and E. Engvall, this series, Vol. 82, p. 803. (1981).

Bovine serum albumin (BSA)
Methylene blue

Equipment

Strips of flat-bottomed wells (Corning, Corning, NY, Cat. No. 24106-8)
Petroff–Hauser chamber
Spectrophotometer
Dissecting microscope

Method Description. Overnight cultures of *S. aureus, E. coli,* and *S. pyogenes* strains are washed in 0.15 *M* NaCl by centrifugation and adjusted to an optical density of 1.0 at 540 nm. One hundred microliters of each bacterial suspension is added to microplate wells and incubated at 37° with horizontal shaking at 100 rpm. A sufficient number of wells are prepared to run triplicate wells for each data point. Every test should provide wells without bacteria to serve as a control. After incubation, nonattached bacteria are removed by washing with 150 μl PBS five times. At this point, the density of immobilized bacteria can be estimated visually. If necessary, determination of the number of bound bacteria can be determined using the growth assay described elsewhere in this volume.[6] After bacteria are immobilized, all wells are filled with 0.1% (w/v) BSA–PBS solution and incubated at 37° for 30 min without shaking to quench any additional binding sites on the plastic.

Meanwhile, the buccal cell suspension should be prepared. Buccal cells are washed with PBS and adjusted to 50,000 cells/ml in 0.1% BSA–PBS. It is important that the cell suspension not clump in the BSA solution. If the cells do clump, the pool will not be satisfactory for the IAA because clumped cells will be impossible to enumerate. After quenching the wells, the BSA solution is discarded, and 200 μl of the buccal cell suspension is added to the wells. Immediately, the tops of the wells should be sealed with Parafilm and the strip overturned, so the cells are not in contact with the bacterial layer until the beginning of the experiment. The strips are fixed onto the rotator so that the long axes are aligned with the axis of rotation. The strips are rotated at 12 rpm for 3 min in one direction and 3 min in the opposite direction in order to ensure adequate mixing. After the strips are taken off the rotator, the Parafilm covering is discarded and the buccal cell suspension is removed by aspiration.

Buccal cells attached to the bottom of the wells can be enumerated at this stage by placing the strip upside down under a dissecting microscope. However, the buccal cells can also be stained for easier visualization by carefully adding 150 μl of 0.01% (w/v) methylene blue in 0.1% BSA/PBS

[6] E. V. Sokurenko, V. A. McMackin, and D. L. Hasty, this volume, p. 519.

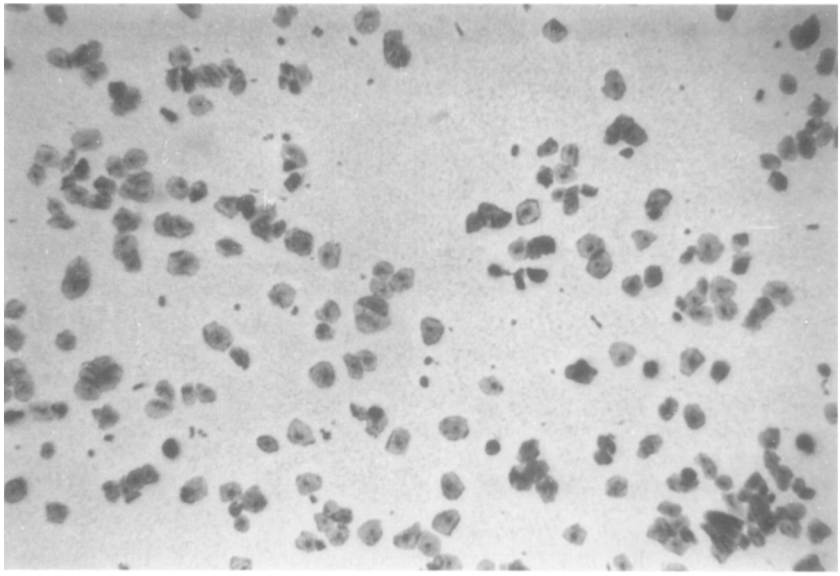


FIG. 1. Photomicrograph of buccal epithelial cells bound to *E. coli* CSH50.

to the wells for a 1-min incubation. Excess stain is removed by aspiration. The strips can be stored in this condition for several days, if necessary, in a humid atmosphere to avoid formation of crystals. Under the dissecting microscope, the attached cells should be spread evenly throughout the field and without obvious clumps (Fig. 1). Results of an experiment performed as described above are presented in Fig. 2. At least for *E. coli* and *S.*

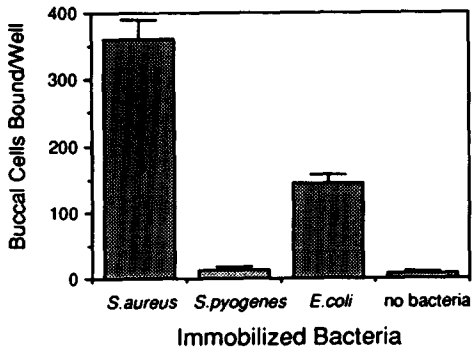


FIG. 2. Binding of buccal epithelial cells in suspension to immobilized *S. aureus* Cowan 1, *S. pyogenes* M5, and *E. coli* CSH50. Values given are means ± S.D. ($n = 4$).

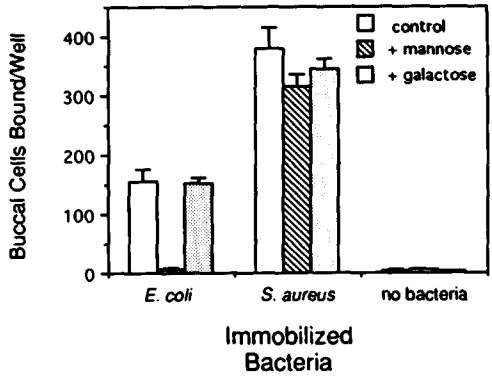


FIG. 3. Binding of buccal epithelial cells to immobilized *E. coli* CSH50 and to *S. aureus* Cowan 1 in the absence (control) and presence of 1% (w/v) mannose or 1% (w/v) galactose. Values given are means ± S.D. ($n = 4$).

pyogenes strains the authors have utilized previously, the results are essentially in agreement with the relative adhesive activities observed using other, more standard types of adhesion assays conducted with buccal cells and bacteria in suspension (i.e., relative adhesion of bacteria and buccal cells is in the order *E. coli* ⩾ *S. pyogenes*).[7]

Method Applications and Restrictions

Inhibition of Adhesion. We used the IAA to study inhibition of *E. coli* adhesion by mannose (Fig. 3). This type of inhibition assay is easily performed in essentially any of the standard adhesion assay formats. We believe that the IAA will be especially useful for studying the effects of molecules that agglutinate bacteria. In many adhesion assays, differential centrifugation is used to separate bound and unbound bacteria, but clumps of aggregated bacteria are difficult or impossible to separate from the cells. In inhibition assays using the IAA, it is of course important that the inhibitory compounds used do not elute the bacterial layer from the assay well surface. The attachment of *E. coli* to plastic, as in the experiment shown (Fig. 3), is not sensitive to D-mannose.

The influence of different treatments of bacteria or cells on adhesion can also be addressed in the IAA. For example, $NaIO_4$ treatment of buccal epithelial cells destroys the receptors for adhesion of type 1 fimbriated *E. coli* (Fig. 4).[8]

[7] D. L. Hasty, H. S. Courtney, and E. V. Sokurenko, unpublished observations.
[8] I. Ofek, D. Mirelman, and N. Sharon, *Nature (London)* **265,** 623 (1977).

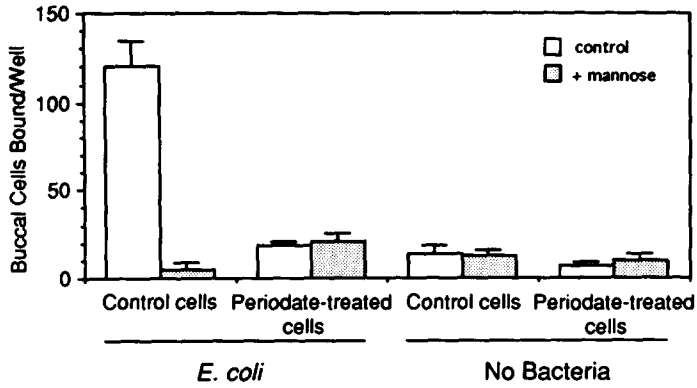


FIG. 4. Binding of control or periodate-treated buccal epithelial cells to immobilized *E. coli* CSH50 in the absence or presence of mannose. The buccal cell suspension (10^5 cells/ml) was incubated with rotation in 20 m*M* $NaIO_4$ for 45 min at room temperature. The cells were then washed with 0.1 *M* Tris–maleate buffer, pH 7.4, followed by washing with 5 m*M* $NaBH_4$ in the same buffer for 30 min at 37°. Values given are means ± S.D.

Adhesion Competition. The IAA is a convenient method for studying the competition of one bacterial species with another for adhesion to the target cell surface. For instance, a suspension of buccal cells can be pretreated with *S. aureus* Cowan 1 or *E. coli* CSH50 and then incubated with an immobilized layer of bacteria, as described above. Suspended *S. aureus,* but not *E. coli,* inhibit adhesion of buccal cells to a layer of *S. aureus* (Fig. 5), suggesting a difference in receptors for the two organisms. As one would expect, *S. aureus* inhibits the adhesion of buccal cells to a layer of *E. coli*

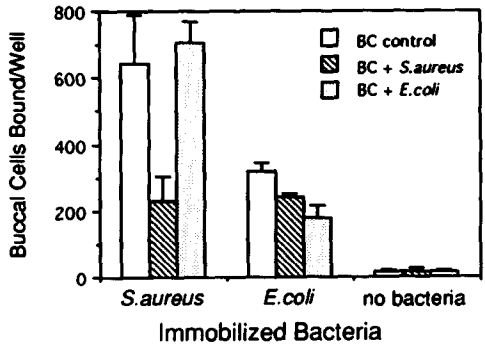


FIG. 5. Binding of buccal epithelial cells to immobilized *S. aureus* Cowan 1 or *E. coli* CSH50. Buccal cells were preincubated with bacteria (final OD of 0.1) for 15 min at room temperature, then rotated at 12 rpm, before being added to wells containing immobilized bacteria. Values given are means ± S.E.M.

only slightly. The relatively low activity of suspended *E. coli* in inhibiting buccal cell binding to an immobilized layer of *E. coli* may be the result of lower overall affinity of *E. coli* to the buccal cell surface, compared with the affinity of *S. aureus*. These speculations need to be addressed experimentally, and the IAA should offer a novel approach. Consideration must also be given to the possible effects of bacteria–bacteria interactions, especially when using different species.

Restrictions. As mentioned above, one requirement is that the cells used, whether buccal epithelial cells or other cell types, should not clump. The density of the adsorbed bacterial layer is another problem that may arise. Binding of target cells during rotation presents problems if the bacterial layer is not sufficiently dense. Therefore, conditions to optimize formation of a sufficient layer of immobilized bacteria should be studied. It may also become necessary to determine the actual numbers of bacteria immobilized under different conditions. The growth assay presented elsewhere in this volume[6] is an effective method to accomplish this goal.

Acknowledgments

The authors thank Jane Hurley for technical assistance. Research was supported by National Institutes of Health Grant DE-07218 (D.L.H.) and by Veterans Administration Medical Research funds (D.L.H.). Dr. Sokurenko is on leave from the Laboratory of Biomedical Technologies, Moscow Sechenov Medical Academy, Moscow, Russia.

[20] Strategies for Employing Molecular Genetics to Study Tip Adhesins

By VIKTORIA VETTER and JÖRG HACKER

Tip Adhesins of Pathogenic Bacteria

Adhesins enable pathogenic bacteria to bind to eukaryotic receptor molecules. The adhesins are important factors, facilitating the colonization and, under special circumstances, the invasion of pathogenic bacteria into eukaryotic cells.[1-4] Adhesins of gram-negative bacteria have been studied extensively. The adhesins may be composed of single proteins, which form nonfimbrial or afimbrial adhesins.[5] Some of the adhesins are visible as thin aggregative fimbriae (also termed fibrillae) by electron microscopy.[6] The best studied adhesins are the so-called fimbrial adhesins, forming long hairlike structures on the bacterial cell surface. The fimbrial rod has a diameter of 2.8–7 nm and is 2–3 μm in length.[1-4,7]

The best studied fimbrial adhesins are the adhesion factors of pathogenic *Escherichia coli* strains. These fimbriae are composed of major subunit proteins, which are identical to the structural proteins, and minor subunit proteins.[2,3] Most of the fimbrial adhesins of intestinal *E. coli* strains (enterotoxigenic *E. coli*, ETEC) are composed of so-called major subunit adhesins, that is, the structural proteins act as adhesion factors.[8,9] In contrast, the fimbrial adhesins of extraintestinal *E. coli* strains (uropathogenic *E. coli*, UPEC; meningitis-causing *E. coli*, MENEC) carry minor subunit adhesins, that is, the minor subunit proteins act as adhesion factors.[1-4] Several laboratories have shown that the minor subunit adhesins are preferentially located

[1] J. Hacker, *Curr. Top. Microbiol. Immunol.* **151,** 1 (1990).
[2] K. A. Krogfelt, *Rev. Infect. Dis.* **13,** 721 (1991).
[3] S. J. Hultgren, S. Normark, and S. N. Abraham, *Annu. Rev. Microbiol.* **45,** 383 (1991).
[4] S. J. Hultgren, S. Abraham, M. Caparon, P. Falk, J. W. St. Geme III, and S. Normark, *Cell (Cambridge, Mass.)* **73,** 887 (1993).
[5] R. Ahrens, M. Ott, A. Ritter, H. Hoschützky, T. Bühler, F. Lottspeich, G. J. Boulnois, K. Jann, and J. Hacker, *Infect. Immun.* **61,** 2505 (1993).
[6] S. K. Collinson, L. Emödy, T. J. Trust, and W. W. Kay, *J. Bacteriol.* **174,** 4490 (1992).
[7] J. P. Duguid, I. W. Smith, G. Dempster, and P. N. Edmunds, *J. Pathol. Bacteriol.* **70,** 335 (1955).
[8] F. K. De Graaf, *Antonie van Leeuwenhoek* **54,** 395 (1988).
[9] D. Bakker, P. T. J. Willemsen, L. H. Simons, F. G. van Zijderveld, and F. K. de Graaf, *Mol. Microbiol.* **6,** 247 (1992).

TABLE I
TIP ADHESINS OF PATHOGENIC *Escherichia coli*

Adhesin	Produced by[a]	Carbohydrate binding specifity	Carbohydrate-specific tip adhesin	Binding to ECM	ECM-specific adhesin
P fimbrial adhesin	UPEC	α-D-Gal-1,4-β-D-Gal	PapG	Fibronectin	PapE, PapF
S fimbrial adhesin	MENEC/UPEC	α-Sialic acid-β-2,3-Lac	SfaS	Laminin	SfaS
Type 1 fimbrial adhesin	Pathogenic and nonpathogenic *E. coli*	D-α-Man-D-α-Man	FimH	Fibronectin, laminin	FimH

[a] UPEC, Uropathogenic *E. coli*; MENEC, *E. coli* causing meningitis.

at the tip of the fimbrial rod.[10-12] We focus our interest on three of the best studied tip adhesin systems among pathogenic bacteria. All three adhesion factors are produced by extrainintestinal *E. coli* strains (Table I). P fimbriae, which are preferentially produced by UPEC, are able to bind to α-D-Gal-1,4-β-D-Gal specific receptor structures.[10,13] The Gal-Gal-specific adhesin, termed PapG, is located at the tip of the fimbrial rod, where it forms a tip-located fibrillum.[10,14] S fimbriae are preferentially produced by UPEC and by strains causing meningitis.[15,16] The S-fimbrial adhesion factors (Sfa proteins) are able to bind to sialic acid-containing glycoproteins. Type 1 fimbriae (Fim proteins) are produced by pathogenic and nonpathogenic bacteria.[17,18] The adhesion protein FimH, which binds to mannose-containing receptors, has been located at the tip of the fimbriae as well as in the fimbrial rod (see Table I; Refs. 12 and 18).

[10] F. Lindberg, B. Lund, L. Johansson, and S. Normark, *Nature (London)* **328,** 84 (1987).
[11] T. Moch, H. Hoschützky, J. Hacker, K. D. Kröncke, and K. Jann, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3462 (1987).
[12] K. A. Krogfelt and P. Klemm, *Microb. Pathog.* **4,** 231 (1988).
[13] B. Lund, F. P. Lindberg, M. Baga, and S. Normark, *J. Bacteriol.* **162,** 1293 (1985).
[14] M. J. Kuehn, J. Heuser, S. Normark and S. J. Hultgren, *Nature (London)* **356,** 252 (1992).
[15] T. K. Korhonen, M. V. Valtonen, J. Parkkinen, V. Väisänen-Rhen, J. Finne, I. Orskov, F. Orskov, S. B. Svenson, and P. H. Mäkela, *Infect. Immun.* **48,** 486 (1985).
[16] M. Ott, L. Bender, G. Blum, M. Schmittroth, M. Achtman, H. Tschäpe, and J. Hacker, *Infect. Immun.* **59,** 2664 (1991).
[17] I. Ofek, D. Mirelman, and N. Sharon, *Nature (London)* **265,** 623 (1977).
[18] S. N. Abraham, D. Sun, J. B. Dale, and E. H. Beachey, *Nature (London)* **336,** 682 (1988).

Cloning of Adhesin Determinants

The isolation of fimbrial subunit proteins,[11,12,18-21] along with genetic experiments, has demonstrated that pathogenic bacteria, especially *E. coli* strains causing infectious diseases, are able to produce more than one fimbrial adhesin system. To analyze the adhesins, genomic libraries have been constructed from several *E. coli* pathogens. The *E. coli* strains J96 (O4:K6), 536 (O6:K15), and AD110 (O6:K2) belong to the best studied uropathogenic *E. coli* isolates. Genomic libraries of the strains were constructed by making use of cosmid cloning systems.[22-24] Hull and co-workers showed for the first time that fimbrial adhesins of different receptor specificity are expressed in the *E. coli* K12 background.[22] On that basis, various determinants coding for different adhesin systems were cloned and subcloned into *E. coli* K12 (for review, see Ref. 1). A DNA fragment of more than 8 kb was shown to be necessary for the production of an intact fimbrial adhesin structure.

Identification of Genes Coding for Tip Adhesins

The adhesin determinants that had been cloned into several cosmid molecules were analyzed by different molecular techniques. First, the adhesin determinants were subcloned into different vector molecules (e.g., pBR322, pACYC184). Particular subclones harboring sequences specific for P fimbriae, S fimbriae, and type 1 fimbriae were able to produce fimbrial structures but did not bind to receptor molecules. Other subclones were able to bind specifically to receptors although no fimbrial structure could be detected. The data support the idea that different genes code for major structural subunits and for proteins mediating binding specificity.[1-4,25,26]

On the basis of subcloning and other mutagenizing techniques, the genomic maps of different adhesion-specific determinants could be charac-

[19] M. S. Hanson and C. C. Brinton, *Nature (London)* **332,** 265 (1988).
[20] H. Hoschützky, F. Lottspeich, and K. Mann, *Infect. Immun.* **57,** 76 (1989).
[21] K. Jann and H. Hoschützky, *Curr. Top. Microbiol. Immunol.* **151,** 55 (1990).
[22] R. A. Hull, R. E. Gill, P. Hou, B. H. Minshew, and S. Falkow, *Infect. Immun.* **33,** 933 (1981).
[23] H. Berger, J. Hacker, A. Juarez, C. Hughes, and W. Goebel, *J. Bacteriol.* **152,** 1241 (1982).
[24] I. van Die, C. van den Hondel, H. J. Hamstra, W. Hoekstra, and H. Bergmans, *FEMS Microbiol. Lett.* **19,** 77 (1983).
[25] B. E. Uhlin, M. Norgren, M. Baga, and S. Normark, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1800 (1985).
[26] J. Hacker, G. Schmidt, C. Hughes, S. Knapp, M. Marget, and W. Goebel, *Infect. Immun.* **47,** 434 (1985).

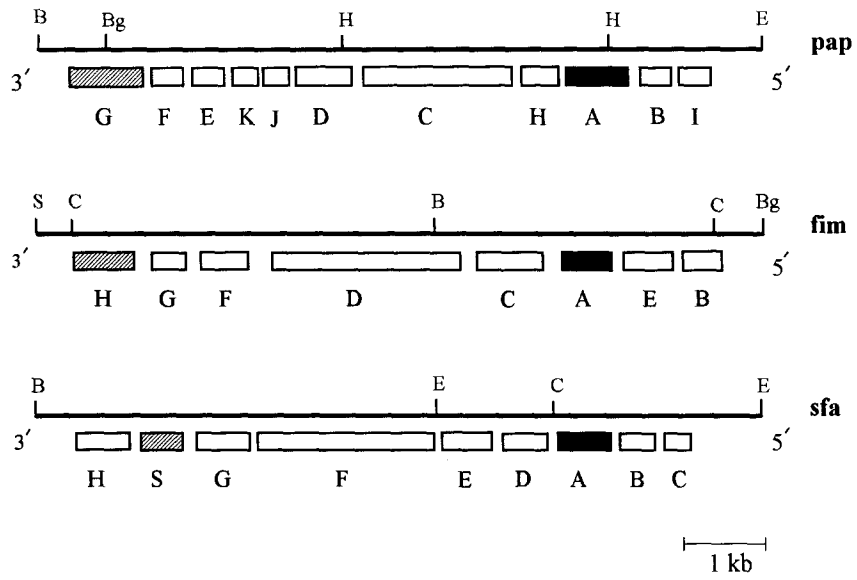


FIG. 1. Genetic maps of *pap*, *fim*, and *sfa* determinants coding for P, type 1, and S fimbriae. Black boxes represent genes coding for major subunits, and hatched boxes represent adhesin specific genes. Restriction endonuclease sites are marked: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; C, *Cla*I.

terized (Fig. 1). The introduction of polar mutations by Tn*5* transposon insertions as well as the construction of frame-shift mutations by site-specific mutagenesis and linker insertion techniques demonstrated that the adhesion-specific genes are located at the 3′ end of the *sfa*, *pap*, and *fim* gene clusters.[11–13,25,27,28] The isolation of adhesion-specific proteins as well as the production of monoclonal antibodies and the usage of immunoelectron microscopy techniques revealed the location of the adhesion molecules to be either solely at the tip (S, P) or preferentially at the tip of the fimbrial structures (type 1; see Refs. 10, 11, and 25–28).

Binding of Tip Adhesins to Receptor Molecules of Extracellular Matrix

The adhesion systems mentioned above bind to well-defined carbohydrate structures, which are parts of glycolipids and glycoproteins on the

[27] K. A. Krogfelt, H. Bergmans, and P. Klemm, *Infect. Immun.* **58,** 1995 (1990).
[28] T. Schmoll, H. Hoschützky, J. Morschhäuser, F. Lottspeich, K. Jann, and J. Hacker, *Mol. Microbiol.* **3,** 1735 (1989).

eukaryotic cell. Korhonen's group and others were able to show that constituents of the extracellular matrix (ECM) can also be used as receptor molecules (Table I). To analyze the binding of P, S, and type 1 fimbria to ECM proteins, clones carrying mutations in the subunit-specific genes were used. With the help of these mutants it was possible to show that the minor subunit proteins PapE and PapF but not PapG are necessary for P-fimbrial binding to fibronectin.[29] Specific binding S fimbriae to laminin is mediated by the tip adhesin SfaS.[30] Type 1 fimbria are able to bind to fibronectin. It appears that binding depends on the type 1-specific adhesion molecule FimH.[31,32] These data allow the conclusion that the minor subunits are proteins capable of binding to different receptor molecules.

Molecular Analysis of Tip Adhesin Genes

To determine the specific binding properties of the tip adhesion molecule of the S fimbriae SfaS, a trans-complementation system with plasmids containing different origins of replication was used.[13,27,33] As shown in Fig. 2a, plasmid pANN801-13, carrying the *sfa* gene cluster as well as the β-lactamase gene (*bla*) of plasmid pBR322, was transformed into the Sfa-negative *E. coli* K12 strain HB101. The recombinant strain expresses S fimbriae, which agglutinate bovine erythrocytes. Figure 2b shows the nonhemagglutinating, *sfaS*-negative clone HB101(pMWW100). The plasmid pMWW100 contains the entire *sfa* determinant carrying a frame-shift mutation in *sfaS* and the chloramphenicol resistance gene (*cat*). As a vector the pACYC derivative pSU2719 was used. Plasmid pMWW100 had been cotransformed with the plasmid pMWW50 (*bla*$^+$), which carries the gene *sfaS* under the control of the inducible *lacZ* promotor of the vector pUC18.

Using this system, it could be demonstrated that the *sfaS*-positive plasmid pMWW50 is able to restore the S-specific binding properties of the nonhemagglutinating *sfaS*-negative mutant clone HB101(pMWW100). The DNA sequence and corresponding amino acid sequence of *sfaS* are given in Fig. 3. Site-directed mutations were introduced into the *sfa* region coding for the C-terminal part of the adhesive molecule SfaS and made it possible to identify an epitope (Lys^{116}–Arg^{122}) involved in binding of the sialic acid-

[29] B. Westerlund, I. van Die, C. Kramer, P. Kuusela, H. Holthöfer, A. M. Tarkkanen, R. Virkola, N. Riegman, H. Bergmans, W. Hoekstra, and T. K. Korhonen, *Mol. Microbiol.* **5,** 2965 (1991).

[30] R. Virkola, J. Parkkinen, J. Hacker, and T. K. Korhonen, *Infect. Immun.* **61,** 4480 (1993).

[31] B. Westerlund and T. K. Korhonen, *Mol. Microbiol.* **9,** 687 (1993).

[32] T. K. Korhonen, R. Virkola, K. Lähteenmäki, Y. Björkman, M. Kukkonen, T. Raunio, A. M. Tarkkanen, and B. Westerlund, *FEMS Microbiol. Lett.* **100,** 307 (1992).

[33] M. Ott, H. Hoschützky, K. Jann, I. van Die, and J. Hacker, *J. Bacteriol.* **170,** 3983 (1988).

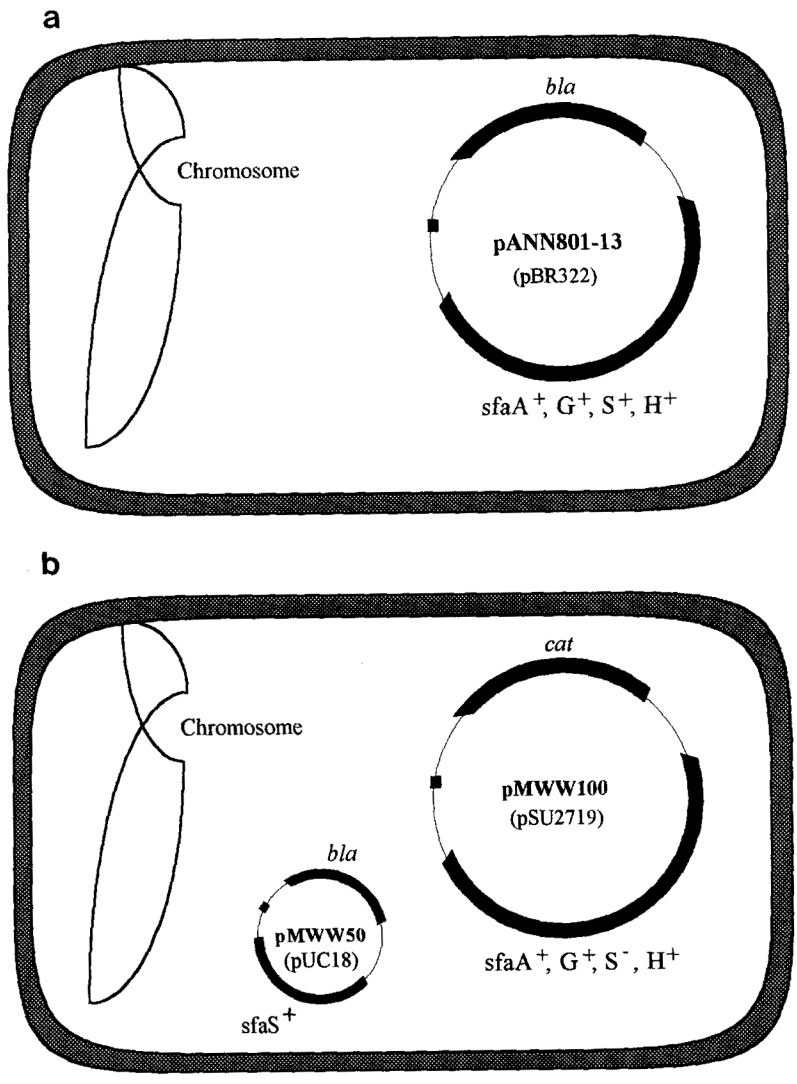


FIG. 2. Trans-complementation system to analyze the *sfa* gene cluster coding for S fimbriae. Plasmid pANN801-13 harbors the entire *sfa* determinant (a), and pMWW100 carries a mutation in *sfaS*, which in turn is complemented by pMWW50 (b). *bla*, β-Lactamase gene; *cat*, chloramphenicol acetyltransferase gene.

FIG. 3. Nucleotide and amino acid sequence of the adhesive molecule SfaS and the primer binding sites upstream and downstream of *sfaS* [T. Schmoll, H. Hoschützky, J. Morschhäuser, F. Lottspeich, K. Jann, and J. Hacker, *Mol. Microbiol.* **3,** 1735 (1989)]. Arrows indicate oligonucleotide primers used for the mutagenesis of SfaS by PCR. Primer D, which is the 5′ complement to primer B and not *sfaS*-sequence-specific, is not shown. Site-specific amino acid exchanges and base pair exchanges are marked at the respective positions. The introduced restriction sites are labeled by black arrow tips.

specific adhesin.[34] To determine other regions of the SfaS protein involved in binding processes, further site-directed mutations were introduced into the adhesin-specific gene *sfaS*.

One single site-specific mutagenic exchange at any position in the double-stranded DNA of the *E. coli* S-fimbrial adhesin gene *sfaS* can be introduced by a modification of the *Thermus aquaticus* polymerase chain reaction (PCR).[35] The method, which is outlined in Fig. 4, requires four synthetic oligonucleotide primers. One primer contains the single-base mismatch, which leads to a specific amino acid exchange, and is thus responsible for the mutagenesis. The other three primers are chosen for selective amplification of the mutated sequence. They contain sequences for the creation of restriction sites. Following digestion, the PCR product can be cloned into the plasmid vector pUC18. The key elements of the technique are two related oligonucleotide primers: (i) a hybrid primer composed of a 3′ segment complementary to a region of the cDNA to be amplified and a 5′ segment whose sequence complements neither target cDNA and (ii) a primer identical to the 5′ segment of the hybrid primer.

In the following example, it was desired to mutagenize the lysine residue in position 83 of the mature S-specific adhesin (Fig. 3). For this purpose six oligonucleotide primers were used (Fig. 4). The first primer, primer A, with one base pair mismatch (5′ GGCGGTATCACGATTGAAATTCAGG 3′; sense forward), was used for directing the mutagenesis, and primer B (5′ GGCCCCTTTTTAACCGCCGTATATTCTGTTCTGCCTGAT 3′; sense inverse) was the hybrid primer. Primer C (5′ CCTGCGATGTTGACATTAAC 3′; sense forward) and primer D (5′ GGCCCCCTTTTTAACCGCCG 3′; sense inverse) were selected to facilitate the selective amplification of the mutated fragment. The oligonucleotide primers E (5′ CGGGAATTCAGACCATAGCGTTTTATGCC 3′; sense forward) and F (5′ CGAGGGATCCGTATGTTTTGGTATCC 3′; sense inverse) were needed to create the *Eco*RI site at the 5′end and the *Bam*HI restriction site at the 3′ end of the 900-bp spanning fragment. Following digestion with the respective enzymes, the resulting restriction sites (sticky ends) were used for the cloning step into pUC18. Sequencing of the resulting insert confirmed that only one amino acid exchange occurred, resulting in a threonine residue at position 83 of the mature S-specific protein.

The resulting construct and plasmid pMWW100 (*sfaS*$^-$, see above), carrying the SfaS adhesin-negative *sfa*determinant, were cotransformed into *E. coli* K12 strain HB101. Phenotypic analyses were studied by performing

[34] J. Morschhäuser, H. Hoschützky, K. Jann, and J. Hacker, *Infect. Immun.* **58,** 2133 (1990).
[35] R. M. Nelson and G. L. Long, *Anal. Biochem.* **180,** 147 (1989).

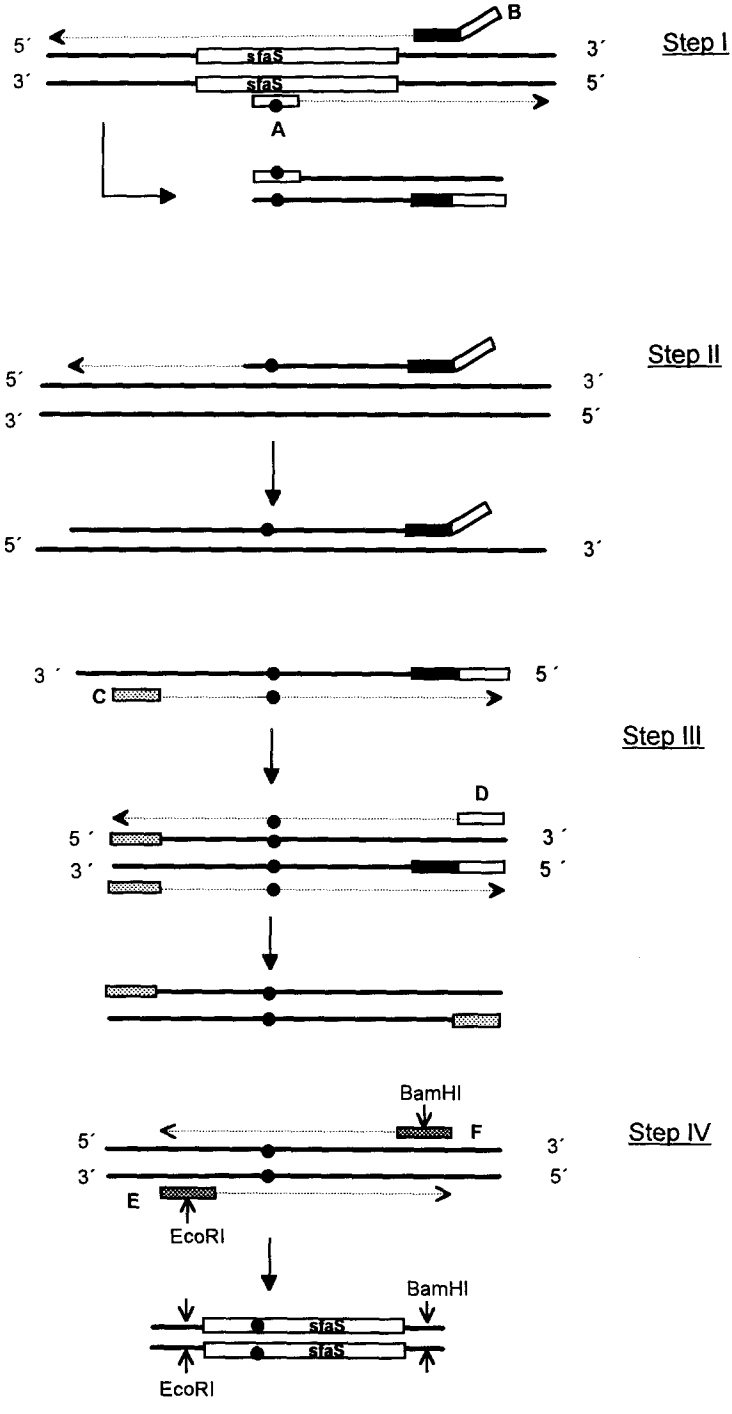
mannose-resistant hemagglutination tests with bovine erythrocytes and enzyme-linked immunosorbent assays (ELISA), employing monoclonal antibodies against the S-specific adhesin and the major fimbrial protein. The assays demonstrated a clear effect of the amino acid exchange at position 83 on the binding properties of the mutagenized tip adhesin of *E. coli* S fimbriae.

Alteration of the standard PCR amplifcation protocol leads to misprimming, misextension, mutations, or heterogeneity owing to misincorporation. The magnesium ion concentration may affect primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, and enzyme fidelity and activity.[36] Consequently, increased magnesium concentration results in random mutated double-stranded PCR products. The randomly mutagenized S-specific mutants were necessary for further analyses of the binding properties of SfaS and also for studies on the interactions of SfaS with other minor subunit proteins of the S-fimbrial adhesin. The random mutations in the adhesin molecules were created by using a 4-fold higher $MgCl_2$ concentration. Otherwise, standard PCR conditions[37] were applied. The primers for the polymerase reaction carried introduced restriction sites (oligonucleotides E and F, see above), which were used for cloning the PCR products into the plasmid vector pUC18. The amplified constructs were sequenced and the effects of the random mutagenesis established. Then, cotransformation of *E. coli* K12 strain HB101 with plasmid pMWW100 (see above) and the amplified constructs, as well as phenotypic analysis, according to the site-specific mutants (see above), made it possible to confirm the importance of the lysine residue at position 83 of the adhesive molecule SfaS. In addition, other amino acids, such as tryptophan at position 43 and arginine at position 89, are involved in binding. Thus, the mutagenized adhesin-specific recombinant plasmids may help to characterize the amino acids necessary for binding of the adhesion factors to eukaryotic receptors (V. Vetter and J. Hacker, unpublished, 1995). In addition, similar experiments were performed to characterize the gene encoding the PapG adhesin of P fimbriae.[38]

Introduction of Mutations into Genome of *Escherichia coli* Strains

Escherichia coli K12 strains carrying recombinant plasmids are the most widely used organisms to analyze tip adhesin determinants. To verify the

[36] M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Academic Press, San Diego, 1990.
[37] K. B. Mullis and F. A. Faloona, this series, Vol. 155, p. 335.
[38] S. J. Hultgren, F. Lindberg, G. Magnusson, J. Kihlberg, J. M. Tennent, and S. Normark, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 4357 (1989).


Step I
B
5′
3′
sfaS
sfaS
A
Step II
Step III
C
D
Step IV
BamHI
F
E
EcoRI
BamHI
EcoRI

contribution of adhesion molecules to virulence, mutations were introduced into adhesion determinants of the chromosome of wild-type strains. For this purpose, different strategies were used. The mutations were introduced by temperature-sensitive replicons[39] or by suicide vectors, which are based on the λ *pir* system and have been developed by Miller and Mekalanos.[40] One simple approach was to clone parts of the adhesion determinants into a vector molecule. An *E. coli* K12 strain containing the cloned adhesion determinant was used for conjugation with another *E. coli* wild-type strain, harboring the adhesion determinant on the chromosome. Homolog recombination led to the disruption of the gene cluster.[41] The disadvantage of this technique is that large pieces of foreign plasmid DNA integrate into the chromosome of the wild-type strains.

The *sacB* system has so far been successfully used by several groups. The *sacB* gene codes for the enzyme levansucrase of *Bacillus subtilis,* which leads to the intracellular accumulation of levansaccharose and accounts for bacterial cell death. Therefore, sucrose-containing media can be used as a positive selection system for strains carrying the *sacB* gene in their chromosome. By this technique, a deletional mutation of the type 1 gene cluster was introduced into the chromosome of *E. coli* K12 strains[42] (see Fig. 5). First, the *sacB* locus as well as the neomycin resistance gene (*neo*) were cloned into a derivative of plasmid pMAK705, a temperature-sensitive pSC101 replicon. Following this, the *sacB-neo* cassette was introduced into the chromosome in the vicinity of the type 1 fimbrial adhesin determinant of strain MG1655. In a second step, the extensive *fim* deletion from the temperature-sensitive plasmid pIB310 was transferred into the chromosome of the intermediate *sacB*-Neo^r-containing MG1655-related strain AAEC064 by allelic exchange. For this, the retaining flanking sequences located upstream of *fimB* (1.4 kbp) and sequences situated downstream of *fimH* (0.6 kbp) were used. The sucrose-resistant mutants were phenotypically confirmed to be afimbriated and not to react with fimbriae-specific monoclonal antibodies. Southern hybridization against the chromosomal

[39] B. Lund, B. I. Marklund, N. Strömberg, F. Lindberg, K. A. Karlsson, and S. Normark, *Mol. Microbiol.* **2,** 255 (1988).
[40] V. L. Miller and J. J. Mekalanos, *J. Bacteriol.* **170,** 2575 (1988).
[41] T. Schmoll, M. Ott, B. Oudega, and J. Hacker, *J. Bacteriol.* **172,** 5103 (1990).
[42] I. C. Blomfield, M. S. McClain, and B. I. Eisenstein, *Mol. Microbiol.* **5,** 1439 (1991).

FIG. 4. Model of site-specific mutagenesis of the S-specific adhesin SfaS by PCR. The coding region of *sfaS* is marked by the long white box. The different filled boxes indicate oligonucleotide primers A to F. The introduced mutation is marked by the black point, and the respective extension directions of the PCR primers are indicated by pointed arrows.

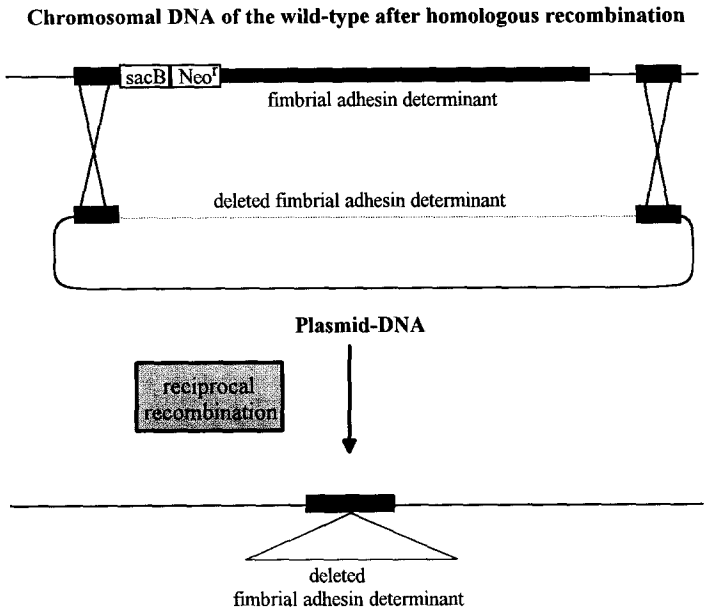


FIG. 5. Introduction of adhesin-specific mutations into the chromosome of *E. coli* strains. For details, see text.

parental DNA confirmed these results. The advantage of this technique is that no additional resistance markers are located in the chromosome.

The technique was also successfully adapted to mutagenize the type 1 fimbrial determinant of the O18:K1 strain.[43,44] Bloch and Orndorff as well as May and co-workers were able to show that the type 1 fimbriae are necessary for binding of bacteria to the oropharyngeal cavity but not for peritonitis.

Eisenstein and co-workers[45,46] used the same strategy of allelic exchange to study the effects of the *fimB* and *fimE* genes as well as the *lrp* locus on both recombination of the *fim* invertible element and fimbrial expression. First, they constructed an intermediate strain with deletion of *fimB* and

[43] C. A. Bloch and P. E. Orndorff, *Infect. Immun.* **58,** 275 (1990).

[44] A. K. May, C. A. Bloch, R. G. Sawyer, M. D. Spengler, and T. L. Pruett, *Infect. Immun.* **61,** 1667 (1993).

[45] M. S. McClain, I. C. Blomfield, K. J. Eberhardt, and B. I. Eisenstein, *J. Bacteriol.* **175,** 4335 (1993).

[46] I. C. Blomfield, P. J. Calie, K. J. Eberhardt, M. S. McClain, and B. I. Eisenstein, *J. Bacteriol.* **175,** 27 (1993).

fimE, thereby locking the invertible element in either the on or off orientation. Following this, they introduced either wild-type or mutant alleles of *fimB* and/or *fimE* into the chromosome of intermediate strains, which contain wild-type or *lrp*-mutant background, by allelic exchange. Analysis of the resulting strains demonstrates the Lrp-stimulated but inversion-independent phase variation of the type 1 fimbriae of *E. coli*.

Moreover, Donnenberg and Kaper[47] constructed EPEC *eae* deletion mutants by also using a positive-selection vector for allelic exchange. The *eae*-determinant codes for an adhesion factor of enteropathogenic *E. coli*, termed *E. coli* attaching and effacing factor. They used the suicide vector pCVD438 with a *pir*-dependent R6K replicon, the *sacB* gene of *B. subtilis*, resistance genes, and parts of the *eae* gene. This construct was termed pCVD441. Via electroporation they introduced the plasmid pCVD441, described above, into the O:127 EPEC strain E2348/69. After growing cultures on sucrose-containing medium, they selected sucrose-resistant colonies and tested them for kanamycin and ampicillin sensitivity, indicating the loss of suicide vector sequences. Colony hybridization with an *eae* DNA probe demonstrated the deletion of the intact *eae* gene.

Mobley and co-workers[48] used a modification of the technique to mutagenize two copies of tip adhesin genes of *pap* determinants by introducing mutations of the minor subunit genes into the chromosome of a wild-type strain. It has been demonstrated that G adhesins of P fimbriae do not contribute to the colonization of *E. coli* strains of the urinary tract of mice.

Conclusion

Molecular genetic approaches make it possible to analyze fimbrial determinants of different species and to determine genes responsible for tip adhesins. To determine the regions responsible for adhesion, specific mutations are introduced into the genes by site-directed mutagenesis. Further results are clearly necessary to confirm these findings in wild-type systems. Various integration systems can be used to introduce adhesin-specific mutations into the chromosome of wild-type pathogens. Thus, the role of tip adhesins *in vivo* can now be analyzed.

[47] M. S. Donnenberg and J. B. Kaper, *Infect. Immun.* **59,** 4310 (1991).

[48] H. L. T. Mobley, K. G. Jarvis, J. P. Elwood, D. I. Whittle, C. V. Lockatell, R. G. Russell, D. E. Johnson, M. S. Donnenberg, and J. W. Warren, *Mol. Microbiol.* **10,** 143 (1993).

[21] Use of Tn*phoA* and T7 RNA Polymerase to Study Fimbrial Proteins

By DIETER M. SCHIFFERLI

Introduction

Molecular cloning and mutagenesis techniques have become essential for studying microbial pathogenesis.[1] Combined with selective or screening procedures for detecting disease-associated phenotypes, they can be utilized to discover new virulence factors and dissect known ones. The aim of this chapter is to focus on two tools which have proved to be particularly useful for studying cloned gene clusters responsible for fimbriation and adhesion of enteric bacteria. Pertinent techniques, concept, strategies, precautions, and advantages of (1) region-directed Tn*phoA* (Tn*5*IS*50*$_L$::*phoA*) mutagenesis[2] and (2) *in vivo* expression of cloned polycistronic genes with a T7 RNA polymerase/promoter system[3] are presented. Broader reviews have included Tn*phoA* mutagenesis techniques for studying bacterial virulence factors in various gram-negative bacteria[4] or the use of transposons in general.[5] Additional information on the mechanisms of transposition can be found in a collection of reviews.[6] Although two expression approaches using T7 RNA polymerase have been developed,[3,7] special emphasis is given to the two-plasmid system of Tabor and Richardson.

Use of Tn*phoA* Mutagenesis for Study of Fimbrial Proteins

Principle

Because of their versatile properties, ease in handling, and disposition to molecular manipulations, transposons are among the most frequently used mutagenic tools. All transposons (abbreviated Tn), Tn*phoA* included,

[1] S. Falkow, *Rev. Infect. Dis.* **10,** S274 (1988).
[2] C. Manoil and J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8129 (1985).
[3] S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1074 (1985).
[4] M. R. Kaufman and R. K. Taylor, this series, Vol. 235, p. 426.
[5] F. J. de Bruijn and S. Rossbach, *in* "Methods for General and Molecular Bacteriology" (P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg, eds.), p. 387. American Society for Microbiology, Washington, D.C., 1994.
[6] D. E. Berg and M. M. Howe, "Mobile DNA." American Society for Microbiology, Washington, D.C., 1989.
[7] F. W. Studier and B. A. Moffat, *J. Mol. Biol.* **189.** 113 (1986).

are mobile discrete DNA segments which carry (1) flanking repeat sequences and one (or more) gene(s) for their own mobility as well as (2) one or more associated genes conferring selective advantages for the host cell (frequently antimicrobial resistance genes). Any transposon, by inserting itself in a new genomic site, acts as a mutagen by altering the local DNA sequence. Usually, transposons affect gene expression at their new insertion locus. Transposition is conveniently selected by using a transposon carrying an antibiotic resistance marker. Technically, one introduces such a transposon in a bacterial host by transformation, conjugation, or transduction using vectors which cannot maintain themselves in the new host. The vectors are typically plasmids or bacteriophages which are replication- and/or integration-defective in certain hosts or under certain growth conditions (suicide vectors). Transposon transposition from the incoming suicide vector to stable genomic DNA (chromosome or a coresident plasmid) can be selected for by growing the recipient bacteria in the presence of the appropriate antibiotic.

Two types of studies, in particular, can benefit from the use of transposon mutagenesis.[5] In the first, the method is used to identify fimbrial or adhesion genes by random mutagenesis of a pathogenic strain followed by screening (if possible, selecting) for nonfimbriated or nonadhesive mutants. This approach, which was described previously in another volume of this series,[4] is then followed by the cloning and further analysis of the mutated locus. In a second type of study, a gene cluster of interest is already cloned on a multicopy number plasmid to be analyzed in *Escherichia coli* in more detail by region-directed mutagenesis,[8] as discussed below. Mutated alleles can then be introduced into the original pathogenic strain by marker exchange experiments.[4] In both types of studies, Tn*phoA*[2] has demonstrated advantages over other transposons, as it can be used to identify exported proteins, as described below. Tn*phoA* is particularly well suited for the study of bacterial fimbrial proteins and adhesins, because such proteins typically cross bacterial membranes to interact with host molecules.

Properties of λTnphoA

Bacteriophage λ Tn*phoA* is λ28(*b*221 *c*I857 *P*am3) carrying Tn*phoA* in or near *rex*, a nonessential gene.[9] Bacteriophage λ28 is an ideal suicide vector because it can neither lysogenize the host strain (*b*221: deletion of the phage attachment site) nor replicate in it (amber mutation in the replication gene *P*). The phage can be propagated in an *amber* suppressor strain (e.g., *E. coli* srain LE392).

[8] D. M. Schifferli, E. H. Beachey, and R. K. Taylor, *Mol. Microbiol.* **5,** 61 (1991).
[9] C. Gutierrez, J. Barondess, C. Manoil, and J. Beckwith, *J. Mol. Biol.* **195,** 289 (1987).

Tn*phoA* is derived from Tn*5*, which is composed of centrally located resistance genes for kanamycin (Km), bleomycin (Bm), and low levels of streptomycin (Sm), and flanked by two insertion sequences designated IS elements.[5] By definition, an IS element encodes a transposase, required for IS or Tn transposition, and terminally repeated DNA sequences which act as a substrate for the transposase. In Tn*5*, only the right IS element (IS*50*$_R$) encodes a full-length and functional enzyme. In Tn*phoA*, a portion of IS*50*$_L$ has been replaced with most of the *E. coli* alkaline phosphatase gene (*phoA*) encoding the mature protein. *Escherichia coli* alkaline phosphatase (PhoA) normally has a signal sequence which is cleaved off on export to the periplasm, where the mature protein dimerizes to an active enzyme. The extreme left side of IS*50*$_L$ is retained in Tn*phoA* for its role in transposition and only slightly modified to produce one continuous open reading frame including *phoA*. Therefore, when Tn*phoA* inserts itself in a new gene in the proper orientation and proper reading frame, a fusion protein consisting of the target gene product at the N terminus and the mature PhoA at the COOH end can be expressed. Most importantly, the fusion protein retains alkaline phosphatase activity only if the N terminus of the target gene product contains the information required for insertion in or/and export across the cytoplasmic membrane (i.e., a signal sequence). This property is thus extremely useful for identifying and studying genes that encode exported proteins, in general, and fimbrial proteins in particular.

Reagents and Media

LB medium: Bacto-tryptone (Difco, Detroit, MI), 10 g; Bacto-yeast extract (Difco), 5 g; NaCl, 5 g; for LB agar, add 15 g Bacto-agar (Difco); add deionized water to 1 liter and autoclave[5,10]

TB medium: Bacto-tryptone, 10 g; NaCl, 5 g; for TB agar, add 15 g agar; for TB soft agar, add 7 g Bacto-agar; add deionized water to 1 liter and autoclave; add 10 ml of sterile 1 *M* $MgSO_4$

TMG buffer: Tris base, 1.21 g; $MgSO_4 \cdot 7H_2O$, 1.2 g; gelatin, 0.1 g; add deionized water to 1 liter and adjust to pH 7.4; autoclave

Kanamycin sulfate (Km) stock: 30 mg/ml dissolved in deionized water, filter sterilized, stored at 4°; unless specified, supplemented to 45 μg/ml

Ampicillin, sodium salt (Ap), stock: 100 mg/ml dissolved in deionized water, filter sterilized, stored at 4°; supplemented to 200 μg/ml

[10] T. J. Silhavy, M. L. Berman, and L. W. Enquist, "Experiments with Gene Fusion." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984.

Chloramphenicol (Cm) stock: 30 mg/ml dissolved in ethanol, stored at 4°; supplemented to 30 μg/ml

XP stock: 20 mg/ml 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt in *N,N*- dimethylformamide (do not filter), stored at −20° in the dark; supplement media to 40 μg/ml. XP is a chromogenic substrate analog of alkaline phosphatase which turns blue when hydrolyzed

Preparation of a λTnphoA Bacteriophage Stock and Titer Determination

1. Grow overnight an *amber* suppressor *E. coli* strain (e.g., LE392) at 37° in 5 ml TB broth.[5,10]

2. Pellet the cells by centrifugation and suspend them in 2.5 ml of 10 m*M* $MgSO_4$. The cells will be used in steps 3 and 7 (bacteria starved in $MgSO_4$ can be stored at 4° and used for several days).

3. Take 0.1 ml of cells in a large (see step 4) sterile tube, add 10 μl of phage (laboratory stock) or, better, 1 to 5 representative plaques (10^5–10^6 phages), removed via a pipette tip from a fresh agar plate lysate prepared as described in steps 6 to 8, and mix well. Incubate at 37° for 10 min.

4. Add 2.5 ml of TB broth and 2.5 ml of TB soft agar (molten and cooled to 45°), mix, and pour immediately on a TB plate. Incubate the plate right side up at 37° for 6 to 8 hr (confluent lysis).

5. Flood the plate with 3 ml of TB broth to help in scraping off the soft agar layer for transfer to a centrifuge tube that can withstand chloroform (e.g., Corex or Teflon). Add 0.1 ml chloroform, vortex, and centrifuge at 5000 *g* for 15 min. Transfer the supernatant to rubber-lined screw-cap tubes (e.g., Wheaton, Millville, NJ, No. 224882), label, date, and store at 4°. Even though the titer will decrease with time, such laboratory stocks can be used for many years to prepare fresh stocks (see step 3).

6. Prepare appropriate dilutions of the new phage stock to evaluate the titer. Take 10 μl of the stock in 1 ml of TMG buffer and vortex (dilution 10^{-2}); take 100 μl of the dilution in 0.9 ml of TMG buffer, vortex, and repeat the last dilution step four times (dilutions 10^{-3} to 10^{-7}).

7. Melt and cool TB soft agar to 45°, add 3 ml of the agar to 0.1 ml of bacterial cells in $MgSO_4$ (from step 2), and pour immediately on a TB plate. Let the agar solidify.

8. Label the back of the plate with 10 small circles, two for each dilution, from 10^{-3} to 10^{-7}; spot 10 μl per circle with each corresponding dilution. Incubate the plate overnight at 37°. Spots of the less diluted samples should have confluent lysis. The presence of ten plaques or more in the 10^{-7} dilution indicates a useful phage titer for successful mutagenesis [$\geq 10^{10}$ plaque-forming units (pfu)/ml].

Mutagenesis of Cloned DNA with λTnphoA

Only a specific region of the cloned DNA may be important for the studied phenotype (e.g., fimbriation or adhesion). Therefore, subcloning steps should be undertaken before mutagenesis. A thorough restriction map of the subcloned DNA will later be required to map the Tn*phoA* inserts. The cloned DNA to be mutagenized can be carried on any convenient plasmid which does not rely on kanamycin for maintenance in its host (e.g., Ap-resistant pBR322 or Cm-resistant pACYC184).[4,5]

1. Grow the host strain overnight at 37° in 2 ml LB broth and the appropriate antibiotic. *Escherichia coli* strain CC118,[2] or an equivalent strain which does not produce alkaline phosphatase (*phoA*) but keeps its resident multicopy number plasmids as monomers (*recA*), should be used for mutagenesis with λTn*phoA*.

2. Use 20 μl of the overnight culture to inoculate 2 ml of LB broth containing 10 m*M* $MgSO_4$ and the appropriate antibiotic. Grow cells at 37° to an OD_{600} of around 1.0 ($\sim 10^9$ cells/ml).

3. Add 200 μl of phage stock to the culture (multiplicity of infection of 1), vortex briefly, and incubate for 15 min at 30°.

4. To increase the probability of isolating different Tn*phoA* mutants, independent transposition events are obtained by preparing separate cultures: add 0.1-ml aliquots of the infected culture into 10 to 20 sterile tubes containing 0.9 ml LB broth and grow the cultures for 5 to 6 hr at 30°.

5. Plate 0.2 ml of each culture on LB agar containing 300 μg/ml kanamycin (high concentration to select for transposition onto the used multicopy number plasmid), XP, and the appropriate antibiotic for the plasmid; incubate for 2 days at 30°.

6. The appearance of white colonies and of colonies of different intensities of blue attest to the integration of Tn*phoA* into different sites. Separately for each plate, combine the bacteria by scraping the colonies with LB broth and isolate plasmids. Transform CC118 with each plasmid pool; grow the bacteria in LB broth without antibiotics for 1 hr at 37°, before plating on LB agar with Km, XP, and the appropriate antibiotic for the plasmid; incubate for 1 to 2 days at 37°.

7. Isolate independent colonies by restreaking the ones of interest on LB agar with Km, XP, and the appropriate antibiotic for the plasmid (e.g., a collection of colonies demonstrating various intensities of blue). Colonies which remain sectored (white and blue) after several rounds of restreaking of blue sectors suggest the combined maintenance of plasmids with different Tn*phoA* inserts.

8. To ensure the presence of only one type of mutated plasmid per isolated colony, an additional plasmid isolation and transformation step is

undertaken (see step 7). Colonies are isolated on LB agar containing only XP and the appropriate antibiotic for the plasmid (no Km).

Identification of Fimbrial Genes in Cloned DNA

Relevant phenotypes, namely, fimbriation and adhesive properties, of all isolated Tn*phoA* mutants should be determined.[8] Because strain CC118 is fimbriated, another host strain, which is nonfimbriated, should be used to study these phenotypes (e.g., SE5000). Potential interference with cloned genes is best avoided by using host strains whose type 1 fimbrial genes have been completely deleted.[11] Bacteria from blue colonies which are altered in their fimbriation or adhesive properties most probably have Tn*phoA* inserted in genes encoding exported fimbrial proteins. This can be confirmed by locating the Tn*phoA* inserts in the cloned fragment by restriction mapping. Using the original plasmid (without a Tn*phoA* insert) as a control, digesting with a restriction enzyme(s) specific for sites flanking the cloned fragment and an enzyme restricting only at one end of Tn*phoA* (e.g., *Dra*I or *Hpa*I) allows the insertion site and the orientation of Tn*phoA* to be determind in one step. Using the restriction map of Tn*phoA*, and the given list of absent sites, alternative approaches may be designed for more precise mapping (Fig. 1). Mapping results will indicate the orientation of transcription for each Tn*phoA* insert.

Tn*phoA* insertion, like the insertion of Tn*5* and other transposons, is not completely random. Moreover, only one insert in six (two orientations for three reading frames) in a gene encoding an exported protein results in the creation of a fusion protein with phosphatase activity. Exported genes which are expressed poorly may not be recognized if only blue colonies are studied. Therefore, one might increase the chance of isolating Tn*phoA* inserts in a new gene by also analyzing nonfimbriated or nonadhesive mutants forming white colonies. The major problem with this approach is that Tn*phoA*, like Tn*5*, can have polar effects on downstream genes of polycistronic transcriptional units.[5,12] An additional difficulty of isolating strains with Tn*phoA* inserts in certain sites or genes may be related to the production of toxic fusion products. DNA areas suspected of including relevant genes can be studied in more detail with the expression system described later.

[11] I. C. Blomfield, M. S. McClain, and B. I. Eisenstein, *Mol. Microbiol.* **5,** 1439 (1991).

[12] D. E. Berg, *in* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), p. 185. American Society for Microbiology, Washington, D.C., 1989.

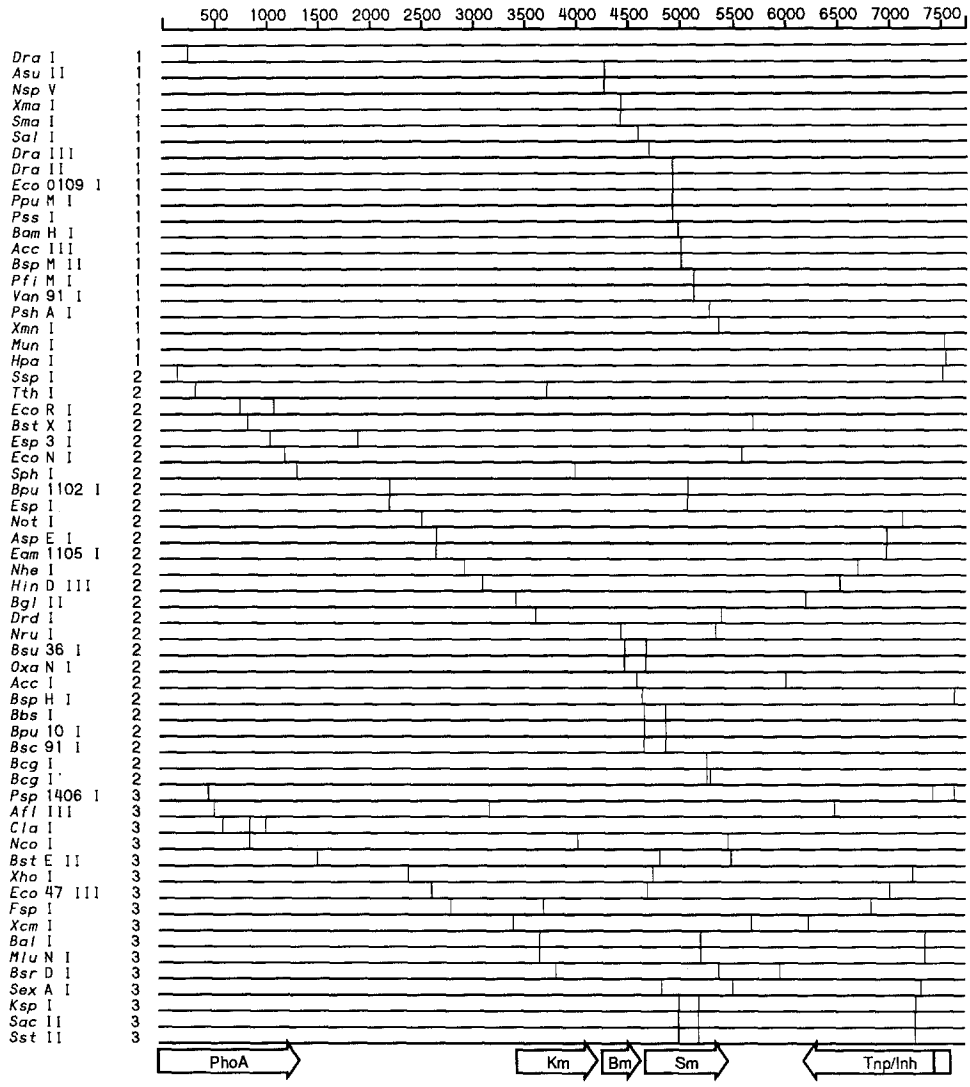


FIG. 1. Restriction map and gene products of Tn*phoA* (7733 bp) based on previously published studies [Manoil and Beckwith[2]; C. S. Hoffman and A. Wright, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 5107 (1985)]. The coordinates are indicated at top in base pairs (bp). Commercially available restriction enzymes cutting Tn*phoA* once, twice, or three times, as indicated on the left of the map, are ranked by the position of their restriction sites beginning with the most leftward site. The genes encoding alkaline phosphatase (PhoA), transposase (Tnp), the trans-acting inhibitor (Inh) protein involved in regulation of transposition, and the proteins for kanamycn (Km), bleomycin (Bm), and low-level streptomycin (Sm, not expressed in *E. coli*) resistance are shown as arrows (noting orientation of transcription) at the bottom of the map. Absent restriction sites in Tn*phoA* are those for *Aat*II, *Acc*65I, *Afl*II, *Apa*I, *Apa*LI, *Asc*I, *Ase*I, *Asp*718, *Avr*II, *Bsi*WI, *Bsp*120I, *Bsp*1407I, *Bst*1107I, *Ecl*136II, *Eco*72I, *Eco*ICRI, *Eco*RV, *Kpn*I, *Mlu*I, *Nde*I, *Nsi*I, *Pac*I, *Pma*CI, *Pme*I, *Ppu*10I, *Pvu*I, *Sac*I, *Sca*I, *Sfi*I, *Sgr*AI, *Sna*BI, *Spe*I, *SPI*I, *Srf*I, *Sse*8387I, *Sst*I, *Stu*I, *Sun*I, *Swa*I, *Vsp*I, and *Xba*I.

Identification of Fimbrial Proteins

Typically, the genes encoding exported proteins in a cloned fimbrial gene cluster are transcribed in the same orientation, suggesting a polycistronic operon structure. A simple way to recognize the various cloned genes is to compare each Tn*phoA* insertion site with the size of the resulting enzymatically active fusion protein. The molecular mass of the products can be determined by Western blot analysis of total bacterial preparations,[8] using commercially available anti-*E. coli* alkaline phosphatase antibodies(e.g., 5 Prime → 3 Prime, Boulder, CO). If a representative number of mutants (e.g., 3 to 4 per kb) ordered from the proximal to the distal end of transcription is analyzed, a progressive increase in the molecular mass of the fusion proteins is observed, corresponding to Tn*phoA* insertions at progressively downstream sites within the same gene. On fusion to a new distal gene, a fusion protein of lower molecular mass will appear on the Western blot. It is common to observe one or two additional proteins of approximately 47 kDa in all mutants. These proteins are related to alkaline phosphatase and result from partial degradation around the fusion joint. The presented technique allows one to map the 5′ end of fimbrial genes and to determine minimal gene sizes.

Western blot analysis[13] and immunoprecipitation[2] can also be used to study the subcellular localization of fusion proteins. Exported fimbrial proteins fused to PhoA typically remain in the periplasm or associate with membrane fractions.

Quantitative Determination of Enzymatic Activities

Although the chromogenic substrate analog XP used in agar is very sensitive for detecting alkaline phosphatase activity, it may be useful to quantify the enzymatic activity of fusion proteins by spectrophotometric methods.[4,14] The activity of alkaline phosphatase is measured in sodium dodecyl sulfate (SDS)- and chloroform-permeabilized cells. A unit of enzymatic activity is usually defined relative to the rate of hydrolysis of *p*-nitrophenyl phosphate (PNPP) per number of bacteria. If mutations affect the cell volume, it is best to express the specific activity as units per milligram protein.[9]

PhoA fusions to fimbrial proteins which are normally present in low amounts may not be detected by Western blotting. However, by comparing enzymatic activities of the various fusion proteins, it is possible to approximate the relative amount of each fimbrial protein.[8]

[13] D. M. Schifferli and M. Alrutz, *J. Bacteriol.* **176,** 1099 (1994).
[14] E. Brickman and J. Beckwith, *J. Mol. Biol.* **96,** 307 (1975).

Isolation of Fusion Proteins for Antibody Production

Fusion proteins can be isolated from soluble periplasmic fractions and purified by affinity chromatography[13] using an anti-PhoA antibody-coupled matrix (e.g., 5 Prime → 3 Prime). A major obstacle to affinity purification is that fimbrial proteins tend to aggregate with membrane fractions. Moreover, the degradation rates of PhoA fusions are often high. Shorter fusion proteins may be more soluble and more stable, but they include fewer epitopes. A pilot experiment should be undertaken with a small collection of allelic Tn*phoA* mutants to evaluate the amount of soluble fusion protein in the periplasmic fraction of each mutant (e.g., by Western blot analysis with anti-PhoA antibodies). This technique can then be coupled to an expression system as discussed later, keeping in mind that proteins which are overexpressed in bacteria may demonstrate altered properties (i.e., solubility and susceptibility to bacterial proteases).

When fusion proteins are used as antigens, the elicited antibodies should be useful for Western blot analysis and may be utilized for immunoprecipitation and immune electron microscopy of negatively stained bacteria, if they recognize native proteins on the bacterial surface. As an alternative, the DNA sequence determined directly from the fusion (see below) can be used to synthesize peptides predicted to be immunogenic.

Determination of Fimbrial Gene Sequences

DNA sequences upstream of insertion sites can be obtained by using appropriate Tn*phoA* primers to sequence through the fusion joints. Two primers have been used successfully for this purpose (R. Taylor, personal communication, 1994). The first primer [5′ d(GCCGGGTGCAGTAATATCG) 3′] hybridizes to *phoA* DNA (nucleotides 80 to 98 of Tn*phoA*) and can be used directly for double-strand sequencing of purified plasmid DNA by the chain-termination method. The second primer [5′ d(GGTTCCGTCCAGGACGC) 3′] hybridizes to the IS*50*$_L$ terminal repeat of Tn*phoA* (nucleotides 21 to 37) and can be used to get more sequence information. For sequencing with this primer, the area of interest has to be subcloned to avoid false priming to the IS*50*$_R$. Theoretically, the complete sequence of a cloned gene cluster could be determined if one could isolate Tn*phoA* inserts every 200–400 bp (if possible, in both orientations). However, because Tn*phoA* insertion may not be sufficiently random, obtaining the appropriate inserts could prove to be more tedious than using other sequencing techniques based on subcloning techniques for template preparation.

TnphoA-Derived Plasmids and Potential Alternatives

The potential excision and transposition of Tn*phoA* in *E. coli* appears to be sufficiently rare so as to not cause problems. However, an alternative mini-Tn*phoA* system has been developed.[15,16] Minitransposons are more stable, because they do not contain the transposase genes. Transposition is achieved by transiently complementing with the appropriate transposase gene in cis or trans on a suicide vector.

Finally, other transposon derivatives of Tn*5* have been engineered with various resistance markers and reporter genes.[5] Elegant genetic techniques have been described for switching translational fusions to transcriptional fusions, and reciprocally.[5,16,17] These tools provide ways to further exploit Tn*phoA* fusions for studying bacterial regulation of fimbrial expression and adhesion.

Use of T7 RNA Polymerase/Promoter System to Study Fimbrial Proteins

Principle

To identify all the genes of a gene cluster required for fimbriation and adhesion, it is imperative to confirm and complete the findings obtained by Tn*phoA* mutagenesis. Although total DNA sequencing would identify open reading frames not recognized by transposon mutagenesis, this approach is tedious and, perhaps more importantly, the deduced proteins would remain hypothetical until their actual detection. Among several techniques which have been exploited to identify proteins encoded by cloned genes, the two-plasmid expression system of Tabor and Richardson[3] has proved to be particularly well suited for *in vivo* expression of fimbrial proteins from genes mostly clustered in one transcriptional orientation.[8] The system takes advantage of the higher processive properties of T7 RNA polymerase in comparison to host (*E. coli*) polymerase. The T7 RNA polymerase is encoded by one of the two plasmids, and its synthesis is heat-inducible. The enzyme directs specific transcription of genes which are cloned downstream from a T7 promoter on the second, compatible plasmid. Because T7 RNA polymerase is resistant to the effect of rifampicin, transcription by the host polymerase can be shut off with

[15] V. de Lorenzo, M. Herrero, U. Jakubzik, and K. N. Timmis. *J. Bacteriol.* **172,** 6568 (1990).
[16] M. R. Wilmes-Riesenberg and B. L. Wanner, *J. Bacteriol.* **174,** 4558 (1992).
[17] C. Manoil, *J. Bacteriol.* **172,** 1035 (1990).

this antibiotic. In actual use, rifampicin may not always be necessary because accumulation of transcripts, which can reach several times the length of the plasmid, rapidly saturates the translational apparatus of the host. This results in near exclusive protein expression of the cloned genes. Proteins expressed from the cloned genes can be specifically labeled with radioactive amino acids for identification by SDS–PAGE and fluorography. A similar expression system, using a T7 RNA polymerase expressed from a λ phage stably integrated into the chromosome, may be simpler to manipulate,[7] but the two-plasmid system more readily enables one to choose the most appropriate host strain for optimizing protein expression.

The T7 RNA polymerase expression system has several advantages over other *in vivo* expression systems, such as minicells[18] or maxicells.[19] The manipulations are simpler and less time-consuming, and expression levels are controllable. Sensitivity can be increased by induction of protein overexpression. However, the levels of each protein synthesized after transcriptional activation of a polycistronic message will vary with the corresponding translational signals (i.e., ribosomal binding sites with properly placed start codons). Therefore, although most proteins of a fimbrial gene cluster will be detected, some may still be missed. To identify such proteins, the same system can be used with high copy number plasmids carrying a T7 promoter and subcloned DNA fragments from the fimbrial gene cluster. When such fragments contain only one or a few weakly translated genes, proteins usually become detectable[8] because stronger translational start signals are not competing for protein synthesis.

Properties of Two-Plasmid System

The system is based on the use of two compatible plasmids,[3] each carrying a different origin of replication. One plasmid, pGP1-2 (Fig. 2), carries (1) the p15A origin of replication, (2) a kanamycin resistance gene, (3) the gene for T7 RNA polymerase under the control of the λp_L promoter, and (4) the heat-sensitive λ repressor *c*I857 expressed from the *lacUV5* promoter. Therefore, the synthesis of T7 RNA polymerase can be activated at high temperature (e.g., 42°), which inactivates the *c*I857 repressor. The polymerase will direct transcription of any gene cloned downstream of a T7 promoter carried on a second plasmid.

[18] G. Dougan and D. Sherratt, *Mol. Gen. Genet.* **151,** 151 (1977).
[19] A. Sancar, R. P. Wharton, S. Seltzer, B. M. Kacinsky, N. D. Clark, and W. D. Rupp, *J. Mol. Biol.* **184,** 45 (1984).

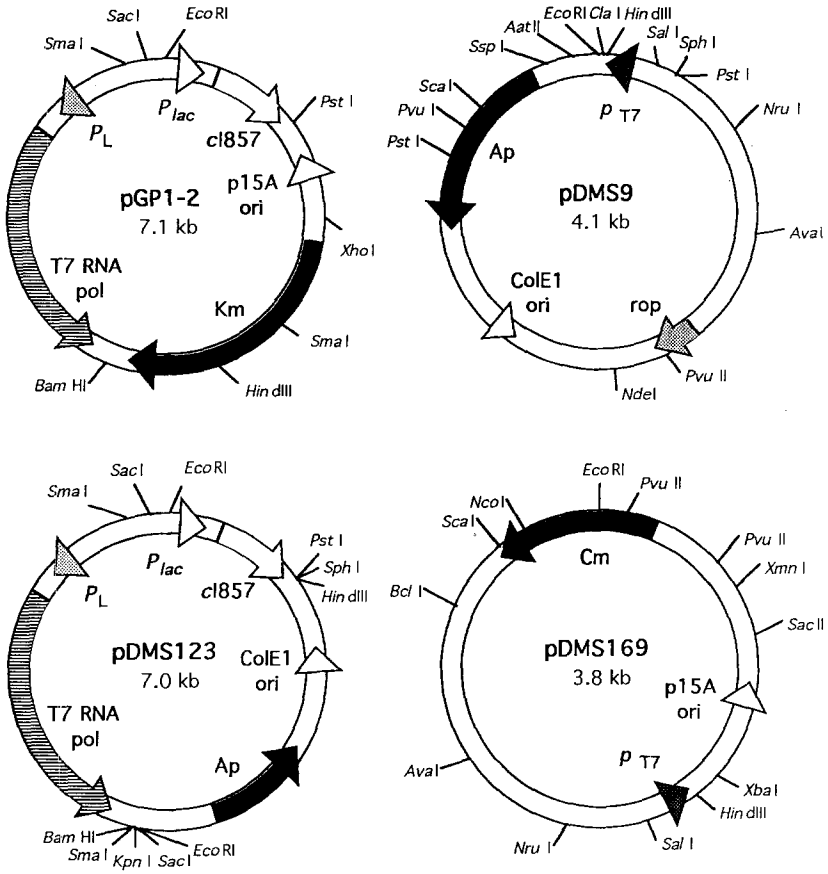


FIG. 2. Useful plasmids for T7 RNA polymerase-mediated transcription of fimbrial genes. Origin and construction of the plasmids are described in the text. Plasmid pGP1-2 has been previously described [S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1074 (1985)]. The following abbreviations are used: p_L, bacteriophage λ left promoter; p_{lac}, *lacUV5* promoter, which is essentially independent of catabolite repression and includes the operator *lacO*; p_{T7}, T7 promoter; ori, origin of replication; Km, kanamycin resistance gene; Ap, ampicillin resistance gene (β-lactamase); Cm, chloramphenicol resistance gene (chloramphenicol acetyltransferase); T7 RNA pol, T7 RNA polymerase.

Useful Plasmids for Cloning and Studying Fimbrial Genes

The original two-plasmid system is based on pGP1-2 (Fig. 2) and any plasmid of the pT7 series,[20] carrying the ColE1 origin of replication, a T7

[20] S. Tabor, *in* "Current Protocols in Molecular Biology" (F. M. Ausubel, ed.), p. 16.2.1. Wiley, New York, 1993.

promoter, and the cloned gene(s). The pT7 series of plasmids do not have an intact *rop* gene to control their copy number. Because of the processive nature of T7 RNA polymerase, even a low level of expression can activate genes behind a T7 promoter to a level of toxicity, which results in cell death or in segregation of strains which have undergone spontaneous mutations. This problem is particularly pronounced with high copy number plasmids carrying a T7 promoter, like many commercially available vectors.

Fimbrial gene clusters are located on the chromosome or on large low copy number plasmids in pathogenic strains of bacteria and cannot be cloned on very high copy number vectors because overexpression of fimbriae has damaging effects on host cells. Whole fimbrial gene clusters can usually be cloned stably in the tetracycline gene or promoter region (in opposite orientation to tetracycline transcription) of medium copy number plasmids like pBR322 (ColE1 origin of replication with the *rop* gene intact) and pACYC184 (p15A origin of replication).[21,22] The pDMS9[8] and pDMS169 vectors are derived from these plasmids and carry a T7 promoter in the tetracycline gene (Fig. 2). Plasmid pDMS9 (Fig. 2) has to be used with pGP1-2, and pDMS123 (Fig. 2) has to be used with pDMS169 (Fig. 2) for reasons of plasmid compatibility. T7 RNA polymerase-mediated transcription of inserted genes into pDMS9 or pDMS169 is oriented in a counterclockwise direction.[7,8] This orientation of transcription is important for stabilizing the constructs and avoiding interference with plasmid replication.[20] In pDMS9 derivatives, β-lactamase is also synthesized, offering an internal control for translation efficiency and transcript stability in the different constructs.[8] Like pDMS9, the pET series of vectors developed by Studier *et al.*[23] are based on the ColE1 origin of replication of pBR322 (with an intact *rop* gene), but they have additional cloning sites and various properties useful for specific applications (e.g., fusion to a well-translated leader peptide, regulatory elements for transcription, and kanamycin resistance).

Plasmid pDMS123 (Fig. 2) carries the T7 RNA polymerase and *c*I857 gene fragment of pGP1-2 in high copy number plasmid pUC19 and can be used with pDMS169 for protein expression, using chloramphenicol acetyltransferase as an internal control. Even though pDMS123 has a higher copy number than pGP1-2, toxic effects are probably avoided because pDMS169 has a lower copy number than pDMS9. Moreover, by using nonfimbriated strain SE5000, which is *recA lac*, the presence of an additional *lac* operator

[21] M. Kehoe, R. Sellwood, P. Shipley, and G. Dougan, *Nature (London)* **291,** 122 (1981).
[22] L. Hagberg, R. Hull, S. Hull, S. Falkow, R. Freter, and C. S. Edén, *Infect. Immun.* **40,** 265 (1983).
[23] F. W. Studier, A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff, this series, Vol. 185, p. 60.

on pDMS123 (from pUC19) does not affect transcription from the *lacUV5* promoter (Fig. 2).

Reagents and Media

Enriched medium: Bacto-tryptone, 20 g; Bacto-yeast extract, 10 g; NaCl, 5 g; 2 ml glycerol; 50 ml 1 *M* KH_2PO_4/K_2HPO_4 pH 7.5; add deionized water to 1 liter and autoclave

10 × M9 medium: Na_2HPO_4, 60 g; KH_2PO_4, 30 g; NaCl, 5 g; NH_4Cl, 10 g; add deionized water to 1 liter and autoclave

Supplemented M9 medium: 100 ml of 10× M9 medium; 0.1 ml of 1 *M* $CaCl_2$, 2 ml of 1 *M* $MgSO_4$; 10 ml of 20% glucose; 60 μl of 1 *M* thiamine; 200 ml of 0.05% tyrosine (poorly soluble) and 20 ml of 0.5% of each other amino acid, except methionine and cysteine (when radiolabeling with these two amino acids); add sterile deionized water to 1 liter. All supplemental stock solutions can be sterilized by autoclaving, except thiamine, aspartate, asparagine, glutamate, tryptophan, and tyrosine stocks, which have to be filter sterilized

Rifampicin (Rif) stock (Sigma, St. Louis, MO, R-3501): 20 mg/ml dissolved in methanol, stored at −20° in the dark; supplemented to 200 μg/ml

Radiolabeled amino acids: 10 mCi/ml of [^{35}S]methionine (or ^{35}S-labeled amino acids containing also [^{35}S]cysteine) (>800 Ci/mmol). If methionine- and cysteine-free proteins are suspected, ^{3}H- or ^{14}C-labeled amino acids can be used instead, adjusting supplemented M9 medium accordingly

Detection of Fimbrial Proteins

1. Transform *E. coli* strain SE5000 (other strains may work as well, but should be Met^+ and Cys^+ and sensitive to the antibiotics required for selecting plasmid maintenance) with pGP1-2 (or pDMS123) and a second compatible plasmid carrying the cloned genes with a T7 promoter.[3,20] As a control, transform a second strain with pGP1-2 and pDMS9 (or pDMS123 and pDMS169). Grow transformed strains overnight at 29° (to avoid inducing protein overexpression) on LB agar with the appropriate antibiotics.

2. Grow one colony of each transformed strain overnight (or to an OD_{590} of 0.5, and continue with step 4) at 29° in 2 ml LB broth with the appropriate antibiotics.

3. Use a 10^{-2} dilution of each overnight culture to inoculate 2 ml of fresh LB broth with the appropriate antibiotics and grow at 29° to an OD_{590} of 0.5.

4. Centrifuge 0.2 ml of each culture in a microcentrifuge tube and wash three times by centrifugation and resuspension (in 1 ml M9 medium) steps.

5. After the last centrifugation step, suspend each pellet in 1 ml supplemented M9 medium (without antibiotics) and grow the bacteria at 29° for 30 min to 3 hr. For this experiment, all the cultures are grown with agitation (e.g., to optimize temperature shifts, use two water bath shakers at 250 rpm, one for steps 5, 8, and 9 at 29° and a second one at 42° for step 6).

6. Shift the temperature to 42° for 20 min.

7. Add rifampicin (10^{-2} dilution of the stock solution) and grow for an additional 10 min.

8. Shift the temperature back to 29° for 20 min.

9. Radiolabel the bacteria by adding 10 μCi of [^{35}S]methionine for 5 min at 29° (*CAUTION*: From this step on, all manipulations have to follow radiation safety protocols authorized by the local radiation safety office.)

10. Centrifuge the cells, discard the supernatant, suspend the pelleted cells in 1× SDS sample buffer, heat-treat (100°, 5 min), and analyze by SDS–polyacrylamide gel electrophoresis and fluorography.[24]

Mapping Genes of Identified Fimbrial Proteins

To map genes encoding the detected proteins, a collection of subcloned fragments from the cloned fimbrial gene cluster, as well as frameshift and deletion mutations, have to be analyzed as described above. Using both pDMS9 and pDMS169 as subcloning vectors will reveal proteins comigrating with the β-lactamase bands (at 33, 31, and 29 kDa) or the chloramphenicol acetyltransferase band (at ~27 kDa). Portions of open reading frames recognized as "new" protein bands can be attributed to the original full-size product by analysis of subclones.[8] If no proteins are detectable on a subcloned fragment, the fragment can be recloned in both orientations in a high copy number plasmid carrying a T7 promoter directing transcription in opposite orientation to the resistance gene (e.g., pBluescript KS, Stratagene, La Jolla, CA) for a more sensitive analysis. As mentioned earlier, a gene product usually becomes detectable when the cloned DNA fragment carries only one weakly translated gene[8] and when the plasmid does not contain stronger translational start signals competing for protein synthesis.

In addition to using the pDMS9 and pDMS169 vectors without cloned genes as controls, experiments without activation at 42° or without rifampicin will help to optimize activation times and rifampicin concentrations when required. In general, rifampicin is more efficient at 42° (increased permeability), whereas bacterial labeling is more efficient at 30° or 37°.[20] Stability of the synthesized proteins can be evaluated by pulse–chase analysis, labeling for 1 min, chasing with nonradioactive methionine (0.5% final

[24] J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.

concentration), and analyzing aliquots at 1 min (just prior to the chase), 10 min, and 40 min. Because most fimbrial proteins have signal sequences that are cleaved off during export, pulse–chase experiments with subclones can be used to match the precursor form of a protein to its processed form. As an alternative, protein export and processing can be inhibited with 9% (v/v) ethanol (just prior to radiolabeling) to identify only precursor protein.[8]

Overexpression of Fimbrial Proteins

If the studied fimbrial gene does not include sufficiently strong signals for translation, the gene may be cloned in an expression vector including such signals (e.g., strong and correctly placed ribosomal binding site relative to the ATG start codon).[3] Plasmid pT7-7 and several plasmids of the pET series (Novagen, Madison, WI) have been developed for this purpose and take advantage of cloning sites like *Nde*I and *Nco*I which include ATG.[20,23] If useful unique restriction sites are not available in the gene to be cloned, the simplest approach is to engineer such sites in primers, which can then be used for polymerase chain reaction (PCR) cloning. For optimizing protein expression, it is worthwhile testing different *E. coli* strains, including protease mutants, like *htpR* and *lon* mutants for cytoplasmic proteins and *degP* or *ompT* mutants for exported proteins[25,26] in a pilot experiment (e.g., 2 ml cultures).

1. Transform *E. coli* strain(s) with pGP1-2 (or pDMS123) and a second compatible plasmid carrying the cloned gene with a T7 promoter. Grow transformed bacteria overnight at 29° on LB agar with the appropriate antibiotics.
2. Use one colony to inoculate LB broth with the appropriate anibiotics; grow overnight at 29°.
3. Use the overnight culture to inoculate enriched broth (10^{-2} dilution) containing the appropriate antibiotics and grow in a shaker at 29° to an OD_{590} of 1.2 to 1.5.
4. Shift to 42° for 30 min.
5. Shift to 37° (30° may be better for exported proteins) for 1.5 hr.
6. Centrifuge the cells at 4° and proceed for the isolation of total soluble proteins, periplasmic proteins, or inclusion bodies.

Alternative Techniques for Toxic Proteins

Using the described protocols, 987P fimbrial protein synthesis arising from basal levels of synthesis of T7 RNA polymerase was not lethal for

[25] S. Gottesman, this series, Vol. 185, p. 119.
[26] K. Sugimura and N. Higashi, *J. Bacteriol.* **170,** 3650 (1988).

the host cells grown at 29°.[8] However, if required, several approaches for studying gene products whose toxic concentrations for the host cells are very low can be tested. For example, some of the pET vectors have been constructed to include the *lac* repressor (*lacI*) and a *lac* operator engineered between the T7 promoter and the studied gene.[23] With such plasmids, LacI downregulates both host transcription of T7 RNA polymerase, which involves the *lacUV5* promoter, and T7-mediated transcription. Another alternative includes the use of plasmids expressing T7 lysozyme, which, in addition to its enzymatic activity, inhibits T7 RNA polymerase.[23] However, such plasmids cannot be adapted to the two-plasmid system and require the use of strains expressing T7 RNA polymerase from a λ phage stably integrated into the chromosome. The most effective solution to toxic proteins consists of introducing the gene for T7 RNA polymerase on a bacteriophage only at the time of induction. Two bacteriophages carrying T7 RNA polymerase, inducible from a *lac* promoter by isopropylthiogalactoside (IPTG), have been described for this purpose: mGP1-2,[20] which is an M13 derivative and requires that the host express the pili receptor, and the λ derivative CE6.[23]

Acknowledgments

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[22] Molecular Cloning of Adhesion Genes

By SHEILA I. HULL and RICHARD A. HULL

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The molecular cloning of genes associated with bacterial adhesion requires methods different from the cloning of single genes. Adhesion of gram-negative bacteria to mammalian tissue is often promoted by complex structures called pili, or fimbriae, on the surface of the bacterium. Expression of fully functional adhesion may require expression of up to 10 genes spaced over a 10- to 15-kb region of DNA. Also, there is usually no reliable direct selection for adherent recombinants so that some sort of physical or functional screening process is required. As a consequence, successful cloning methods must result in chimeric molecules with high molecular weight inserts that represent contiguous regions of the donor chromosome. Inserts

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must be sufficiently random such that the entire chromosome is represented in 1000–2000 recombinant molecules. In this chapter, methods are presented for preparing recombinant DNA libraries that have been used successfully for cloning large bacterial operons and functional gene clusters in the 10- to 25-kb range such as adhesion organelles and carbohydrate antigens. Methods useful in screening for adhesin gene clones are also presented.

Purification of High Molecular Weight DNA from Bacteria

Materials

1 liter Sterile Luria (L) broth in a 2-liter flask
50 ml Overnight L broth culture of the DNA donor bacterium
25% (w/v) Sucrose in 50 m*M* Tris, pH 8.0, 1 m*M* EDTA
10 mg/ml Proteinase K in 10 m*M* $CaCl_2$
0.5 *M* EDTA, pH 8.0
10% Sodium lauryl sarcosinate (sarkosyl)
50 mg/ml Egg white lysozyme
Autoclaved 10 m*M* Tris, pH 8.0, 1.0 m*M* EDTA (TE)
Autoclaved 10 m*M* Tris, pH 7.3, 100 m*M* NaCl, 0.1 m*M* EDTA (TES)
Cesium chloride solution: 23.4 g CsCl per 20.3 ml solution in TE containing 50 μg/ml phenylmethylsulfonyl fluoride (PMSF); the PMSF stock is prepared at 10 mg/ml in 95% (v/v) ethanol
Cesium chloride solution, 868.1 mg/ml

Procedure

One liter of L broth is inoculated with 50 ml overnight growth of bacteria and incubated at 37° with aeration until the OD_{660} is 0.2. Bacteria are harvested by centrifugation and washed once in 20 ml TE. The bacterial pellet is suspended in 20 ml of 25% sucrose solution, and 5-ml samples are dispensed into screw-cap polypropylene tubes on ice. Although the yield from one 5-ml sample is sufficient, processing of at least two tubes is recommended. One hundred microliters of lysozyme is added and incubation on ice continued for 5 min. Proteinase K (25 μl) is added and mixed followed by 1 ml 0.5 *M* EDTA. Lysis is initiated on ice by adding 0.6 ml sarkosyl and gently twirling and inverting the tube to promote mixing. By keeping the sample cool, lysis is delayed, permitting more uniform mixing. After mixing is complete, the tubes are capped and incubated overnight at 50°. The viscous sample is then transferred to a Beckman (Fullerton, CA) 60Ti ultracentrifuge tube or equivalent, by means of a funnel if heat

sealing tubes are used. DNA samples should not be transferred using a syringe or small bore pipette. Alternately, the lysis procedure may be done directly in the ultracentrifuge tube; however, adding reagents to heat sealing tubes is difficult.

Cesium chloride–PMSF mixture (20.3 ml) is added to the lysate. The density of this solution is based on the density of *Escherichia coli* DNA and assumes a lysate volume of 6.7 ml; the same density gradient has been used successfully for a lower %G+C organism, *Proteus mirabilis*. Each tube is filled to the top with CsCl (868.1 mg/ml solution) and sealed. Prior to centrifugation, the sealed tubes are stored at room temperature overnight. The concentration of CsCl in the gradients is near saturation; by allowing the CsCl and lysate to equilibrate overnight, the likelihood of CsCl precipitating during centrifugation is reduced. The tubes are centrifuged in a Beckman 60Ti rotor for 46 hr at 35,000 rpm and 20°. Shorter centrifugation times or use of vertical rotors have proved inferior. Ethidium bromide is not included in the gradient because of difficulty in subsequently removing it from purified DNA without subjecting the DNA to shear forces.

After centrifugation, the DNA may appear as a cottonlike puff near the middle of the gradient. The tube is first vented with an 18-gauge needle near the top; the DNA is collected directly into boiled 1 cm diameter dialysis bags through a 12-gauge syringe needle (Hamilton, Reno, NV) by side puncture of the tube below the DNA. The 12-gauge needle should be inserted swiftly to minimize loss of sample. The flow rate through the needle can be regulated using the 18-gauge needle inserted at the top either manually with an attached rubber tube or by pumping light oil to displace the gradient. When the fraction containing DNA passes through the needle, the solution will become viscous, and positive pressure may be required to force the DNA through the 12-gauge needle. Care should be taken to collect only the viscous DNA with minimal additional CsCl solution. The DNA samples are dialyzed versus 2 liters TES with three changes. Finally, the samples are transferred to a screw-cap plastic tube and stored at 4°. DNA concentration is about 300 μg/ml, although accurate measurement is difficult due to high viscosity of the sample.

Alternate Methods

The chemical extraction method for DNA purification described by Marmur[1] is still frequently used for preparing recombinant DNA libraries. It is less desirable for cloning of large inserts, however, because the product is of lower molecular weight owing to limited but unavoidable mechanical

[1] J. Marmur, *J. Mol. Biol.* **3**, 208 (1961).

shear associated with this procedure. Column chromatography-based systems for purification of high molecular weight DNA are available commercially (Boehringer Mannheim), but their usefulness is limited by the finite capacity of the columns.

Although the CsCl procedure described here was originally designed for the enteric bacteria, it has also been used with *Neisseria gonorrhoeae* and should be easily adaptable to other genera. Lysozyme may be replaced with other lytic enzymes as appropriate.

Partial Digestion of DNA

Materials and Procedure

Partial digests are prepared by varying the digestion time while using an excess of restriction endonuclease and a constant temperature. Digestion times are determined empirically for each DNA batch prior to setting up preparative reactions. DNA (100 μl) is mixed on ice with 11 μl of 0.1 m*M* $MgCl_2$, 1 μl bovine serum albumin (BSA; 10 mg/ml) and 1 μl *Sau*3A (10 U/μl). The undiluted, undigested DNA stock cannot be pipetted with a standard micropipette tip and is best transferred with a large bore tip. A 5-μl sample is immediately mixed with electrophoresis sample buffer, and incubation is continued at room temperature. Additional samples are taken at 2, 4, and 6 min and at 5- to 10-min intervals thereafter. The viscosity of the digest should noticeably decrease after 10–15 min. The samples are subsequently analyzed by electrophoresis through a 0.7% agarose gel. In the example shown in Fig. 1, the time points selected for library preparation were 2, 3, 4, and 6 min. For preparative reactions, four fresh 100-μl digests are set up as before; at each selected time point, one tube is transferred to ice and mixed with 5 μl of 0.5 *M* EDTA. When all partial digests are completed, the samples are heated to 70° for 20 min to terminate the reaction.

Alternate Methods

In place of using partial restriction endonuclease digestion to produce a representative collection of fragments, the chromosomal DNA can be mechanically sheared using sonication. The fragments are then methylated with *Bam*HI methylase, the sheared ends repaired with T4 polymerase and deoxynucleoside triphosphates (dNTPs), ligated with *Bam*HI linkers, and finally ligated into the *Bam*HI site of a suitable vector. Although this procedure will likely result in a more random library, in practice the enzymatically fragmented DNA is satisfactory and easier to produce.

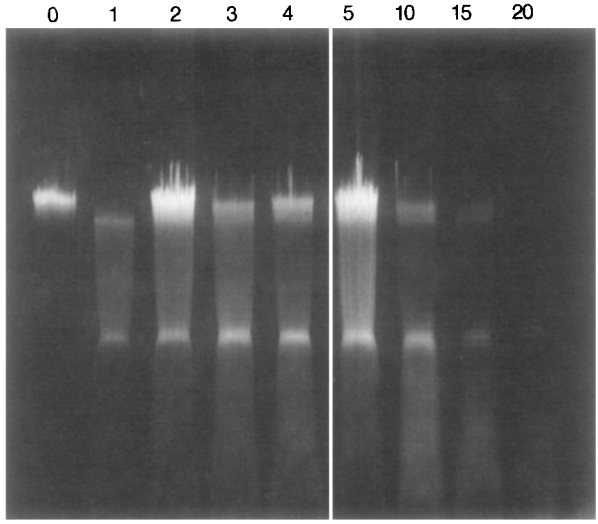

FIG. 1. Partial digest of bacterial DNA with the restriction endonuclease *Sau*3A. DNA was digested with *Sau*3A at room temperature for the time intervals shown (in minutes), and samples from each time point were examined using 0.7% agarose gel electrophoresis.

Size Fractionation

Materials and Procedure

Digested DNA is size fractionated using centrifugation through 5–20% NaCl gradients. The gradients are prepared using sterile solutions in diethyl pyrocarbonate (DEPC)-treated Beckman SW41 ultracentrifuge tubes and chilled on ice prior to use. The four partial digests are pooled, and 200 μl is loaded on each of two gradients. The gradients are centrifuged in a Beckman SW41 rotor for 5 hr at 35,000 rpm and 4°. Fractions (0.5 ml) are collected. If fractions are to be collected from the bottom of the gradient, it is helpful to include a 50% sucrose cushion; this will prevent larger DNA fragments from forming a pellet and plugging the collection needle.

Samples (5 μl) of alternate fractions are examined using electrophoresis through an 0.35% agarose gel for 18 hr at 1 V/cm. After samples for electrophoresis are taken, 1 ml of 95% ethanol is mixed with each fraction, and the DNA is allowed to precipitate overnight at −20°. The fractionated DNA can be stored indefinitely in the freezer at this step. In the example in Fig. 2, DNA in fractions 12–17 is greater than 30 kb and would be suitable for preparing cosmid libraries. DNA in fractions 9–11 is in the 20- to 30-kb range and would be suitable for use with λ vectors.

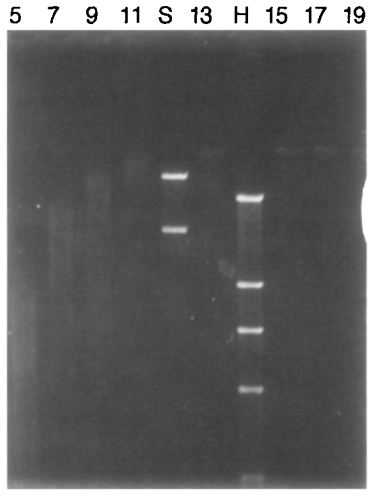

FIG. 2. Agarose gel showing size-fractionated DNA obtained from a 5–20% NaCl gradient. Fraction numbers from the top of the tube are indicated. Molecular weight standards are bacteriophage λ DNA digested with *Sal*I (S) (33 and 15 kb) and *Hin*dIII (H) (23.7, 9.5, 6.7, 4.3, and 2.3 kb).

Alternate Methods

The sizing step is included to remove low molecular weight DNA fragments which would otherwise ligate together randomly to form an insert sufficiently large to be packaged. This is especially important when cloning large operons because the presence of clones composed of random rearrangements will both reduce cloning efficiency and lead to potentially confusing results. The formation of randomly associated molecules can also be prevented by dephosphorylating the insert DNA prior to ligating with vector. However, under such conditions, correct insert-to-vector molar ratios are difficult to calculate as the majority of insert DNA in the ligation mix, although capable of ligating to vector, is too small to be packaged. As a consequence, much of the vector is consumed in formation of unproductive molecules.

DNA may also be size fractionated by preparative electrophoresis through 0.35% agarose gels. This procedure is limited by the capacity of the gels used. DNA fragments of the appropriate size for cosmid cloning represent a small fraction of the total partial digest; DNA recovered from several gels is needed to provide a sufficient amount of high quality product for a single ligation.

Ligation and Packaging

DNA from selected size fractions is collected by centrifugation and suspended in 0.4 ml TE. The DNA concentration of the undiluted sample is determined spectrophotometrically in DEPC-treated semimicrocuvettes. The concentration is usually about 2–3 μg/ml for fractions greater than 30 kb and 8–10 μg/ml for the 20- to 30-kb fractions. For ligation, fractions in the appropriate size range may be pooled to supply 2.5–3.5 μg and coprecipitated with *Bam*HI-digested cosmid vector such as pHC79 or phage vector such as EMBL3 at a 1:1 molar ratio. Prior dephosphorylation of the vector is not beneficial. Dephosphorylation of the insert is also not required but may be beneficial if the insert DNA is not size fractionated as discussed above. Ligation is done using standard conditions in a final volume of 11 μl. After ligation, the sample will become viscous. Two to three microliters of ligated DNA is then packaged *in vitro* into bacteriophage λ transducing particles.[2] The packaged phage can be stored several years at −70° with minimal loss of viability.

Selection of a host strain is based primarily on its susceptibility to bacteriophage λ. HB101 is a suitable host for either cosmid or phage cloning of adhesin genes. However, this host expresses type 1 pili under some growth conditions, which may potentially complicate interpretation of results. *Escherichia coli* P678-54, and its *recA* derivative, JW369, lack genes for type 1 pili and may therefore be more useful. For experiments using phage replacement vectors, *E. coli* Q359 is used as a host.

The transducing titer (vector antibiotic resistance phenotype) for cosmid libraries is about 10^3/μl phage mixture. If one assumes an average insert size of 40 kb in a cosmid clone and an *E. coli* genome size of 4700 kb, the frequency of any particular gene in a library would be 1 in 118 ampicillin-resistant transductants. In practice, the frequency of complete adhesin gene clusters among ampicillin-resistant transductants varies between 0.1 and 1%. The titer of recombinant phage using λ replacement vectors is usually about 10^2–10^3/ml. The frequency of recombinant phage containing at least part of an adhesin gene cluster is 0.1–1%. However, the frequency of complete adhesin gene clusters is lower as compared with cosmid cloning (about 0.01–0.2%) owing to the smaller size of the phage vector inserts.

Functional Screening Methods for Identifying Adhesin Clones

If the adhesion phenotype of interest is a hemagglutinin, hemagglutination of suseptible erythrocytes is the most direct way to screen for recombi-

[2] L. Enquist and N. Sternberg, this series, Vol. 68, p. 281.

nant cosmid clones. Growth conditions for maximal expression of hemagglutination in the donor strain should be established prior to screening recombinant clones. Conditions to consider include incubation temperature, growth with or without aeration, liquid versus solid media, minimal media versus L broth or other complex media, and glucose effects.

Hemagglutination

Ampicillin-resistant transductants are picked and inoculated as a patch, 50–100 per plate, onto two L plates containing ampicillin and incubated overnight. Patches should be tested the following day as the hemagglutination capacity of recombinant clones is often diminished after prolonged incubation. Erythrocytes are washed and suspended at 3% (v/v) in phosphate-buffered saline (pH 7.0) containing 0.01% gelatin (BSG). Human erythrocytes should be used within 1 week of collection and washed and diluted just prior to testing hemagglutination. If the desired adhesion phenotype is D-mannose-resistant, 10 m*M* D-mannose may be included in the erythrocyte mixture. Bacteria from an individual patch are picked with a sterile toothpick and mixed with a drop of erythrocyte solution on a glass plate chilled over ice. Enough bacteria should be used so that the drop of erythrocytes becomes visibly turbid. Patches are tested in batches of 10–20; after each batch, the glass plate is gently rocked 5–10 times to enhance visualization of hemagglutination, which may appear immediately or only after rocking.

Gradient Enrichment

The frequency of hemagglutinating clones can also be enriched, if necessary, using glycerol step gradient centrifugation. Step gradients are prepared in sterile 15-ml clear centrifuge tubes, using 3 ml of 20% glycerol in BSG for the bottom step and 3 ml of 5% (v/v) glycerol in BSG for the top step. Ampicillin-resistant recombinant bacteria are collected from overnight growth (pool 10^4–10^5 colonies), suspended gently in BSG to an OD_{600} of 1.5, and diluted 1 : 10 in BSG. One-half milliliter diluted bacteria is mixed with 0.5 ml 3% erythrocytes and incubated at room temperature for 20 min with occasional gentle mixing. The bacteria–erythrocyte suspension is then layered onto the gradient and centrifuged at 600 rpm (relative centrifugual force of 77) for 12 min in a Sorvall GLC centrifuge with a swinging bucket rotor. The 5% glycerol step and sample load volume are removed with a syringe and bent 18-gauge needle and gently replaced with 3 ml sterile BSG as a wash. The BSG and remaining gradient are removed, the erythrocytes suspended in BSG, and dilutions spread on antibiotic-containing agar plates. A single gradient cycle provides approximately 10-fold enrichment. The

maximum enrichment achieved using serial gradients with intermediate regrowth steps was 10^5-fold.

Advantages

The hemagglutination procedure is simple and direct. It assures that at least the minimum number of genes required for the adhesion phenotype are present on the cloned molecule.

Disadvantages

(1) Cloned genes, especially from genera other than *Escherichia,* may not be expressed or their products may not be assembled into adhesin organelles in *E. coli.* (2) In general, this method will not work for adhesins that are not hemagglutinins. However, if the tissue receptor for the adhesin of interest is known, latex beads coated with receptor may be substituted for erythrocytes. (3) Clones that contain only part of the adhesin gene cluster will not be detected.

Antigen Screening Methods for Identifying Adhesin Clones

Recombinant DNA libraries may also be screened for the presence of adhesin-associated antigens without regard to expression of functional adhesion. The methods used for screening either cosmid or phage libraries for adhesin-associated antigens do not differ significantly from general published procedures.[3,4] In the following a method is described that is useful in identifying appropriate antigens for preparation of antibody when the nature of the adhesin organelle is unknown; this method is applicable to adhesins that are not hemagglutinins.[5]

Materials

10 ml Minimal medium: 60 m*M* potassium phosphate (pH 7.4); 2 m*M* disodium citrate; 0.8 m*M* $MgCl_2$; 15 m*M* NH_4Cl; 80 μM $(NH_4)SO_4$; 20 μg/ml of required amino acids; 0.4% glycerol; and 0.5 mg/ml yeast extract

$H_2{}^{35}SO_4$

12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel and associated buffers

[3] J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.

[4] M. Snyder, S. Elledge, D. Sweetser, R. A. Young, and R. W. Davis, this series, Vol. 154, p. 107.

[5] S. K. Wray, S. I. Hull, R. G. Cook, J. Barrish, and R. A. Hull, *Infect. Immun.* **54,** 43 (1986).

BSG (phosphate-buffered saline, pH 7.0, with 0.01% gelatin)
Target tissue to which bacteria adheres dispersed as single cells or groups of two or three cells

Procedure

Bacteria are grown overnight in 10 ml minimal medium containing 50 μCi/ml $H_2{}^{35}SO_4$ (or [^{14}C]glucose and additional unlabeled SO_4 if the adhesin is thought not to be a protein). Other growth conditions may be used to enhance the adhesion phenotype. The bacteria are washed once with 1 ml BSG and suspended in 1 ml BSG at a density of $1-2 \times 10^{10}$ cells/ml. Outer membrane material is sheared from the bacterial surface by multiple passage through a 26 gauge needle. Whole bacteria are removed by centrifugation for 10 min at 10,000 $\times$ *g*, 4°. Target cells are washed in BSG, and pellets containing 2×10^5 cells are suspended in BSG containing radiolabeled outer membrane material. After incubation at 37° for 1 hr with continuous mixing, the reaction mix is centrifuged, and both the pellet and supernatant are retained. The cell pellet is washed 3 times with BSG and suspended in 1 ml BSG. The radioactivity of the cell and original supernatant fractions is determined, and an equal number of counts per minute (cpm) from each are dialyzed versus distilled water and freeze-dried. Samples are then examined using SDS–polyacrylamide gel electrophoresis and autoradiography. Adhesin antigens will be those enriched in the cell fraction as compared to the supernatant. This method may also be used in development of purification strategies for adhesin molecules. Presence of the adhesin in fractions obtained at different purification steps can be monitored by including a sample of the fraction in the reaction mix and measuring its capacity to competitively reduce binding of radiolabeled adhesin to tissue.

Advantages

(1) The antigen screening method does not require assembly of a functional adhesin organelle. Adhesin genes from other genera of bacteria that may be cloned into *E. coli* but not assembled and clones that contain only part of an adhesin operon can be identified. (2) This method does not require that the adhesin-associated molecules be proteins, only that they be antigenic. (3) The method can be used with both phage and cosmid libraries.

Disadvantages

(1) The adhesin molecule must be purified in advance for production of antibody; alternatively, a homogenic mutant derivative of the donor

strain that does not express the adhesin antigen must be available to adsorb irrelevant antibodies. (2) Clones that express cross-reactive antigens may be inadvertently selected.

DNA Hybridization Screening Method for Identifying Adhesin Gene Clones

Recombinant DNA libraries may also be screened for the presence of adhesin-associated gene sequences without regard to the expression of any cloned gene. For screening cosmid libraries, the colony blot method described by Maas[6] is both sensitive and convenient. Filters for hybridization are prepared directly from the transduced colonies, and hybridizing colonies can be recovered from growth remaining after blotting.

Materials

Whatman (Clifton, NJ) No. 3 paper and autoclaved Whatman 541 paper
0.5 *M* NaOH, 1.5 *M* NaCl
1 *M* Tris (pH 7.0), 2 *M* NaCl

Procedure

Sterile Whatman 541 paper is placed on agar plates containing 100–200 colonies. The filters are gently massaged to remove any bubbles. The orientation of the filter is marked with a pencil. The Whatman 541 papers are placed bacteria side up in a glass petri dish on three layers of Whatman No. 3 paper saturated with NaOH/NaCl solution. Papers are steamed for 4 min in a covered beaker of boiling water. The Whatman 541 paper is removed and immersed in 300–500 ml of 1 *M* Tris (pH 7.0), 2 *M* NaCl for 4 min. Air dry.

Prior to hybridization, the filters are washed once briefly in hybridization solution and incubated with hybridization solution containing the selected probe and competitor DNA. No filter baking or prehybridization step is required. For screening phage libraries, standard plaque hybridization methods are appropriate.[3]

Advantages

(1) No gene expression is required so adhesin genes that are neither functional nor expressed in *E. coli* can be cloned. (2) The procedure can be used to screen phage libraries so adhesin genes producing products that inhibit growth of *E. coli* can be cloned.

[6] R. Maas, *Plasmid* **10,** 296 (1983).

Disadvantages

The DNA hybridization method is useful only when DNA or amino acid sequence information is available for the gene of interest. Unless the adhesin genes share DNA sequence similarity with previously cloned genes wherein the previous clone can be used as a hybridization probe, it is necessary to design synthetic oligonucleotide probes based on the amino acid sequence of an adhesin-associated protein. As a consequence of the degeneracy of the triplet code, such probes are usually ambiguous at one or more positions and have reduced specificity. Isolation of incorrect cross-reactive clones is possible and even likely, leading to considerable wasted effort. Alternately, converging oligonucleotides designed on the basis of the amino acid sequence from different regions of the protein can be used as polymerase chain reaction (PCR) primers to produce a DNA probe. For this method, the DNA sequence of any PCR product should be determined to confirm its appropriateness prior to using it as a probe. The deduced amino acid sequence predicted from the PCR product should match the actual amino acid sequence of the target protein.

Acknowledgments

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[23] Molecular Analysis of *Streptococcus pyogenes* Adhesion

By EMANUEL HANSKI, GEORGE FOGG, AVIVA TOVI, NOBUHIKO OKADA, ISRAEL BURSTEIN, and MICHAEL CAPARON

Introduction

Streptococcus pyogenes (the group A streptococcus) is one of the most versatile human pathogens as regards the number of different tissues it can infect and the wide range of different diseases it can cause. *Streptococcus pyogenes* can cause disease through three basic pathogenic mechanisms. Multiplication on the surface of or within a tissue results in destruction of host cells and is accompanied by an intense inflammatory response. These types of diseases can range from the more mild and self-limiting infections of the throat (e.g., pharyngitis, commonly known as "strep throat") and the skin (e.g., impetigo) to infections which involve increasingly deeper

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layers of tissue (e.g., erysipelas, cellulitis, necrotizing fasciitis, and myositis). The bacterium also has the capacity to penetrate into the vasculature from these initial lesions to produce life-threatening sepsis. A second pathogenic mechanism involves the ability of *S. pyogenes* to produce a number of different toxins which then disseminate from a local site of infection. Pathology results from the systemic intoxication of different types of host cells and is exemplified by scarlet fever and the streptococcal toxic shock-like syndrome. A third pathogenic mechanism results in the generation of a number of diseases referred to as nonsuppurative sequelae which are apparently immunopathological diseases that are triggered by an infection with *S. pyogenes*. Acute glomerulonephritis and rheumatic fever are two examples of streptococcal nonsuppurative diseases, the latter of which is apparently the result of a host immune response to streptococcal antigens which cross-react with host tissues.

To cause this wide range of disease, *S. pyogenes* has evolved the ability to interact with a wide variety of host cells and tissues, and it is likely that these interactions play a significant role in the development of different pathologies. For example, rheumatic fever is a sequel to infection of the throat, but not infection of the skin.[1] The reason for this is unclear, but it may reflect differences in the cellular composition of the tissues or may reflect alterations in bacterial gene expression in response to differences in the microenvironments encountered in the two compartments. It is also likely that the streptococci will have specific mechanisms for interaction with different populations of host cells during the course of a single infection. For example, each streptococcal infection is initiated by the interaction of the bacterium with epithelial cells of either the oropharynx or the skin.[1] The inflammatory response which accompanies these early adhesion events will then bring the organism into contact with phagocytic cells that have been recruited to the site of infection. Infection of deeper tissues will require the organism to interact with yet additional populations of host cells that are present in these different compartments. For those infections which become systemic, the organism must have the capacity both to interact with and to penetrate the extracellular matrix, as well as to interact with those host cells which make up the vasculature. Thus, the identification of adhesins required at each of these stages of infection and the mechanisms which regulate expression of the adhesins is expected to provide considerable insight into the pathogenesis of the different streptococcal diseases.

In gram-negative bacteria, the best characterized adhesion mechanisms involve the interaction of long fibrous organelles, usually called pili, with

[1] L. W. Wannamaker *N. Engl. J. Med.* **282,** 23 (1970).

specific host molecules.[2] *Streptococcus pyogenes* is a gram-positive bacterium and has not been shown to produce pili. Instead, considerable evidence has accrued to suggest that its ability to bind to various host proteins which, in turn, can themselves be associated with host cells, or tissues, plays an important role in adhesion. Many of the host proteins bound by *S. pyogenes* include proteins of the extracellular matrix, including fibronectin,[3,4] laminin,[5] vitronectin,[6] and collagen.[7] Other host proteins that are bound include fibrinogen,[8] salivary glycoproteins,[9] plasmin,[10] albumin,[11] and the Fc domains of immunoglobulin G (IgG) and IgA.[12] In many cases the streptococcal adhesins that recognize these various proteins are poorly characterized, whereas in other cases multiple streptococcal molecules have been implicated in binding, so that the contribution of any one specific adhesin to adhesion is not clear. Perhaps the best example of the latter case is binding to fibronectin, where numerous proteins[13–15] as well as the glycolipid lipoteichoic acid (LTA)[16] have been implicated in binding. In addition, several surface structures of *S. pyogenes* have been implicated as adhesins for host cells, but it is not clear just what host structure serves as the microbial receptor (e.g., M protein or LTA).[17]

It has been suggested that adhesion of *S. pyogenes* may involve at least two distinct kinetic steps.[17] The first step involves a readily reversible event mediated primarily by hydrophobic interactions between the streptococcal

[2] S. J. Hultgren, S. Abraham, M. Caparon, P. Falk, J. W. St. Geme III, and S. Normark, *Cell (Cambridge Mass.)* **73,** 887 (1993).
[3] E. B. Myhre and P. Kuunsela, *Infect. Immun.* **40,** 29 (1983).
[4] L. M. Switalski, L. Å. C. Rydén, K. Rubin, M. Höök, and T. Wadström, *Eur. J. Clin. Microbiol.* **1,** 381 (1982).
[5] L. M. Switalski, P. Speziale, M. Höök, T. Wadström, and R. Timpl, *J. Biol. Chem.* **259,** 3734 (1984).
[6] G. S. Chhatwal, K. T. Preissner, G. Müller-Berghaus, and H. Blobel, *Infect. Immun.* **55,** 1878 (1987).
[7] P. Speziale, B. Raucci, S. Meloni, M. L. Meloni, and T. Wadström, *FEMS Microbiol. Lett.* **48,** 47 (1987).
[8] E. Whitnack, and E. H. Beachey, *J. Clin. Invest.* **69,** 1042 (1982).
[9] H. S. Courtney and D. L. Hasty, *Infect. Immun.* **59,** 1661 (1991).
[10] R. Lottenberg, C. C. Broder, M. D. P. Boyle, S. J. Kain, B. O. Schroeder, and R. Curtiss III, *J. Bacteriol.* **174,** 5204 (1992).
[11] I.-M. Frick, P. Åkesson, J. Cooney, K. Söbring, K. Schmidt, H. Gomi, S. Hattori, C. Tagawa, F. Kishimoto, and L. Björk, *Mol. Microbiol.* **12,** 131 (1994).
[12] P. Cleary, and D. Retnoningrum, *Trends Microbiol.* **2,** 131 (1994).
[13] E. Hanski and M. G. Caparon, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6172 (1992).
[14] V. Pancholi and V. A. Fischetti, *J. Exp. Med.* **176,** 415 (1992).
[15] S. R. Talay, E. Ehrenfeld, G. S. Chhatwal, and K. N. Timmis, *Mol. Microbiol.* **5,** 1727 (1991).
[16] W. A. Simpson, H. S. Courtney, and I. Ofek, *Rev. Infect. Dis.* **9,** S351 (1987).
[17] D. L. Hasty, I. Ofek, H. S. Courtney, and R. J. Doyle, *Infect. Immun.* **60,** 2147 (1992).

and host cell surfaces, which may involve the hydrophobic LTA molecule. The second step occurs only after the cells have come into contact and involves a high affinity recognition of a specific host cell molecule by a streptococcal adhesin. In keeping with this model, it is likely that many of the binding interactions between *S. pyogenes* and different host components play important roles at some stage of the pathogenic process. What has been lacking is a comprehensive strategy for the evaluation of streptococcal binding of a specific host component in adhesion to different cells and tissues, as well as a strategy for defining the contribution of a defined streptococcal product to adhesion to different tissues and cells. In this chapter, we outline methods used to study these phenomena, using as examples our work with the M protein and a fibronectin-binding protein called protein F. Emphasized are the important role of genetics and the use of relevant *in vitro* models of streptococci–host cell interaction.

Cloning and Mutagenesis of Adhesins

Initially it is to be assumed that the contributions of a known streptococcal binding activity to adhesion is to be examined, and that a streptococcal protein demonstrating the binding activity has been cloned. If the binding protein has not been cloned, then a number of cloning techniques are available which take advantage of the binding reaction for detection of the streptococcal protein, and the reader is referred to a review which describes many of these techniques in detail.[18] Assays for studying specific *S. pyogenes*–tissue interactions which do not require any knowledge of possible host cell receptors are described in a latter section (see below).

The only conclusive way to study the role of a putative streptococcal binding protein in the recognition of a host protein or tissue is through the analysis of an isogenic mutant which differs from the wild type by only a single defined mutation in the gene which encodes the putative adhesin. To illustrate the importance of this technique, we have generated insertional mutations in the gene which encodes protein F and have shown that the resulting mutants were considerably defective in the ability to bind fibronectin and to adhere to certain host cells.[13] On the other hand, we have identified several streptococcal fibronectin-binding proteins through analysis of cloned streptococcal DNA in *Escherichia coli* that subsequently were found not to contribute to the ability of the streptococcal cells to bind to fibronectin, as isogenic mutants defective in expression of the putative binding proteins were not altered in ability to bind fibronectin, even under conditions where protein F was not expressed by the bacteria. Owing to

[18] A. Olsén, E. Hanski, S. Normark, and M. G. Caparon, *J. Microbiol. Methods* **18,** 213 (1993).

their importance for the analysis of streptococcal adhesion, the different methods available for the construction of isogenic mutants are described in detail.

Conjugative Transposon Tn916 Mutagenesis

The conjugative transposon Tn*916* encodes the machinery that allows a gram-positive host within which it resides to serve as a conjugal donor during a mating event.[19] Conjugative transposition involves excision of the transposable element from the donor DNA molecule, transfer to the recipient cell through a circular intermediate, and insertion of the element into numerous different loci of the recipient genome.[19] Tn*916* mutagenesis results in a pool of mutants that contain insertions throughout the entire chromosome. Therefore, this strategy is most useful for generating mutations in genes that contribute to the phenotype under study but that have unknown chromosome locations. Note that Tn*916* mutagenesis can also generate mutations in regulatory genes which affect the adhesin under analysis and which also may have pleiotropic effects on expression of other adhesins. Thus, the Tn*916*-inactivated gene must be carefully characterized to ensure that the transposon insertion lies within the coding region of the gene which encodes the putative adhesin. The methods for Tn*916* mutagenesis in *S. pyogenes* have been extensively outlined in a previous volume.[20]

Transformation of Streptococcus pyogenes by Electroporation (Glycerol Method)

The other methods of insertional mutagenesis to be discussed rely on the ability to transform *S. pyogenes* with exogenous DNA. As previously described, we have successfully used electroporation to transform *S. pyogenes*.[20] Here we present a more versatile method in which the bacteria are prepared in a glycerol solution that is suitable for either immediate transformation or storage of competent cells.

1. The *S. pyogenes* strain is cultured overnight at 37° in Todd–Hewitt yeast extract (THY) broth (see recipe in Table I) supplemented with 20 m*M* glycine (glycine is prepared as a 2.0 *M* stock solution which is filter sterilized and added to sterile medium just prior to use). A different glycine concentration may be required for optimal transformation of various streptococcal strains.

[19] J. R. Scott, *J. Bacteriol.* **174,** 6005 (1992).
[20] M. G. Caparon and J. R. Scott, this series, Vol. 204, p. 556.

TABLE I
RECIPE FOR TODD-HEWITT YEAST EXTRACT MEDIUM[a]

Component	Amount (g)
Beef–heart infusion (Difco, Detroit, MI, Cat. No. 0132-01-0)	500.0
Neopeptone	20.0
Sodium carbonate	2.5
Glucose	2.0
NaCl	2.0
Na_2HPO_4	0.4
Yeast extract (Difco, Cat. No. 0127-01-7)	2.0

[a] Todd–Hewitt yeast extract (THY) medium is prepared by adding the components to distilled water and bringing the final volume to 1.0 liter. The medium should be approximately pH 7.8. Place 100-ml portions of medium into 125-ml culture bottles that have Teflon-lined screw tops (e.g., Wheaton, Millville, NJ, Cat. No. 219815). Sterilize by autoclaving at 15 psi for at least 25 min. Once the bottles have been removed from the autoclave, they should immediately be tightly sealed until they are to be used to prevent absorption of excessive O_2. Store at room temperature for no longer than 1 week. All medium components, as well as a premixed powder base (Cat. No. 0492-01-4) (which requires the addition of yeast extract), are available from Difco.

2. The overnight culture is diluted 1:20 in THY broth supplemented with 20 m*M* glycine and incubated at 37° with no aeration. A culture with a total volume of 100 ml yields enough cells for two transformations and a negative control.

3. Growth of the cells is followed spectrophotometrically by measuring the absorbance at 600 nm (A_{600}). The cells are harvested shortly after the culture enters exponential phase (A_{600} between 0.15 and 0.2), which usually occurs after 2 to 3 hr. It may be necessary to plot a growth curve for each strain to be transformed in order to determine the A_{600} of an early exponential phase culture.

4. Harvest the cells by centrifugation (6500 *g*, 10 min, 14°) and save both the pellet and the conditioned supernatant.

5. Suspend the pellet in 5 ml of conditioned supernatant and transfer the suspension to a 15-ml conical centrifuge tube. Heat-shock the cells in a 43° water bath for 9 min and centrifuge the cells as above (Step 4).

6. Wash the cells twice by suspending the pellet in 10 ml of 15% (v/v) glycerol and centrifuging as above (Step 4). The final pellet is suspended in 0.6 ml of 15% glycerol, transferred to a 1.5-ml microcentrifuge tube, and placed on ice.

7. At this point the cell suspension may be divided into 200-μl portions and frozen immediately in a dry ice/ethanol bath for storage at −80°. To obtain the highest transformation efficiencies, however, newly prepared cells should be used.

8. The amount of DNA used to transform *S. pyogenes* depends on the nature of the DNA and on the strain of bacteria. In general, transformation of circular plasmid DNA containing a streptococcal replicon requires approximately 1 to 5 μg DNA per transformation, whereas transformation of DNA used in integrational and allelic replacement studies may require more. The DNA should be dissolved in a low salt buffer (e.g., 10 m*M* Tris-HCl, 1.0 m*M* EDTA, pH 8.0) and placed into a chilled 1.5-ml microcentrifuge tube. For each transformation, a 200-μl sample of the cell suspension is placed into the tube containing the DNA and mixed by pipetting up and down several times. The cells and DNA are then transferred to a chilled 0.2-cm Gene Pulser electroporation cuvette (Bio-Rad, Richmond, CA). The negative control sample is not subjected to electroporation. Instead, it is placed directly into 10 ml of THY broth and incubated at 37° for 1 hr.

9. The Gene Pulser apparatus (Bio-Rad) is set with the voltage at 1.75 kV, the capacitance at 25 μF, and the Pulse Controller at 400 Ω resistance.

10. The electroporation cuvette containing the cells and DNA is placed in the electrode chamber, and current is applied. The time constant should be between 7 and 8 msec.

11. The cell suspension is removed immediately with a microcapillary pipette tip and placed in a chilled 1.5-ml microcentrifuge tube. The cells are left on ice for 30 to 60 min.

12. The cell suspension is transferred to 10 ml of THY broth (with no antibiotics or glycine) and incubated for 1 hr at 37°.

13. The cells from the transformation and the negative control are harvested as above (Step 4) and suspended in 1 ml THY broth.

14. Aliquots are plated on THY agar supplemented with the appropriate antibiotics and incubated at 37°. Colonies are usually visible in 24–48 hr.

Integrational Plasmid Mutagenesis

The use of integrational plasmids to modify the host chromosome has been well established in the gram-positive bacterium *Bacillus subtilis,* and the ability to transform *S. pyogenes* by electroporation (see above) has made this technique available to the study of many streptococcal genes.[21]

[21] N. Okada, R. T. Geist, and M. G. Caparon, *Mol. Microbiol.* **7,** 893 (1993).

Inactivation of a cloned streptococcal gene involves the construction of a chimeric integrational plasmid that contains an internal fragment of the gene of interest, an *E. coli*-specific replicon, and an antibiotic-resistance determinant that can be selected for in both *E. coli* and *S. pyogenes* hosts. Based on experience, the internal fragment of the gene should be at least 400 bp in length and must not include either the 5′ or 3′ end of the gene. Note that because no natural group A streptococcal isolate resistant to penicillin has been reported, no integrational vector should be constructed which contains an intact β-lactamase gene. After the integrational plasmid is constructed and purified from *E. coli*, it is used to transform the *S. pyogenes* strain from which the gene was originally cloned. Because an *E. coli* plasmid replicon will not replicate in an *S. pyogenes* host, stable antibiotic-resistant transformants are only obtained when homologous recombination occurs between DNA sequences contained on both the integrational plasmid and the streptococcal chromosome. The resulting transformants will contain an inactive mutant allele in which the coding sequence of the gene is interrupted by vector-derived sequences (see Fig. 1A).

We have constructed pCIV2 (Fig. 1A),[21] a pUC-based integrational vector that contains an *aphA3* kanamycin resistance gene in place of the β-lactamase gene of the original vector, which is suitable for use in both *E. coli* and *S. pyogenes*. More recently, pJRS233, a temperature-sensitive shuttle vector that is maintained in low copy number in *E. coli* and is temperature-sensitive for replication in *S. pyogenes*, has been used to improve the efficiency of chromosomal integration in several studies.[22] During mutagenesis with pJRS233-derived plasmids, the population of transformants containing the plasmids is expanded by growing them at the permissive temperature for plasmid replication. This expansion increases the probability that chromosomal integration will occur when the cells are shifted to the nonpermissive temperature, while maintaining selection for the antibiotic resistance marker (erythromycin) of the plasmid. In addition to inactivation of streptococcal genes, strategies involving integrational plasmids have been used for promoter analysis,[21] introduction of reporter gene fusions into the chromosome,[23] and determining whether a regulatory element functions at a distance from its target gene.[24] Therefore, once a streptococcal gene has been cloned and partially characterized, integrational plasmids offer a powerful tool for genetic analysis in *S. pyogenes*.

[22] J. Perez-Casal, E. Maguin, and J. R. Scott, *Mol. Microbiol.* **8,** 809 (1993).
[23] R. T. Geist, N. Okada, and M. G. Caparon, *J. Bacteriol.* **175,** 7523 (1993).
[24] G. C. Fogg, C. M. Gibson, and M. G. Caparon, *Mol. Microbiol.* **11,** 671 (1993).

Cassette Mutagenesis Using ΩKm-2 Interposon

The function of a cloned gene can be evaluated by allelic replacement analysis in which an insertionally inactivated allele is exchanged for the wild-type gene. The mutant and its isogenic parent can subsequently be compared for their adhesion capabilities or any other phenotype under investigation. Once a gene has been cloned and its restriction map determined, it is often convenient to construct an inactivated mutant allele of the gene by inserting an antibiotic resistance cassette into the coding region at a unique restriction site. Digestion of the plasmid containing the mutant allele by a restriction endonuclease that cuts only in the vector sequences yields a linear molecule of DNA which can be used to transform a strain of *S. pyogenes* containing a wild-type gene. Homologous recombination between the DNA flanking the antibiotic resistance cassette and the identical sequences in the chromosome results in an exchange of the mutant allele for the wild-type gene.

In *S. pyogenes*, we have successfully used the kanamycin resistance cassette, ΩKm-2, to construct disrupted mutant alleles for allelic replacement.[25] The ΩKm-2 interposon consists of a set of strong transcriptional and translational termination signals in all reading frames that bracket both ends of an *aphA3* kanamycin resistance gene which is active in both *E. coli* and *S. pyogenes* (Fig. 1B). Allelic replacement with an ΩKm-2-disrupted gene results in a polar mutation in which genes that are located in the same operon and lie downstream of the insertion are not transcribed (Fig. 1C). Thus, one should be aware that insertional inactivation with ΩKm-2 may affect the expression of more than one gene in the resulting mutant strain. If possible, the wild-type gene should be cloned onto a streptococcal cloning vector, introduced into the insertionally inactivated mutant, and tested in a complementation analysis for restoration of the adhesion phenotype under analysis. The streptococci–*E. coli* shuttle vector pLZ12[25] is useful for complementation of ΩKm-2-disrupted genes.

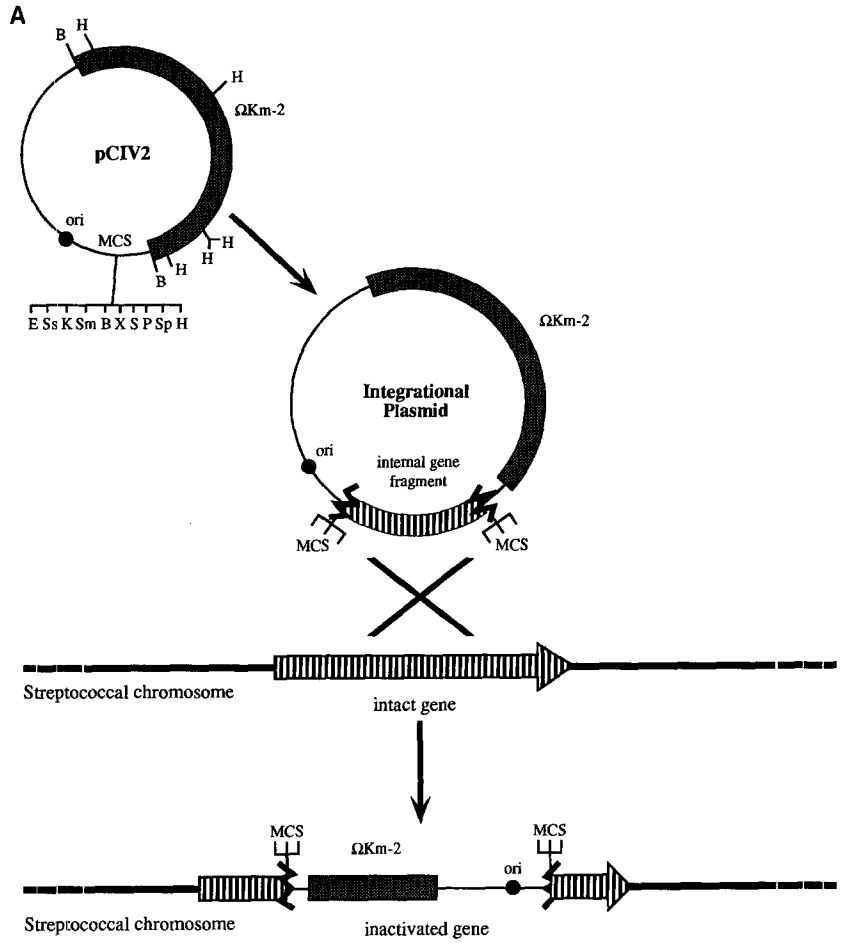
Shuttle Mutagenesis Using mγδ-200 in Streptococcus pyogenes

Unfortunately, both integrational plasmids and the ΩKm-2 interposon require extensive knowledge of the target sequences and unique restriction sites. Furthermore, Tn*916* mutagenesis is not always amenable to this type of genetic analysis because transposition occurs randomly throughout the entire genome and is not targeted to the region of interest. Thus, if a DNA fragment which encodes a putative adhesin has been cloned into

[25] J. Perez-Casal, M. G. Caparon, and J. R. Scott, *J. Bacteriol.* **173,** 2617 (1991).

E. coli, it is desirable to have a rapid method for mutagenesis which specifically targets this chromosomal locus that also does not require any preliminary restriction or sequence analyses. We have developed a shuttle mutagenesis strategy using a modified form of the replicative transposon γδ (Tn*1000*) to analyze an 8-kb *S. pyogenes* chromosomal fragment for regulatory genes that affect the level of fibronectin binding and protein F expression.[24]

FIG. 1. Insertional mutagenesis of streptococcal adhesins. (A) Insertional mutagenesis using an integrational plasmid. The plasmid pCIV2[21] contains the *aphA3* gene which encodes resistance to kanamycin that can be selected for in both *E. coli* and *S. pyogenes*. A DNA fragment that lacks both the 5′ and 3′ ends (internal gene fragment) of the adhesin-encoding gene to be inactivated is cloned into the multiple cloning site (MCS) of pCIV2 in *E. coli*. The plasmid is then used to transform *S. pyogenes*. Because the ColE1 origin of replication (ori) of pCIV2 is not capable of replicating autonomously in *S. pyogenes*, all kanamycin-resistant transformants will have arisen via a single homologous recombination event between the adhesin gene DNA of both the plasmid and chromosome, which is represented by the X between the two molecules. As a consequence of integration, the adhesin gene is insertionally inactivated. (B) Structures of ΩKm-2 (I) and mγδ-200 (II). The ΩKm-2 interposon contains the *aphA3* kanamycin resistance gene for selection in both *E. coli* and *S. pyogenes* (the location and direction of transcription of *aphA3* are indicated by the open bar and arrow) that is flanked at both ends by strong terminators of translation in all three reading frames (▶), strong terminators of transcription (○), and several restriction enzyme sites. *In vitro* cloning techniques are used to insert ΩKm-2 into the coding region of the adhesin gene using an available unique restriction site. The *E. coli* plasmid which contains the inactivated allele is converted to a linear fragment by restriction digest and used to transform *S. pyogenes* to resistance to kanamycin (see below). In mγδ-200, ΩKm-2 (shown in the same orientation as above) is flanked by the inverted repeats (⋙) of the *E. coli* transposon γδ (Tn*1000*) and contains the γδ *res* sequence that is required for transposition of the element. (C) Shuttle mutagenesis using mγδ-200. A nonconjugative plasmid which contains a cloned adhesin gene (T) is used to transform *E. coli* CBK, which possesses a conjugative plasmid (MGD) which contains mγδ-200 (⋙) and a separate plasmid which contains γδ transposase (XRD). The resulting strain is mated to *E. coli* LW49, which results in the transfer of a cointegrate plasmid (C) formed between the target and mγδ-200 donor plasmid that is generated by transposition of mγδ-200 by a replicative mechanism. The LW49 recipient contains a source of γδ resolvase in its chromosome (R) which catalyzes a site-specific recombination event at the *res* sequence that results in the resolution of the cointegrate into the original MGD plasmid and a mutated adhesin plasmid (M) which now contains a single randomly inserted copy of mγδ-200. The site of insertion is mapped, and the plasmid purified from *E. coli*, converted to a linear molecule by digestion with a restriction enzyme (E), and used to transform *S. pyogenes* to resistance to kanamycin. Homologous recombination between the adhesin gene sequences which flank mγδ-200 on both the transformed DNA and the streptococcal chromosome (indicated by the lines between the two molecules) results in insertional inactivation of the adhesin gene in the streptococcal chromosome. The method for mutagenesis using mγδ-200 is presented in detail in the text. Restriction enzyme sites are as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xba*I.


A
B
H
H
ΩKm-2
pCIV2
ori
MCS
H
H
H
B
E Ss K Sm B X S P Sp H
ΩKm-2
Integrational Plasmid
ori
internal gene fragment
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MCS
Streptococcal chromosome
intact gene
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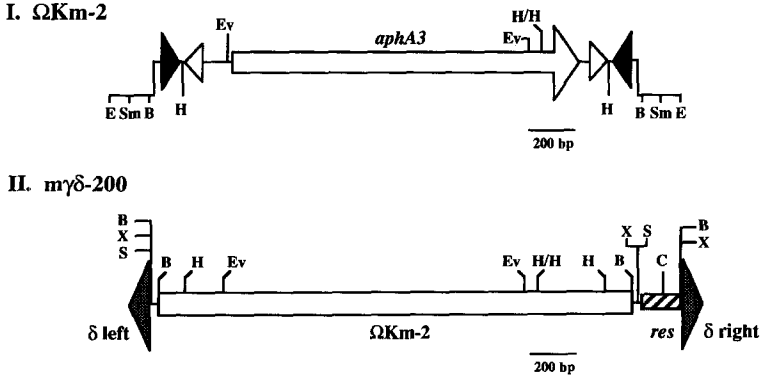
MCS
ori
Streptococcal chromosome
inactivated gene


B
I. ΩKm-2
Ev
aphA3
H/H
Ev
E Sm B
H
H
B Sm E
200 bp
II. mγδ-200
B
X
S
B
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δ left
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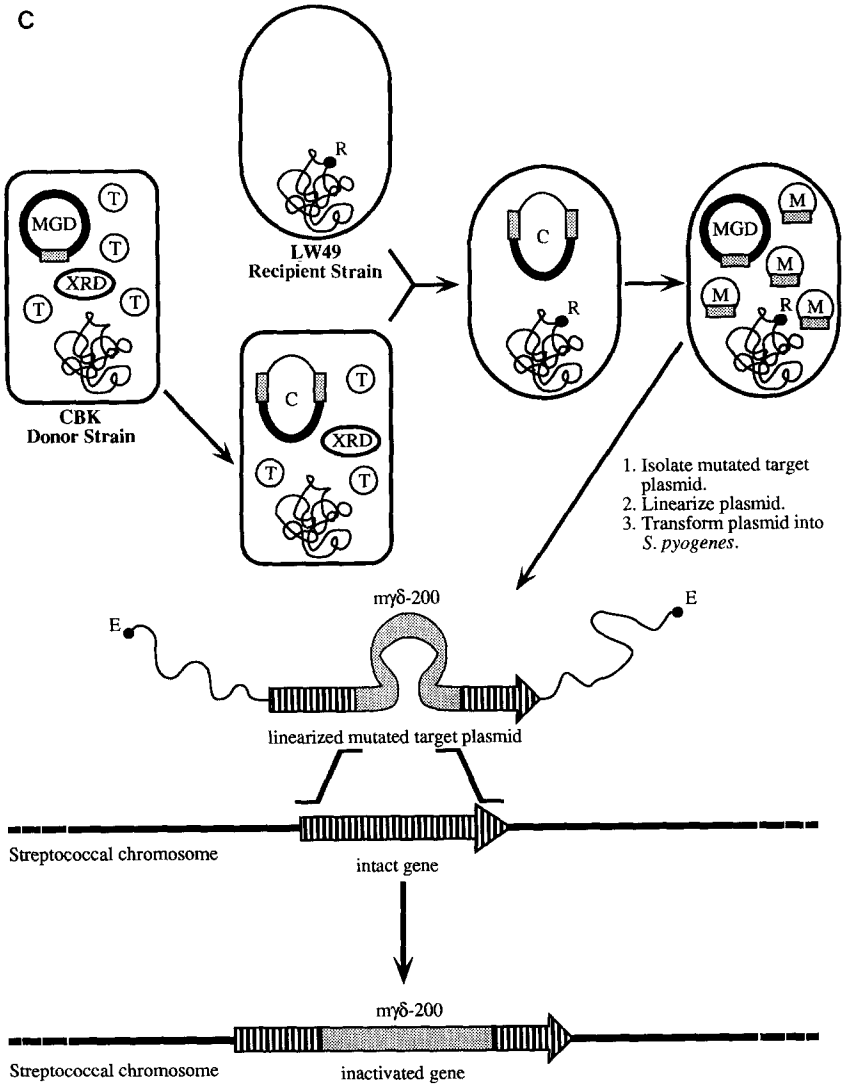

FIG. 1. (*continued*)

This transposon, mini-γδ-200 (mγδ-200), contains inverted repeats from the δ end of γδ that flank both the γδ *res* site and the ΩKm-2 interposon (Fig. 1B).[24] To make random polar insertions in a cloned fragment, a target plasmid containing the cloned sequences of interest is transformed into an

E. coli mutagenesis strain that harbors the mγδ-200 element on a conjugation-proficient donor plasmid (pMGD5) and a source of γδ transposase (pXRD4043). To avoid the use of a β-lactamase-containing plasmid, we use pCL1921, a vector with a spectinomycin resistance marker, to clone the streptococcal fragments.[26] However, any compatible nonconjugative cloning vector that does not encode resistance to ampicillin, chloramphenicol (which is present on pXRD4043, see below), or kanamycin (which is encoded by mγδ-200) can be used for constructing the target plasmid. The plasmid cloning vector must also be compatible with the F plasmid, from which pMGD5 is derived, and pACYC184, from which pXRD4043 is derived (see below).

During the replicative mechanism of transposition, the activity of transposase and DNA polymerase results in a stable structure called a cointegrate in which the donor plasmid and the target plasmid are fused together by directly repeated copies of mγδ-200 located at each of the junctions of the two plasmids. Subsequently, the cointegrate is conjugally transferred from the mutagenesis strain to a recipient *E. coli* strain (LW49) that contains a source of γδ resolvase (see below). Successful transfer and resolution of the cointegrate into the original donor plasmid and a target plasmid with a transposon insertion is detected by selecting transconjugants that have kanamycin resistance (selection for mγδ-200), nalidixic acid resistance (selection for the LW49 recipient strain and against the CBK donor strain), and the resistance carried on the target plasmid. Each transconjugant contains identical mutated target plasmids with a single mγδ-200 insertion. Thus, several different transconjugants must be selected for further evaluation in order to obtain a series of insertions along the entire length of the cloned fragment. The location of the mγδ-200 insertion into the target plasmid can be determined by analysis with restriction endonucleases or by the polymerase chain reaction (PCR). Mutated plasmids containing insertions at desired locations in the cloned fragment are linearized with a restriction endonuclease that only recognizes sites in the vector, and these linear molecules are used to transform *S. pyogenes* for allelic replacement studies as described above. This mutagenesis strategy serves as a powerful genetic technique to analyze a specific region of the streptococcal chromosome. The use of mγδ-200 has been summarized in Fig. 1C.

The following method adapted from Berg *et al.*[27] describes the use of mγδ-200 to make insertional mutations in streptococcal fragments that have

[26] C. G. Lerner and M. Inouye, *Nucleic Acids Res.* **18,** 4631 (1990).

[27] C. M. Berg, N. B. Vartak, G. Wang, X. Xiaoxin, L. Liu, D. J. MacNeil, K. M. Gewain, L. A. Wiater, and D. E. Berg, *Gene* **113,** 9 (1992).

been cloned into the pCL1921 vector. However, the method can be modified to suit any nonconjugative cloning vector that has an appropriate selectable marker.

1. *Escherichia coli* strain CBK(pMGD5, pXRD4043) is transformed with a target plasmid (pCL1921 derivative) containing the cloned streptococcal DNA of interest by standard methods. The resulting transformants serve as the male donor strain in the mating reaction and are resistant to kanamycin (selection for pMGD5), chloramphenicol (selection for pXRD4043), and spectinomycin (selection for target plasmid).

2. Select one donor strain colony and grow it in 1 ml Luria–Bertani (LB) broth supplemented with 1 m*M* isopropylthiogalactoside (IPTG) at 37° without shaking. Mating efficiency is optimal when the culture is between 5×10^7 to 1×10^8 colony-forming units (cfu)/ml, which is reached after approximately 3 hr. Concurrently, the recipient strain, LW49, should be grown to early stationary phase (2×10^8 cfu/ml) by adding 0.2 ml of an overnight culture to 30 ml LB broth and incubating at 37° for 2 to 2.5 hr with vigorous shaking.

3. Prepare the mating mixture by combining 0.5 ml of the donor culture with 0.2 ml of the recipient culture in a large test tube and incubate at 37° without shaking for 30 min.

4. Add 5 ml of prewarmed LB broth supplemented with 1 m*M* IPTG to the mating mixture and incubate for an additional 3 hr at 37° without shaking.

5. Plate 100 μl of the mating mixture on LB agar containing kanamycin, spectinomycin, and nalixidic acid. Grow up several candidate transconjugants in LB broth, extract the plasmid DNA, and determine the position of the m$\gamma\delta$-200 insertion by restriction mapping and PCR. Select a group of plasmids for allelic replacement studies that have nested insertions covering the entire cloned fragment of streptococcal DNA.

6. For allelic replacement, 50 to 100 μg of plasmid DNA must be linearized by digestion with a restriction endonuclease that cuts only in the vector-derived (pCL1921) region of the target plasmid. After digestion, the DNA is extracted with phenol/chloroform and precipitated by ethanol according to standard methods.

7. Dissolve the linearized DNA in a low salt buffer and run a small sample on an agarose gel to verify complete digestion of the plasmid DNA. The remaining DNA is used to transform *S. pyogenes* as described above. Cells that have exchanged alleles are selected on THY agar with kanamycin, and the chromosomal structure of the mutant strains is confirmed by Southern blot analysis using a m$\gamma\delta$-200 probe.

8. The resulting mutants are analyzed with regard to the phenotype under study.

Expression of Adhesins in Heterologous Streptococci

Unfortunately, it is not possible to subject all strains of *S. pyogenes* to the mutagenesis techniques described above because it is not yet possible to transform all strains of *S. pyogenes*. If an interesting adhesin is identified in a nontransformable strain, it still may be possible to use a genetic approach for the analysis of this adhesin through expression of the adhesin in a heterologous streptococcal strain. In this case, the gene which encodes the putative adhesin is cloned onto a broad host range streptococci–*E. coli* shuttle vector (e.g., pLZ12 or pLZ12-Km)[25,28] and used to transform an *S. pyogenes* strain which lacks this gene. The plasmid-containing strain which expresses the cloned gene can then be compared to the host strain which contains only the vector with no cloned insert (see below). In certain cases, it may be possible to introduce the plasmid into a different streptococcal species for analysis. This method has been used to analyze protein F following introduction of a plasmid which contains the gene encoding protein F into *Enterococcus faecalis* (a group D streptococcus).[28] One potential pitfall to this method would involve adhesins which require a strain-specific regulatory factor for their expression. If the structural gene encoding the adhesin is introduced into a heterologous strain which lacks the regulatory factor, it will not be possible to obtain expression of the adhesin. These additional determinants could involve additional genes for secretion and assembly of the adhesin, or involve regulatory elements required to activate the promoter of the adhesin gene. If this appears to be the case, it may be possible to utilize a method in which the putative adhesin is introduced into a heterologous strain as a translation fusion to a surface protein that is normally strongly expressed on the surface of the heterologous strain.[29,30]

Regulation of Adhesin Expression

Because many, if not all, the binding activities described for *S. pyogenes* may play some role in host cell interaction, an important question then becomes at which stage of the infectious process or in which host compartment does the binding activity contribute to pathogenesis. One way in which the question can begin to be addressed is through an analysis of the regulation of expression of the binding activity or adhesin under study. Much effort has been directed at defining the gene products required at a

[28] E. Hanski, P. A. Horwitz, and M. G. Caparon, *Infect. Immun.* **60,** 5119 (1992).

[29] M. Hansson, S. Ståhl, T. N. Nguyen, T. Bächi, A. Robert, H. Binz, A. Sjölander, and M. Uhlén, *J. Bacteriol.* **174,** 4239 (1992).

[30] G. Pozzi, M. Contorni, M. R. Oggioni, R. Manganelli, M. Tommasino, F. Cavalieri, and V. A. Fischetti, *Infect. Immun.* **60,** 1902 (1992).

specific stage of an infection (e.g., entry, attachment, colonization, and invasion) in terms of the environmental signals which the microorganism sense to control the coordinate regulation.[31] It has become clear that bacteria have evolved sophisticated signal transduction systems to sense environmental cues that vary between different host compartments. In *S. pyogenes*, it appears that the tensions of different gases in the environment constitute important cues for virulence gene regulation. Transcription of the gene which encodes M protein (*emm*) is regulated in response to the concentration of CO_2,[32] whereas transcription of the gene which encodes protein F (*prtF*) is regulated in response to O_2 tension.[33] These regulatory pathways apparently allow the streptococcus to distinguish between the surface of the skin and the deeper tissue layers of the epidermis, where the concentrations of CO_2 and O_2 vary significantly.[34] Further analysis of environmental signal transduction may reveal yet other signaling pathways which may control adhesin expression.

Streptococcus pyogenes has a complex nutritional requirement, and a number of media are available for culture. However, careful use of Todd–Hewitt yeast extract medium (THY; Table I) results in differential expression of CO_2 and O_2 regulated genes. Differential expression of CO_2- and O_2-regulated genes can be obtained depending on whether *S. pyogenes* is cultured on liquid or solid media, apparently as the result of the differential diffusion of the gases from the two media.[32] Solid medium is prepared by adding Bacto agar (Difco, Cat. No. 0140-05-6) to THY liquid medium to a final concentration of 1.4% prior to autoclaving. The medium is then autoclaved as described above, allowed to cool to 55°, and augmented with any required medium supplements at that time. Add 35-ml portions to 100 × 15 mm petri dishes and allow to solidify at room temperature. Solid medium can be stored at 4° for up to 1 month.

Expression of CO_2-regulated genes is maximally obtained in liquid medium.[32] For most adhesion assays, a 10-ml culture usually provides sufficient numbers of cells. For liquid culture, a 10-ml volume of liquid THY medium is added to a sterile 13 × 100 mm round culture tube that has a rubber or Teflon-lined screw top (e.g., Corning Pyrex tube, Corning, NY, Cat. No. 98513, or equivalent). The culture is then inoculated with 30 μl of a partially thawed frozen stock of the *S. pyogenes* strain of interest. The top is tightly sealed, and the culture is incubated at 37° for 18 hr without agitation. If

[31] J. J. Mekalanos, *J. Bacteriol.* **174,** 1 (1992).
[32] M. G. Caparon, R. T. Geist, J. Perez-Casal, and J. R. Scott, *J. Bacteriol.* **174,** 5693 (1992).
[33] T. VanHeyningen, G. Fogg, D. Yates, E. Hanski, and M. Caparon, *Mol. Microbiol.* **9,** 1213 (1993).
[34] M. E. McBride, *in* "The Skin Microflora and Microbial Skin Disease" (W. C. Noble, ed.), p. 73. Cambridge Univ. Press, Cambridge, 1992.

more cells are required, the technique can be scaled up by using larger culture bottles; however, care should be taken to ensure that the medium occupies at least three-quarters of the total volume of the bottle. Following culture, the streptococci are harvested by centrifugation (7000 *g*, 5 min, 14°) and washed once in the appropriate adhesion buffer (see below), and the density of bacterial cells is determined and adjusted to the required cell number (see below) by determining the optical density (OD_{600}) or by direct examination using a Petroff–Hauser slide chamber and phase-contrast microscopy. A frozen *S. pyogenes* stock is prepared by adding sterile glycerol to an overnight culture to a final concentration of 25% (v/v). The mixture is then divided into sterile freezer vials and stored at −80°.

Expression of O_2-regulated genes is maximally obtained by culture on solid medium.[33] A solid culture is most conveniently inoculated by placing a 50-μl volume of a partially thawed frozen stock of the *S. pyogenes* strain of interest at the center of the plate. The inoculum is then carefully spread over the entire surface of the medium using a sterile glass rod. The petri dish which contains the incoulated solid medium is inverted and then placed in a 37° incubator and cultured for 18 hr under an ambient atmosphere. Following incubation, the plate is flooded with 5 ml of phosphate-buffered saline (PBS, 0.14 *M* NaCl, pH 7.2) and the streptococcal cells harvested by carefully suspending the growth using a sterile glass rod. The cells are collected by centrifugation (7000 *g*, 5 min, 14°), and washed once in the appropriate adhesion buffer (see below), and the density of bacterial cells is determined and adjusted to the required cell number as described above. The growth from two dishes is sufficient for most adhesion experiments. If more cells are required, additional dishes can be inoculated. Additional control over the expression of O_2- and CO_2-regulated genes can be obtained by incubation of the solid cultures under atmospheres altered in the tension of CO_2 and O_2. This is most easily accomplished utilizing a CO_2 incubator equipped with O_2 and CO_2 monitors for precise control of atmosphere. In the absence of an incubator, excellent results can be obtained using a commercial gas generator in a sealed jar. Gas generators capable of generating atmospheres which consist of a wide range of CO_2 and O_2 concentrations are available from a number of manufacturers (e.g., GasPak systems, BBL Microbiology Systems, Cockeysville, MD). Culture of solid media under CO_2-rich anaerobic conditions (GasPak, Cat. No. 70304) will result in the stimulation of expression of CO_2-regulated genes and the repression of O_2-regulated genes.[20]

A final note of caution should be added. It has been a common practice to passage *S. pyogenes* strains serially through mice or in the presence of fresh human blood *in vitro*. This has been necessary because many virulence phenotypes of *S. pyogenes* (e.g., expression of M protein) decrease on serial

in vitro culture on most microbiological media.[35] Passage through blood or animals selects for strains which express high levels of certain virulence factors.[35] However, this practice can also produce strains which have become aberrant in regulation of many virulence factors.[24,35] Therefore, every effort should be made to reduce the number of *in vitro* passages to which any *S. pyogenes* strain may be subjected. In addition, any regulatory phenomenon observed in an extensively passaged strain should also be examined in several recently obtained clinical isolates to ensure that the regulatory response is also characteristic of a number of non-*in vitro* passaged strains.

Structure–Function Analyses of Adhesins

Comparison of isogenic mutants in models of adhesion will address what role a specific adhesin plays in interaction with host cells. Once an interaction has been identified it is then useful to determine and characterize the domains of the protein responsible for the adhesion to target cells. This information then becomes invaluable for the development of specific adhesin inhibitors. Two general strategies are useful for the identification of adhesin domains. The first strategy involves constructing a set of fusion proteins which express different domains of the protein and testing the ability of each of the chimeric proteins to inhibit adhesion. Comparison of a large panel of fusion proteins will allow the domain(s) responsible for adhesion to be localized to a specific subset of amino acids of the intact adhesin protein. An alternative approach is to express domains of the adhesins within the exposed region of a different surface protein of the streptococcus (or heterologous bacterial species). Comparisons of the abilities of bacteria which express different domains of the adhesin on their surfaces to adhere to target cells will lead to the definition of the adhesive domains. The advantage of the latter approach is that it is unaffected by any nonspecific interactions of the chimeric proteins with either the bacteria or the target cells that may occur during an inhibition assay, and thus provides the most direct method for evaluating the role of a specific adhesive domain in adhesion. The expression of hybrid proteins should be conducted in a mutant strain in which the original genes involved in hybrid formation have been inactivated or deleted from the chromosome or should be conducted in a heterologous streptococcal species that lacks either gene. The streptococcal mutants expressing the hybrid proteins are then tested for the adhesion to target cells and compared to both the wild-type strain and to the mutant strain used as the exression host. The results obtained from

[35] R. C. Lancefield, *J. Immunol.* **89,** 307 (1962).

these studies allow direct assessment and quantification of the contribution of a specific domain of the protein to adhesion.

Construction of Fusion Proteins

Standard structure–function analyses of a cloned adhesin requires the construction, expression, and purification of mutant adhesin proteins using an *E. coli* host. However, this process can often be time-consuming and inefficient. An alternative approach which can greatly simplify this process is to express domains of the protein of interest as a translational fusion to a peptide or protein which provides a characteristic that allows for relatively fast and simple affinity purification of the hybrid. Examples of widely used fusion partners include maltose-binding protein, β-galactosidase, and glutathione *S*-transferase. In analyses of the fibronectin-binding protein, protein F, extensive use of vectors which place a short affinity tag consisting of several histidine residues at the N or C terminus of the polypeptide have been made. The resulting hybrid is readily purified in a single step by affinity chromatography by virtue of the high affinity of the polyhistidine motif to bind to affinity matrices which contain immobilized Ni^{2+}. The presence of the affinity tag has only rarely been shown to influence the activity of the resulting hybrid protein. Both the vectors for construction of the polyhistidine-tagged proteins and the affinity reagents for their purification are available commercially. For analysis of protein F extensive use of the vectors and reagents available from Qiagen (Chatsworth, CA) has been made, although similar materials are available from other vendors (e.g., Novagen, Madison, WI). It should be noted that it will be necessary to determine the DNA sequence of the adhesin to be studied for successful construction of the fusion proteins.

1. The polymerase chain reaction (PCR) is used to amplify a specific domain of interest from the adhesin gene. The primers should be carefully designed to include a restriction site which will allow the amplified fragment to be ligated into a restriction site of the polyhistidine fusion vector so as to construct an in-frame fusion between the histidine residues and the adhesin gene. For example, we have utilized the expression vector pQE30 (Qiagen) which contains an affinity tag consisting of six consecutive histidine residues that is fused at the N terminus of the hybrid polypeptide. The primers for PCR amplification are designed to preserve the reading frame of different amplified protein F segments utilizing the *Kpn*I restriction site of the vector. The PCR should be conducted according to the recommendations of the vendor providing the polymerase. It is not necessary to utilize PCR amplification of specific domains if suitable restriction sites are present in the adhesin gene that will allow for the construction of in-fame fusions.

2. The sequence of the amplified segments should be verified following cloning to ensure that amplification has not introduced any mutations into the cloned fragment and that an in-frame fusion between the polyhistidine tag and the adhesin domain has been generated. Sequence analysis can utilize any commercial sequencing kit (e.g., Sequenase 2.0 kit from United States Biochemicals, Cleveland, OH) and oligonucleotide primers which anneal to vector sequences flanking the cloning site (available from Qiagen).

Purification of Fusion Proteins

The fusion protein constructed as described above is purified from extracts of the *E. coli* host by affinity chromatography. The method utilized should follow the recommendations of the manufacturer of the Ni^{2+} affinity matrix. It should be noted that the initial solubilization of the protein produced from *E. coli* can be conducted under denaturing conditions, because the ability of the polyhistidine tag to bind to Ni^{2+} is not impaired even in the presence of high concentrations of denaturants. Experience shows that purification under denaturing conditions provides the fastest and most reliable way to purify many different fusion proteins since it is not necessary to evaluate the solubility characteristics and cellular location of each individual hybrid in the *E. coli* host.

1. The fusion construct present in the pQE30 vector is used to transform *E. coli* SG13009 which contains pREP4 (Qiagen), a plasmid which provides a source of the $LacI^q$ repressor. This will allow precise control overexpression of the fusion protein, whose transcription in pQE30 is under the control of the *lac* promoter. Transformants are selected on Luria agar plates containing both 100 μg/ml of ampicillin (to select for pQE30 chimera) and 25 μg/ml of kanamycin (to maintain selection for pREP4).

2. Colonies can be screened for optimal conditions for expression of the fusion protein by varying the concentration of the IPTG inducer (which will derepress the *lac* promoter) and the time following induction for harvesting the cells using small-scale cultures (e.g., 10 ml). The proteins are purified using denaturing conditions as described below and the yield of purified protein determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The optimal conditions are then applied in large-scale purification.

3. For a typical large-scale purification, 20 ml of LB broth supplemented with 100 μg/ml of ampicillin and 25 μg/ml of kanamycin is inoculated with the appropriate clone and grown overnight with vigorous shaking. The 20-ml culture is used to inoculate 1 liter of LB broth containing the same antibiotics and the incubation continued at 37° until an OD_{600} of 0.8 is reached. Then, 2 m*M* IPTG (final concentration) is added, and the culture

is grown for another 5 hr. The pellet is collected by centrifugation and stored frozen (−70°) until used for the purification of the hybrid proteins.

4. For standard purification under denaturing conditions, the method recommended by the manufacturer of the Ni^{2+} resin (Qiagen) is used. The cell pellet is suspended in 5 ml of lysis buffer A (6 *M* guanidine hydrochloride, 0.1 *M* NaH_2PO_4, 10 m*M* Tris-HCl, pH 8.0) per gram weight of bacteria and slowly stirred for 1 hr at room temperature.

5. The resulting lysate is clarified by centrifugation at 10,000 *g* for 15 min at room temperature, and the supernatant is mixed with 8 ml of a 50% slurry of the Ni-NTA resin (Qiagen) which has been preequilibrated in lysis buffer A.

6. The mixture is slowly stirred at room temperature for 1 hr and then carefully loaded into a 1.6-cm-diameter column. The resin is washed with 10 column volumes of lysis buffer A and 5 column volumes of buffer B (8 *M* urea, 0.1 *M* NaH_2PO_4, 10 m*M* Tris-HCl, pH 8.0). If the OD_{280} of the flow-through is greater than 0.01, continue washing with buffer B.

7. Wash with buffer C (8 *M* urea, 0.1 *M* NaH_2PO_4, 10 m*M* Tris-HCl, pH 6.3) until the flow-through once again has an OD_{280} less than 0.01.

8. The fusion protein is then eluted with 20 ml buffer B containing 0.25 *M* imidazole. Fractions (2 ml each) are collected and analyzed by SDS–PAGE. Fractions which contain the fusion protein are pooled and dialyzed against buffer B without imidazole.

Analysis of Fusion Proteins

The strategy and methods for using fusion proteins for the analysis of the structure and functional relationships of a streptococcal adhesin is best illustrated using the fibronectin-binding protein, protein F, as an example. The domain structure of protein F cloned from *S. pyogenes* JRS75 is shown in Fig. 2.[36] It contains cell wall-anchoring motifs at the C terminus typical of other streptococcal surface proteins. Unique domains include a 37-amino acid motif (RD2) that is repeated four times completely and one time partially and UFBD, comprising a nonrepetitive stretch of 43 amino acids, which is located immediately N-terminal to RD2. The combination of RD2 and UFBD is referred to as UR. A series of fusion proteins were constructed which included various regions of protein F, including hybrid proteins which just contained RD2, UFBD, and UR (Fig. 2A). The proteins were purified as described above with typical yields of 3–5 mg of purified protein from only 1 liter of culture that were greater than 95% pure when analyzed by SDS–PAGE (Fig. 2B).

[36] S. Sela, A. Arvik, I. Burstein, A. Tovi, M. G. Caparon, and E. Hanski, *Mol. Microbiol.* **10,** 1049 (1994).

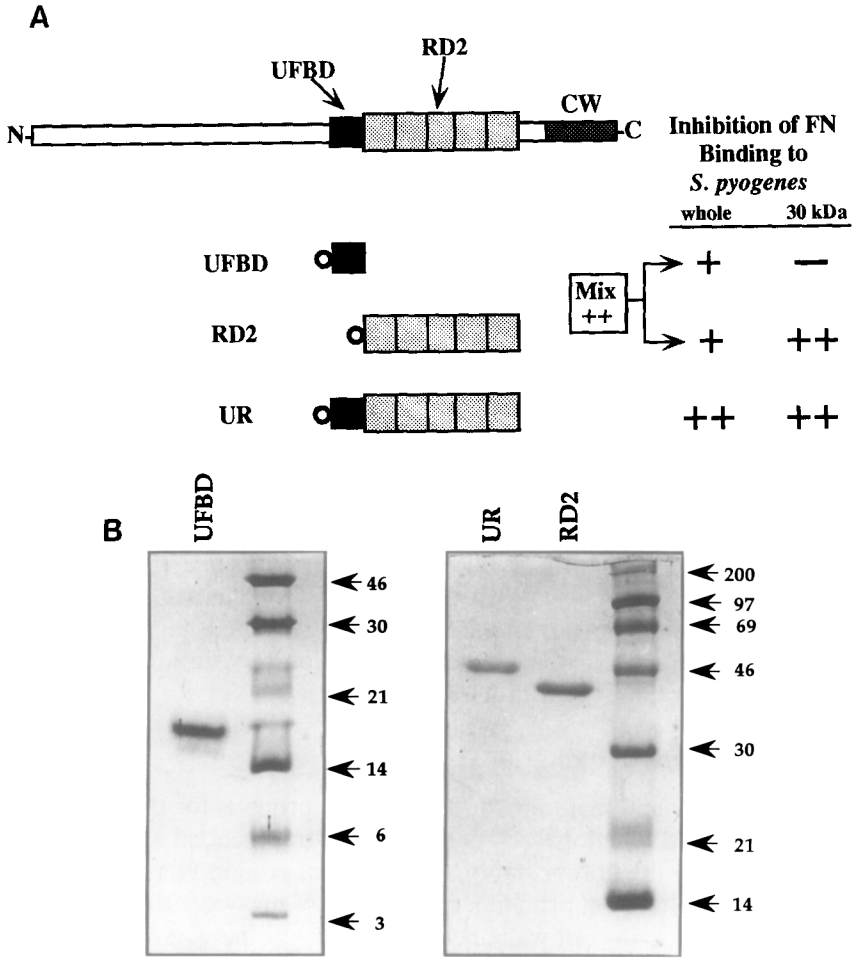

FIG. 2. Use of fusion proteins in the structure–function analysis of a streptococcal adhesin. (A) The domain structure of protein F is shown. RD2 consists of a sequence of 37 amino acids that is repeated in tandem four times and partially a fifth time. UFBD consists of a single stretch of 43 amino acids located immediately N-terminal to RD2. The combination of UFBD and RD2 is referred to as UR. A region involved in attachment of the protein to the cell wall is indicated as CW. Fusion proteins were constructed which contained UFBD, RD2, and UR linked in-frame to an N-terminal affinity tag which consisted of six consecutive histidine residues (○). The polyhistidine affinity tag allows for a simple single-step purification based on the ability of the tag to bind to an affinity matrix which contains immobilized Ni^{2+} (consult the text for details). The results obtained from purification of the fusion proteins are illustrated in (B). Purified fusion proteins RD2 and UR were subjected to 10% and UFBD was subjected to 15% SDS–PAGE, respectively, and stained with Coomassie brilliant blue. The molecular masses of the standard proteins are indicated by the arrows on the right-hand side and are denoted in kilodaltons. When the abilities of the purified proteins to inhibit the

When the purified proteins were used to probe the ability of protein F to bind to fibronectin, it was found that only the proteins which contained RD2, UFBD, or UR were capable of inhibiting the ability of fibronectin to bind to intact cells of an *S. pyogenes* strain that expressed the wild-type protein on its surface. However, fusion proteins which contained UFBD or RD2 alone were capable of only partial inhibition, even when assayed at very high concentration. Complete inhibition required both domains (UR, Fig. 2A). Furthermore, complete inhibition could be obtained when both UBFD and RD2 were introduced into the binding reaction on separate molecules (Fig. 2A). Finally, only RD2 could inhibit the binding of the purified N-terminal fragment of the fibronectin molecule to *S. pyogenes*; UFBD could not (Fig. 2A). Thus, this example illustrates that through careful analysis of fusion proteins it was possible to determine that protein F contains two distinct domains (RD2 and UFBD) capable of binding to two separate regions of fibronectin.[36] Similar analyses of fusion proteins derived from other host protein-binding streptococcal adhesins should prove to be invaluable for both mapping the domains of the adhesin responsible for binding to the host protein as well as analyzing domains responsible for adhesion to target cells.

Expression of Adhesin Domains in Hybrid Surface-Exposed Proteins

As described in the preceding section, the construction and analysis of purified fusion proteins can provide important information of the structure–function relationships of a streptococcal adhesin. However, because assays for function of the purified hybrid proteins rely on the inhibition of an adhesion phenotype, careful consideration of possible artifacts associated with inhibition assays must always be made. These can include steric interference with an adjacent binding motif or inhibitions which result from interactions between different streptococcal proteins and the fusion protein, rather than direct recognition of a host receptor by the fusion protein. A more powerful and direct technique to obtain structure–function informa-

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binding of the whole fibronectin (FN) molecule to a wild-type *S. pyogenes* strain was tested, it was found that although UR was an effective inhibitor, both UFBD and RD2 exhibited partial inhibition (++, complete inhibition; +, partial inhibition; −, no inhibition). Complete inhibition could be obtained when both UFBD and RD2 were introduced into the binding reaction on separate molecules ("mix"). In addition, RD2, but not UFBD, was an effective inhibitor of the binding of an N-terminal fibronectin 30-kDa fragment. These results indicate that protein F contains two distinct domains that bind to two separate regions of the fibronectin molecule.[36] This example illustrates how fusion proteins can be used to provide high resolution structure–function information concerning a streptococcal adhesin.

tion involves the presentation of defined domains of the adhesin on the streptococcal surface, using the secretion and anchoring domains of a different streptococcal surface protein. The following method[37] is based on the technique of Pozzi *et al.*[30] for the expression of foreign protein domains with the surface-exposed regions of the serotype 6 M protein molecule and method of Perez-Casal *et al.*[22] for the introduction and expression of mutated M protein molecules in *S. pyogenes*.

1. The polymerase chain reaction is used to amplify a specific domain of interest from the adhesin gene. The primers should be carefully designed so that the PCR product is flanked by a *Kpn*I restriction site at the 5′ end and a *Hin*dIII site at the 3′ end (Fig. 3A). Furthermore, the sequence of the primers should also be designed so that the insertion of the fragment between *Kpn*I and *Hin*dIII sites of *emm*6.1, the gene which encodes a serotype 6 M protein (GenBank Accession No. M11338),[38] will conserve the *emm*6.1 reading frame across both the *Kpn*I and *Hin*dIII junctions (Fig. 3A).

2. The PCR product is then cloned between the *Kpn*I and *Hin*dIII sites of *emm*6.1 contained on the streptococci–*E. coli* shuttle vector pJRS233A (Fig. 3A). This will result in the exchange of the adhesin domain segment for approximately 550 bp of *emm*6.1 sequence which includes the B and C repeat domains of M6.1 (Fig. 3B).[39]

3. The resulting plasmid is used to transform an *S. pyogenes* host which contains both the *emm*6.200 allele (Fig. 3C)[32] and a mutation which inactivates the gene for the adhesin under analysis. If a host with these characteristics is not available, then it may be possible to insert the PCR fragment into the vector pVMB20 for transformation of *S. gordonii* GP232 as described by Pozzi *et al.*[30]

4. The pJRS233A vector is temperature-sensitive for replication in *S. pygoenes* (but not for replication in *E. coli*). Therefore, the transformation of *S. pyogenes* proceeds as outlined above with the following modification as described by Perez-Casal *et al.*[22] Transformation proceeds exactly as described above until the cells are incubated on ice following electroporation (step 11 in transformation protocol). The cells are then suspended in 10 ml of THY broth (Table I) and incubated for 1 hr at 30°. The cells are harvested by centrifugation (8000 *g*, 10 min, 20°) and resuspended in 1 ml THY broth, and a 0.1-ml portion is mixed with 3 ml of THY containing 0.7% agar (which has been cooled to 47° following sterilization). The mixture

[37] A. Tovi, I. Burstein, and E. Hanski, submitted for publication (1995).
[38] S. K. Hollingshead, V. A. Fischetti, and J. R. Scott, *J. Biol. Chem.* **162,** 1677 (1986).
[39] V. A. Fischetti, *Clin. Microb. Rev.* **2,** 285 (1989).

is poured over the surface of a THY plate (each plate contains 35 ml medium) and incubated at 30° for 2 hr in an anaerobic atmosphere (see above, Regulation of Adhesion Expression). Erythromycin is added to 3 ml of THY plus 0.7% agar to give a final concentration for the entire plate of 1 μg/ml (i.e., add 41 μg), and the mixture is poured over the surface of the plate. The plates are returned to an anaerobic environment and incubated at 30° for 48 hr.

5. Erythromycin-resistant transformants are then passaged 4–5 times at 37° for 18 hr each by inoculating 10^6 cfu into 10 ml of THY medium which contains 1 μg/ml erythromycin. An aliquot from the final passage is plated on THY agar with 1 μg/ml erythromycin and incubated at 37° so as to obtain single colonies. Because the pJRS233A vector is temperature-sensitive for replication in *S. pyogenes*, passage at 37° in the presence of erythromycin results in selection for cells where the plasmid has integrated into the streptococcal chromosome by virtue of the fact that the pJRS233A contains a DNA segment upstream of *emm*6.1 in pJRS233A that is homologous to the corresponding segment upstream of *emm*6.1 on the chromosome (Fig. 3C). This introduces the chimeric fusion protein under the control of the *emm*6.1 upstream regulatory apparatus,[21] which has been shown to be the most efficient method for generating high level expression of mutant alleles of M protein.[22,39a]

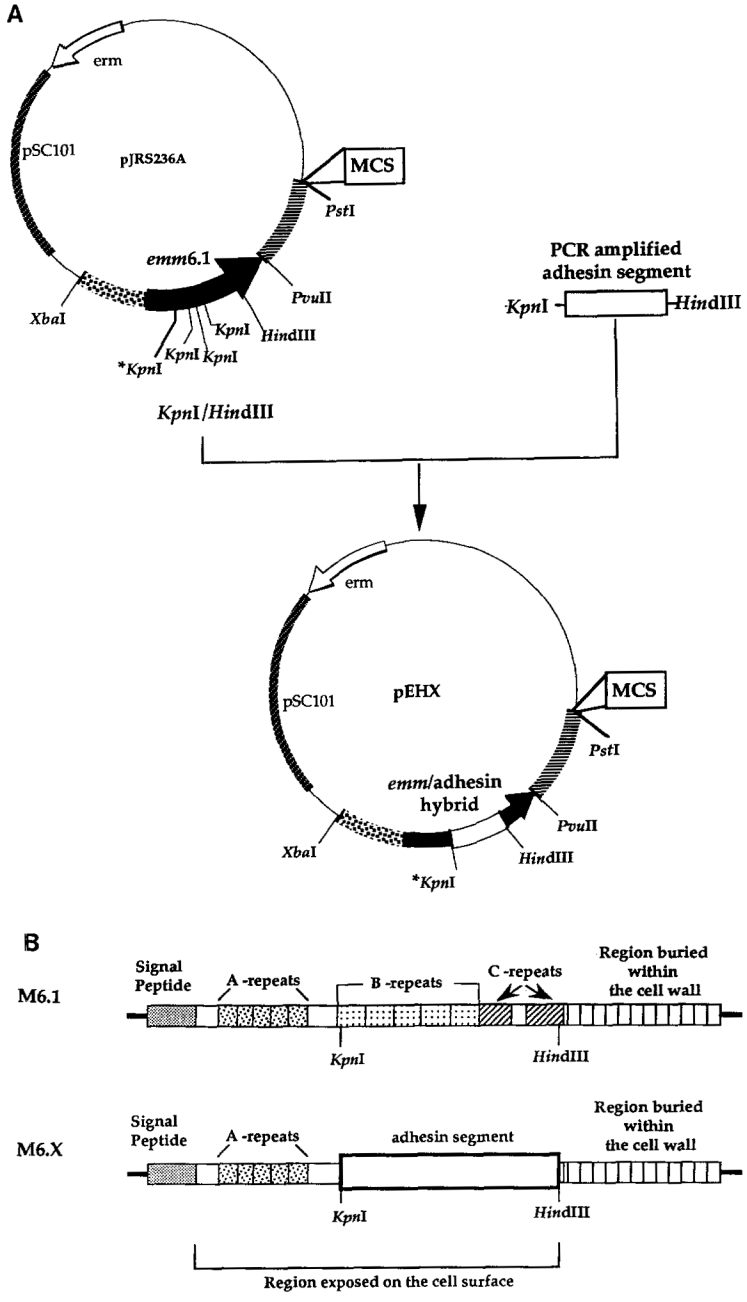
6. Because the introduced fusion protein will also contain sequence homologous to the adhesin under study, recombination may also occur at the native locus of the adhesin gene, which would not result in the production of the desired hybrid proteins. Therefore, it is essential that the resulting erythromycin-resistant transformants are screened for integration into the correct locus. This can be done using Southern blots on chromosomal DNA that has been digested with *Pvu*II and probes which can distinguish between recombination at the two loci (Fig. 3C). A more convenient method for screening multiple transformants utilizes PCR and amplimers which anneal to sites immediately upstream and downstream of the *Kpn*I and *Hin*dIII sites of *emm*6.1 into which the adhesin segment was inserted ($P_{5\text{-}3}$ and $P_{3\text{-}3}$, Fig. 3C). The amplified PCR fragments should have the same size as the inserted adhesin segment if the desired recombination event occurred. If, however, recombination occurred through the adhesin locus, the same primers must be now oriented at the opposite direction to one another, and thus no amplification can be obtained. The method of Hynes *et al.*[40] enables rapid and convenient screening of multiple transformants. Individual colonies of *S. pyogenes* are grown overnight at 37° in 1.5 ml THY

[39a] J. Perez-Casal, M. G. Caparon, and J. R. Scott, *Res. Microbiol.* **143,** 549 (1992).

[40] W. L. Hynes, J. J. Ferretti, M. S. Gilmore, and R. A. Segarra, *FEMS Microbiol. Lett.* **94,** 139 (1992).

supplemented with 1.5% glycine. The cells are harvested by centrifugation in a microcentrifuge (5 min) and the cell pellet suspended in 1 ml of solution I [20% (w/v) sucrose; 10 m*M* Tris-HCl, pH 8.0; 100 m*M* NaCl; 1 m*M* EDTA] with 25 mg of lysozyme and 50 units of mutanolysin. After incubation for 1 hr at 37°, the pellet is harvested by centrifugation and suspended in lysis buffer (50 m*M* KCl; 10 m*M* Tris-HCl, pH 8.3; 0.1 mg/ml gelatin; 0.45% Nonidet P-40; and 0.45% Tween 20) supplemented with proteinase K (100 μg/ml). Following incubation at 60° for 60 min, the samples are heated at 95° for 10 min. A portion of each lysate (2 μl) is subjected to PCR amplification using standard methods and the amplimers $P_{5\text{-}3}$ (5′-CTAATTTGCTGTTTGCTTCTT-3′, bp 1509–1489 of *emm*6.1) and $P_{3\text{-}3}$(5′-GAGCGAGAAAATAAAGAAGCC-3′, bp 912–932 of *emm*6.1). Strains that are positively identified by the first stage of screening are confirmed by Southern blot analysis. This ensures that the hybrid genes

FIG. 3. Expression of adhesin domains in hybrid proteins exposed on the streptococcal cell surface. (A) The *E. coli*-streptococci shuttle plasmid pJRS236A contains a low copy pSC101 replicon for maintenance in *E. coli*, a second replicon that is temperature-sensitive for replication in *S. pyogenes*, and an erythromycin-resistance gene (*erm*) that can be selected for in both *E. coli* and *S. pyogenes*. Also present is the gene which encodes a type 6 M protein of *S. pyogenes* (*emm*6.1) and approximately 500 bp of DNA sequences that are located immediately 5′ to *emm*6.1 in the streptococcal chromosome (checkered band). The plasmid pJRS236A[37] is essentially a derivative of pJRS236[22] from which a *Kpn*I site and a *Hin*dIII site were removed from a multiple cloning site (MCS). Note that the MCS contains sites for the enzymes *Pst*I, *Eco*RI, *Eco*RV, *Cla*I, *Sal*I, and *Xho*I. To construct a hybrid expression protein, a domain of the adhesin gene is amplified by the PCR so that it is flanked at the 5′ end by a *Kpn*I site and at the 3′ end by a *Hin*dIII site. The PCR amplimers are carefully designed so that the amplified fragment can be inserted between the most 5′ *Kpn*I site (**Kpn*I) and *Hin*dIII site of *emm*6.1 so as to conserve the *emm*6.1 reading frame to generate the *emm*/adhesin hybrid gene, as is illustrated on the plasmid pEHX. (B) Structures of the M6.1 and M6.X proteins. Insertion of the adhesin domain between the *Kpn*I and *Hin*dIII sites essentially replaces the B and C repeat regions of M6.1 with a defined domain of the adhesin. The region of M6.1 that it encoded 5′ to the *Hin*dIII site of *emm*6.1 is the region of the protein which is exposed on the streptococcal cell surface.[39] (C) The plasmid containing the *emm*/adhesin hybrid is used to transform an *S. pyogenes* strain from which the wild-type gene encoding the adhesin has been inactivated (consult the text for details) and which contains an allele of *emm*6.1 (*emm*6.200)[32] in which the 3′ region of the gene has been replaced by a chloramphenicol resistance gene (*cat*86). The initial transformants are propagated at a temperature permissive for replication of the temperature-sensitive replicon. However, on a shift to a nonpermissive temperature, antibiotic resistance can only be maintained by the integration of the plasmid into the chromosome via homologous recombination (illustrated by the X between the plasmid and chromosome). This will now place the hybrid at the *emm*6.1 locus of the chromosome, which has been shown to result in much stronger expression of the *emm*6.1 gene.[22] The chromosome structure of the resulting strain is shown, which can be verified by both Southern blotting (the expected band sizes obtained from a *Pvu*II digest are shown at bottom) and by PCR using primers $P_{3\text{-}3}$ and $P_{5\text{-}3}$ as described in the text.


A
erm
pSC101
pJRS236A
MCS
PstI
emm6.1
PvuII
XbaI
KpnI
HindIII
*KpnI
KpnI
KpnI
PCR amplified
adhesin segment
KpnI
HindIII
KpnI/HindIII
erm
pSC101
pEHX
MCS
PstI
emm/adhesin
hybrid
PvuII
XbaI
HindIII
*KpnI
B
M6.1
Signal
Peptide
A -repeats
B -repeats
C -repeats
Region buried
within
the cell wall
KpnI
HindIII
M6.X
Signal
Peptide
A -repeats
adhesin segment
Region buried
within
the cell wall
KpnI
HindIII
Region exposed on the cell surface

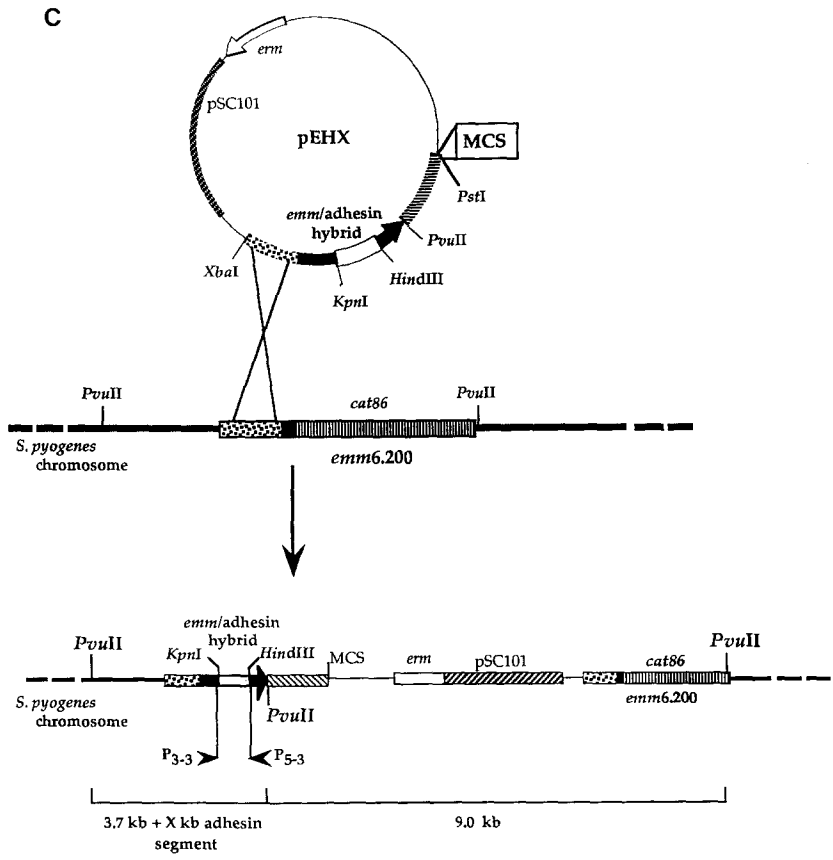


FIG. 3. (*continued*)

are indeed crossed into the chromosome and are not located on a freely replicating plasmid.

Adhesion to Target Cells and Tissues

The methods presented in the preceding sections will result in the production of a number of reagents and mutant streptococcal strains that can be used as powerful tools for the analysis of streptococcal adhesion. As closely as possible, the model of adhesion used in the analyses should resemble the target cells and tissues that are naturally colonized and infected by *S. pyogenes*. The most rigorous analyses of adhesion utilize relevant animal models in which the bacterium infects the animal by a route which resembles the human infection, displays cell and tissue tropism analogous

to the human cells and tissues infected, and produces disease with pathology that is similar to the human infection. Unfortunately, no animal model with all these features has yet been developed for the analysis of streptococcal adhesion and infection. Therefore, it is necessary to evaluate the role of any potential streptococcal adhesin utilizing relevant *in vitro* models of adhesion. Because *S. pyogenes* only infects humans, the most meaningful data are obtained through experimentation on human tissues. As discussed, it should be kept in mind that all *in vitro* models have certain limitations as well as strengths. Thus, it is always desirable to utilize and compare a number of different methods whenever possible.

In evaluation of a known streptococcal protein with regard to adhesion, the wild-type strain should always be compared to the isogenic mutant constructed as described above that no longer can express the potential adhesin. If a difference is observed, the interaction between the streptococcal cell and the host receptor can be examined in greater detail by including the fusion proteins that have been constructed as competitive inhibitors in the adhesion assays, or it can be characterized through evaluation of the strains which express the hybrid adhesin–surface-exposed protein. In the latter case, the hybrid protein should be expressed in a strain which normally does not bind to the target cell under analysis.

Techniques Utilizing Isolated Tissue

Buccal and pharyngeal epithelial cells isolated from healthy human subjects and tonsilar epithelial cells prepared from tissue removed from patients undergoing tonsillectomies for medical indications have frequently been used in the evaluation of streptococcal adhesion. The method presented here refers specifically to buccal epithelial cells; however, it can be applied to the other two cell types with slight modifications.[41] Be sure to comply with all appropriate institutional and federal regulations concerning informed consent when conducting any experiments involving human subjects.

1. For each experiment, buccal cells are obtained from several healthy adult volunteers. The buccal mucosa is wiped with sterile gauze and then scraped twice using sterile wooden tongue depressors.
2. Material from the second scraping is suspended in 10 m*M* phosphate-buffered saline (PBS) (pH 7.4) and then filtered through sterile gauze.
3. The filtered cell suspension is vortexed vigorously for 30 sec and the epithelial cells collected by centrifugation (170 *g*, 3 min, 20°).
4. The buccal epithelial cells are suspended in PBS and washed an additional three times in PBS. The cells are then resuspended in PBS following the final wash to a density of 2×10^5 cells/ml.

[41] M. G. Caparon, D. S. Stephens, A. Olsén, and J. R. Scott, *Infect. Immun.* **59,** 1881 (1991).

5. Streptococci (growth conditions are discussed above) are harvested by centrifugation (5000 *g*, 14°, 5 min), washed once in PBS and adjusted to a density of 2×10^7 bacterial cells/ml in PBS (note that the streptococci will form chains) using a Petroff–Hauser chamber adapted for phase-contrast microscopy. Alternatively, a near-single-cell suspension of the streptococcal strain can be prepared by brief sonication to disrupt the cells from the chains. Subject the washed bacterial suspension to sonication until microscopic examination reveals at least 90% single cells or diplococci. The amount of energy required to obtain disruption of the chains typically does not result in the lysis of the streptococcal cells.

6. Equal volumes of the streptococcal cell and buccal cell suspensions are mixed together in a microcentrifuge tube, which is then rotated end-over-end at 7 rpm for 30 min at 37°. This represents a 100:1 ratio of streptococcal cells to buccal epithelial cells.

7. The mixture is then subjected to centrifugation to remove unattached bacteria. Centrifuge at 170 *g* for 3 min at ambient temperature and carefully remove the supernatant fluids. Suspend the pelleted material in 1 ml of PBS and repeat the centrifugation for a total of three times.

8. The final pellet is resuspended in 0.5 ml PBS and a drop (20 μl) placed on a glass slide and allowed to dry in air. The sample is fixed by immersion in methanol for 30 sec and then subjected to the Gram stain.

9. Bacterial adhesion is then evaluated by light microscopy (magnification, ×1000) and quantiated as the number of streptococcal cells that have attached to at least 30 epithelial cells. The streptococcal cells will appear an intense blue color, whereas the epithelial cells will stain a dull orange. A control includes epithelial cells that were not incubated with streptococci, which should contain fewer than one attached gram-positive bacterium per epithelial cell.

It is highly recommended that adhesion be evaluated by some direct method (e.g., by microscopy) rather than an indirect method for detection of the presence of bacteria (e.g., using radiolabeled streptococci). The reasons for this include the fact that some streptococcal isolates have been reported to adhere as large aggregates.[41] In this case, the adhesin under analysis may not be contributing to adhesion through the recognition of a host cell receptor, but rather through its ability to promote interbacterial interactions. In addition, it also may be the case that different strains exhibit different degrees of cytotoxicity against the epithelial cells.[41] This could lead to a conclusion that the strain under analysis does not adhere, when in fact, the absence of adhesion actually reflects the destruction of the epithelial cells.

A number of important limitations to data obtained from cells isolated from tissue should be considered. Because the cells are heavily colonized by the normal microbiota, it is necessary to use cells from deeper tissue layers in order to obtain uncolonized cells. Thus, the cells are likely to differ from the surface-exposed cells both with regard to the state of differentiation they represent and the degree to which they have interacted with components of oral secretions, both of which may play key roles in interacting with a particular adhesin. Cells isolated from oral tissues are also notoriously heterogeneous, as can be seen by the large variations in size obtained among the cells isolated in the same preparation. These factors may contribute to reports that streptococci adhere in large numbers to certain cells in the epithelial cell population, but do not adhere at all to other cells.[41a]

Techniques Utilizing Cultured Cells

The use of cultured cells can also provide a valuable technique for the evaluation of streptococcal adhesion. Uniformity among the cells in a population, the ability to culture large numbers of cells under reproducible conditions, and the ability to process large numbers of cells simultaneously also make the use of cultured cells attractive. The cultured cells chosen for analysis should closely resemble a target cell from the issue of interest. For this reason, the use of transformed cells is discouraged. Although transformed cells may be easy to manipulate in culture, they are usually considerably altered in the repertoire of receptors that they will express relative to cells present in the native tissue. A more attractive approach is to utilize primary cultures of cells prepared from the tissue of interest. To provide an example of the usefulness of this technique, the use of epidermal tissue will be presented, because the epidermis is an important target tissue of initial adhesion in streptococcal infection of the skin and because the epidermis represents a readily available and manipulable source of material for primary culture.

1. Epidermal tissue is obtained under informed consent from normal individuals who are undergoing tissue reduction surgeries. The underlying fat layer is removed from the tissue, which is then placed dermal side down on a piece of sterile gauze that has been soaked in 0.25% (w/v) trypsin in PBS.

2. After incubation overnight at room temperature, the skin is rinsed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS), 5 U/ml penicillin, and 5 μg/ml streptomycin.

[41a] H. S. Courtney, C. von Hunolstein, J. B. Dale, M. S. Bronze, E. H. Beachey and D. L. Hasty, *Microbial Path.* **12,** 199 (1992).

3. The epidermal sheet is separated from the underlying dermis with sterile forceps and placed in DMEM supplemented with 5% FCS, 5 μg/ml streptomycin, and 25 m*M* HEPES (pH 7.4).

4. The epidermal cells are pipetted up and then expelled from the pipette to break up the clumps of tissue into a single cell suspension.

5. The freshly isolated epidermal cells are cultured in 24-well plates (Costar, Cambridge, MA, Cat. No. 3524) on 12-mm round coverslips (Fisher Scientific, Pittsburgh, PA) coated with a solution of type I collagen (Vitrogen, Flow Laboratories, McLean, VA) that has been diluted in PBS (1:3, v/v). The epidermal cells are plated at a density of 7.5×10^5 cells/cm^2 and are maintained in a humidified atmosphere of 5% CO_2, 95% air (v/v) at 37° for 3 to 4 days before use.

6. For analysis of streptococcal adhesion, the epidermal cells are washed three times in DMEM containing 25 m*M* HEPES, pH 7.4 (DMEM/HEPES) that has been prewarmed to 37°.

7. The *S. pyogenes* strains to be analyzed are cultured as described above, harvested by centrifugation (6000 *g*, 10 min, 14°), and washed once in DMEM/HEPES medium.

8. The final bacterial pellets are resuspended in DMEM/HEPES to densities which represent multiplicities of infection of 100, 10, and 1 streptococcal cell per epidermal cell (see above).

9. A 0.5-ml portion of the bacterial suspension is added to a well of the plate which contains the epidermal cells and incubated at 37° for 2 hr in 5% CO_2, 95% air.

10. To remove nonadherent streptococci, the coverslips are carefully removed from the well using forceps and immersed in PBS in a 200-ml beaker with gentle agitation. This procedure is repeated for a total of 12 consecutive immersions.

11. The washed cells are fixed in methanol at room temperature for 3 min and allowed to dry in air.

12. Adherent streptococci are visualized by staining with gram stain and then examined under a light microscope as described in the preceding section.

An additional advantage of epidermal cells is that their state of differentiation can be readily controlled *in vitro* by alteration of culture conditions, so that the cells can be cultured to resemble cells from more or less differentiated regions of the tissue.[42] However, such well-described techniques for the control of differentiation state of primary cell cultures from other tissues

[42] E. Fuchs, *J. Cell Biol.* **111,** 2807 (1990).

are not always available. Cells in primary culture can lose many of the traits that are characteristic of the cells *in vivo*. In addition to differentiation state, many tissues are also complex collections of different unrelated cell types. Thus, it should be kept in mind that primary cultures may not always be representative of all the cells that may be present in a given tissue.

Techniques Utilizing Fixed Tissue

In situ screening of histological sections provides a powerful technique that allows the evaluation of the role of a streptococcal adhesin in attachment to any cell that is present in any particular tissue. In its simplest form, the method involves overlaying a streptococcal strain onto a thin section of tissue which has been fixed to a glass slide. The slide is then washed and stained to reveal the binding pattern of the bacteria to cells and structures within the tissue. Thus, this technique can be thought of as using the bacteria themselves as histological probes to uncover host receptor specificities within a potential target tissue. The identities of the revealed target cells and/or cellular receptors can be further investigated directly using the tissue sections in combination with other well-described histological techniques, including double labelings with cellular and receptor-specific probes, inhibition assays using purified receptors, and defined biochemical modifications of the tissue.[43,44]

Reagents

10% Buffered formalin: Prepare 4 g NaH_2PO_4 and 6.5 g Na_2HPO_4 and dissolve in 900 ml distilled water. Add 100 ml of 37% formaldehyde (formalin solution; Sigma, St. Louis, MO, Cat. No. F1635)

PBS: Prepare 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, and 0.2 g KH_2PO_4 and add 900 ml distilled water; adjust to pH 7.2 using 50% (w/v) NaOH, and then bring final volume to 1000 ml

Blocking buffer: Add 0.2 g bovine serum albumin (BSA; Sigma, Cat. No. A3350) and 50 μl of Tween 20 (Sigma, Cat. No. P1379) in a final volume of 100 ml PBS

Acridine orange stock solution: Add 1 g acridine orange (Sigma, Cat. No. A6014) to 100 ml distilled water; store in the dark at 4°. The working solution should be prepared fresh daily by adding 50 μl of stock solution to 5 ml of 0.2 *M* acetate buffer (pH 4.0)

[43] P. Falk, T. Borén, D. Haslam, and M. Caparon, *Methods Cell Biol.* **45,** 161 (1994).
[44] P. Falk, T. Borén, and S. Normark, this series, Vol. **236,** 353.

Preparing Paraffin-Embedded Tissue Sections

1. Cut a small block of tissue (3 to 4 mm in thickness).
2. Place in 10% buffered formalin and incubate overnight at room temperature.
3. Follow standard paraffin embedding procedures (consult Luna[45]).
5. Collect 5-μm sections onto clean glass slides.
6. Place in a 60° oven for 30 min.
7. Just prior to use, deparaffinize by two immersions in xylene, for 10 and 3 min, respectively. After deparaffinization, take care not to allow the tissues to dry at any time during an experiment.
8. Rehydrate by three immersions in 100% 2-propanol (3 min each).
9. Rinse in running tap water (5 min) followed by PBS three times (5 min each) in a histology staining jar.

Streptococcal Adhesion to Tissue Sections

1. After blotting the slides, circle the tissue by using grease pencil or PAP pen (Daido Sangyo, Co. Ltd., Japan) and overlay 200 μl of blocking buffer to the tissue section on each slide.
2. Place the slides in a humified chamber and incubate at room temperature for 30 min.
3. The *S. pyogenes* strains from overnight cultures (grown as described above) are harvested by centrifugation (6000 *g*, 10 min, 14°) and washed once in PBS.
4. The bacterial pellets are suspended in blocking buffer, and the density of streptococcal cells is adjusted spectrophotometrically to an OD_{600} of 1.0.
5. A 200-μl portion of the bacterial suspension is overlaid on a tissue section. Incubate at room temperature for 2 hr in a humidified chamber.
6. The slides are washed six times for 5 min each with PBS in a staining jar.
7. Remove the slide from the staining jar and place 200 μl of the acridine orange staining solution on the sections.
8. After incubation for 2 min, wash the slides in three changes of PBS.
9. Mount a coverslip on the tissue using PBS/glycerol (1:1, v/v) and examine the sections under a fluorescence microscope with the appropriate filters to observe emission from acridine orange. The streptococcal cells will appear a bright orange, and nucleated host cells will appear a dull orange. Nonnucleated host structures will appear green.

[45] L. G. Luna (ed.), "Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology," 3rd Ed. McGraw-Hill, New York, 1968.

The advantage of this technique is that, by using tissue sections, the architecture of the intact tissue is preserved, including both cellular and noncellular (e.g., extracellular matrix) structures. Also, all representative cell types will be present, as will cells in all relevant stages of differentiation. A limitation is that the assay employs fixed tissue, so it cannot be assumed that all receptor specificities will be preserved in native conformation during fixation and embedding. However, the use of fixed tissue has proved useful for identification of both glycoconjugate and protein receptors.[46–48] If fixation is a concern, then the assay can be conducted using frozen sections. Another potential problem may concern acridine orange staining. The stain is useful because it reveals both the location of the streptococci within the tissue as well as the architecture of the tissue itself. However, it has been observed that some *S. pyogenes* strains do not stain well with acridine orange. If this proves to be the case, a number of alternative detection methods are available, including conjugating fluorescent dyes to the bacterial surface or using fluorescent dye–antibody conjugate probes. Readers are referred to a review which describes these alternative methods in detail.[43] The assay can also be used to characterize and identify the host cell receptors recognized by the streptococcal adhesin using well-characterized histochemical staining techniques, inhibition assays with purified receptors, and biochemical modifications of tissue. These assays have been described in detail in a review[43] and are not discussed further here.

"Adaptor" Activity

The methods presented in the preceding sections have outlined approaches to evaluate the potential role in adhesion of streptococcal structures which have known binding specificities for different host proteins. However, it has been assumed that the host proteins will be present on the cells and tissues used in the assays that have been described. This may not always be the case, particularly in instances where the streptococci come into contact with the protein in soluble form prior to its interaction with host cells or tissues. For example, this can occur if the organism comes into contact with saliva in the oral cavity or with serum proteins in the bloodstream during severe invasive disease. In this case, by binding a host protein to the surface, the streptococci can acquire specificities for additional host tissues by virtue of the fact that the host tissues themselves can bind the host protein in question while it is present on the streptococcal

[46] P. Falk, K. A. Roth, T. U. Westblom, G. I. Gordon, and S. Normark, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2035 (1993).
[47] I. Ofek, E. H. Beachey, W. Jefferson, and G. L. Campbell, *J. Exp. Med.* **141,** 990 (1975).
[48] N. Okada, A. Pentland, P. Falk, and M. Caparon, *J. Clin. Invest.* **94,** 965 (1994).

cell surface. Thus, the host protein functions as an "adaptor" to bridge a streptococcal adhesin to a host cell receptor, even though the adhesin does not directly recognize the receptor.

Adaptor activity can be screened for by preincubation of the host protein in question with the *S. pyogenes* strain prior to its incubation with host cells in any of the assays described above. Care should be taken that the concentration of host protein used does not lead to aggregation of the streptococci during the course of the assay, which can occur with multimeric host proteins presenting multiple binding domains to the streptococcal cells. As an example of this technique, the *in situ* assay has revealed that *S. pyogenes* binds very poorly to the dermis of the skin, which consists predominantly of collagen- and elastin-containing structures.[48] However, if the streptococci are preincubated with soluble fibronectin, such as they might encounter during infection of a wound, binding to the dermis is significantly enhanced.[48] This presumably occurs via the ability of collagen to bind fibronectin.[49]

Concluding Remarks

This chapter has presented a comprehensive strategy for the evaluation of known streptococcal surface proteins in adhesion to host cells and tissues. Central to the analyses is the construction of defined mutant strains that are defective in expression of the protein under analysis and the comparison of the mutant and wild-type strains in relevant *in vitro* models of adhesion. However, it is possible to use the same techniques to characterize adhesion to a given cell or tissue when the identity of the streptococcal adhesin is unknown. To perform that analysis, the order in which the different techniques are used will be slightly different. The *in situ* assay for adhesion to cells in tissue will be ideal for the establishment of a defined interaction with a specific host cell or tissue. Once the host cell or tissue is identified, further analyses might utilize primary cultures of cells derived from the tissue which are representative of the target cell. Further histological and biochemical characterization of adhesion to the cell in primary culture and the *in situ* assay[43,44] may reveal the identity of the host cell receptor, which can then be utilized to identify a clone expressing the adhesin in a genomic library of streptococcal DNA in *E. coli*.[18] The cloned gene can then be used in the construction of defined mutants as well as fusion and hybrid proteins for further analyses. If the receptor is not identified, then a genetic approach which utilizes Tn*916* transposon mutagenesis can be employed[20] to generate a mutant no longer able to adhere to the target cell. The

[49] R. O. Hynes, and K. M. Yamada, *J. Cell Biol.* **95,** 369 (1982).

transposon-marked gene can be easily cloned into *E. coli* for sequence analysis and the generation of additional mutations and fusion proteins.

As a final note, once a specific interaction between a streptococcal adhesin and a host cell receptor has been identified, further studies directed at understanding the mechanisms by which the streptococcus regulates expression of the adhesin become of interest as it is likely that other virulence determinants required for survival in the host compartment where the receptor is located will be coregulated with the adhesin. These experiments will help reveal the strategies utilized by the microorganism to regulate expression of genes coordinately at defined stages of the infectious process and may lead to the identification of new virulence factors. Thus, the combination of receptor and adhesin identification and gene regulation studies should prove rewarding in understanding the molecular pathogenesis of streptococcal infections, as well as provide essential information for the development of novel therapeutic strategies to combat infection by *S. pyogenes.*

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[24] Bacterial Adhesion to and Penetration of Intestinal Mucus *in Vitro*

By PAUL S. COHEN and DAVID C. LAUX

Introduction

Intestinal epithelial cells are covered by a relatively thick (up to 400 μm) mucus layer consisting of mucin, a 2-megadalton (MDa) gel-forming glycoprotein, and a large number of smaller glycoproteins, proteins, glycolipids, and lipids.[1-4] Presumably, shed epithelial cells are a source of many of the smaller mucus components.[5] The mucus layer itself is in a dynamic state constantly being synthesized and secreted by specialized goblet cells and degraded to a large extent by indigenous intestinal microbiota.[6] Degraded mucus components are shed into the intestinal lumen and are excreted in feces.[6,7]

Bacterial enteropathogens must traverse the mucus layer in order to approach and adhere to intestinal epithelial cells. For this reason, the role of bacteria–mucus interaction in intestinal colonization and pathogenesis has been of interest to many investigators. As a result of several studies, the mucus layer has been implicated in interacting with bacteria in a number of ways. These include involvement of the mucus layer as an initial site for bacterial adhesion, as a protective barrier which the bacteria must penetrate, and as a source of nutrients and matrix for bacterial replication, colonization, and infection.[1,6,7,8] Methods of studying adhesion to and penetration of the mucus layer *in vitro* are the subjects of this chapter.

[1] M. R. Neutra and J. F. Forstner, *in* "Physiology of the Gastrointestinal Tract" (L. R. Johnson, ed.), 2nd Ed., p. 975. Raven, New York, 1987.
[2] Y. S. Kim, A. Morita, S. Miura, and B. Siddiqui, *in* "Attachment of Organisms to the Gut Mucosa" (E. C. Boedecker, ed.), Vol. 2, p. 99. CRC Press, Boca Raton, Florida, 1984.
[3] A. Allan, *in* "Physiology of the Gastrointestinal Tract" (L. R. Johnson, ed.), p. 617. Raven, New York, 1981.
[4] A. Slomiany, S. Yamo, B. L. Slomiani, and G. B. J. Glass, *J. Biol. Chem.* **253,** 3785 (1978).
[5] M. M. Weiser, *in* "Attachment of Organisms to the Gut Mucosa" (E. C. Boedecker, ed.), Vol. 2, p. 89. CRC Press, Boca Raton, Florida, 1984.
[6] L. C. Hoskins, *in* "Attachment of Organisms to the Gut Mucosa" (E. C. Boedecker, ed.), Vol. 2, p. 51. CRC Press, Boca Raton, Florida, 1984.
[7] J. R. Vercelloti, A. A. Salyers, W. S. Bullard, and T. D. Wilkins, *Can. J. Biochem.* **55,** 1190 (1977).
[8] R. Freter and G. W. Jones, *Rev. Infect. Dis.* **5,** S647 (1983).

Isolation of Mucus

Undiluted Intestinal Mucus

After sacrificing the animal, undiluted crude mucus can be obtained from any section of the intestine in the same way. The mouse cecum is used as an example. The cecum is cut away from the rest of the intestine, and any liquid luminal contents are allowed to drain out. Next, the cecum is rinsed with sterile distilled water until the eluate appears free of fecal matter and debris. After the cecum is opened with a pair of scissors, it is rinsed again with sterile distilled water to remove any remaining fecal material and placed in a fresh sterile petri dish, and the wall is gently scraped with a rubber spatula. The procedures are all carried out on ice. Typically, 1 ml of extremely viscous mucus is obtained from 10 animals. Although undiluted mucus cannot be used for adhesion or mucus penetration assays, it can be used to study whether a specific bacterium can utilize mucus for growth. Generation times as short as 35 min have been observed for specific bacteria growing in undiluted intestinal mucus at 37°.[9]

Diluted Intestinal Mucus

Intestinal sections are cleaned and opened as described above, and mucus is scraped from intestinal walls into HEPES–Hanks' buffer (pH 7.4). Contaminating epithelial cells and membranes are removed by centrifugation, once at 12,000 *g* for 10 min at 4° and once at 27,000 *g* for 15 min at 4°. Typically, scraping ceca from six mice (5–8 weeks old) into 5 ml of HEPES–Hanks' buffer (pH 7.4) results in a concentration of about 6 mg mucus protein/ml. HEPES–Hanks' buffer (pH 7.4) contains in grams per liter of water (weight to volume): NaCl, 8.0; KCl, 0.40; $CaCl_2 \cdot H_2O$, 0.05; KH_2PO_4, 0.35; $MgSO_4 \cdot 7H_2O$, 0.20; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2.6. The buffer is adjusted to pH 7.4 with 1 *N* NaOH. Intestinal mucus in HEPES–Hanks' buffer (pH 7.4) can be kept frozen indefinitely (−70°) without loss of receptor activity.

Adhesion to Mucus

The adhesion assay can be broken down into three parts. These are immobilization of the mucus in polystyrene tissue culture wells, growth of the bacteria, and performance of the assay.

[9] B. A. McCormick, B. A. D. Stocker, D. C. Laux, and P. S. Cohen, *Infect. Immun.* **56,** 2209 (1988).

Immobilization of Intestinal Mucus

Routinely, intestinal mucus is diluted to a concentration of 1 mg/ml with respect to protein. Samples (0.2 ml) are added to 24-well polystyrene tissue culture plates (Linbro, Flow Laboratories, McLean, VA, or Nunclon, Nunc Inter Med., Roskilde, Denmark), and the plates are incubated overnight at 4°. After incubation the wells are washed twice with 0.5 ml of HEPES–Hanks' buffer (pH 7.4) to remove unbound mucus components. As a control, plates containing bovine serum albumin (BSA, 1 mg/ml) are prepared. As further controls, immobilized mucus can be treated with BSA for 1 h at 4° to fill empty space in the polystyrene, or wells can be prepared that have only been treated with HEPES–Hanks' buffer (pH 7.4). During the washing procedure, buffer is removed by aspiration using a Pasteur pipet.

Growth of Bacteria

We have chosen to grow bacteria in the presence of radioactive compounds for adhesion assays. At times it may be important to grow the bacteria in a minimal medium and at other times either in a rich medium such as Trypticase soy broth or in intestinal mucus itself. In each case it is possible to achieve labeling levels between 5×10^{-3} and 1×10^{-2} disintegrations/min (dpm) per colony-forming unit (cfu) in *Escherichia coli* by growing the cells in the presence of [*methyl*-^{3}H]thymidine (10 μCi/ml, 120 Ci/mmol). Standing cultures are inoculated at about 5×10^6 cfu/ml and incubated for 18 hr at 37°. The cells are then centrifuged and washed twice at room temperature. Centrifugation is for 5 min at 5000 *g* and cells are washed in an equal volume of HEPES–Hanks' buffer (pH 7.4). Final suspension is in the same buffer at an A_{600} of 0.5 (about 5×10^8 cfu/ml for *E. coli*). *Escherichia coli* growing in either intestinal mucus or in minimal medium can also be labeled in the presence of Tran^{35}S-label, a mixture of [^{35}S]methionine and [^{35}S]cysteine (10 μCi/ml, 1100 Ci/mmol; ICN Biomedicals, Costa Mesa, CA). Tran^{35}S-label is much less expensive than [^{35}S]methionine. For assays involving growth in intestinal mucus, it is important to note that the mucus should be sterilized before inoculation. We have found that exposure to ultraviolet light (254 nm) serves the purpose quite well (15 cm from the 15 Watt source for 10 min). Sterilization by filtration should be avoided because much of the mucus binds to the filters.

Performance of Adhesion Assay

Prewarmed radioactively labeled bacterial cells (0.2 ml) are added to polystyrene wells containing immobilized intestinal mucus, immobilized

BSA, and to wells that have been treated with buffer, but do not contain any immobilized substance (plastic controls). The plates are incubated for 1 hr at 37°, and the cells are then washed twice with HEPES–Hanks' buffer (pH 7.4) to remove unbound bacteria. Adherent bacteria are released by adding 0.5 ml of 0.5% (w/w) sodium dodecyl sulfate (SDS) to each well and then incubating the polystyrene plates for 3 hr at 37°. The SDS is removed from each well, and the level of radioactivity is determined by scintillation counting. All assays are performed in triplicate. In our hands, radioactive bacteria release less than 0.5% of the total incorporated label during the 1-hr incubation period. Furthermore, subsequent incubation of released radioactivity with immobilized components fails to produce significant levels of adherent radioactivity ($\leq$400 dpm). Results are expressed as the mean $\pm$ standard deviation of the mean.

It has been found that many *E. coli* and *Salmonella typhimurium* strains bind significantly better (Student's *t* test) to immobilized mucus than to BSA and plastic.[9–12] In some cases, although adhesion to mucus is found to be far greater than to BSA, adhesion to plastic is found to be greater than adhesion to mucus. In such cases, wells containing immobilized mucus are incubated with BSA for 1 hr at 4° to fill in any possibly exposed plastic and the adhesion assay is repeated. The control wells contain immobilized BSA that has been exposed to BSA again for 1 hr at 4°.

It should be mentioned that the adhesion assay can be performed using nonradioactive bacteria as long as they can survive 0.5% SDS, as is the case with some gram-negative bacteria. In such instances, viable counts can be determined after the adherent bacteria are removed from the mucus, BSA, and plastic.

Additional Applications

Once it is determined that a bacterium binds specifically to mucus, it may become of interest to determine the nature of the mucus receptor. Immobilized mucus can be treated with proteases prior to performing adhesion assays to determine whether the receptors may possibly be proteinaceous. Trypsin and Pronase [1 mg/ml in HEPES–Hanks' buffer (pH 7.4), 0.2 ml per well] are equally effective. Wells are incubated for 1 hr at 37° and are then washed twice with HEPES–Hanks' buffer (pH 7.4). Immobilized mucus treated with BSA instead of a protease serves as the control. Similarly, immobilized mucus can be treated with sodium metaperiodate prior to performing adhesion assays to determine whether the receptors might

[10] D. C. Laux, E. F. McSweegan, and P. S. Cohen, *J. Microbiol. Methods* **2,** 27 (1984).
[11] J. J. Nevola, D. C. Laux, and P. S. Cohen, *Infect. Immun.* **55,** 2884 (1987).
[12] P. L. Conway, A. Welin, and P. S. Cohen, *Infect. Immun.* **58,** 3178 (1990).

contain oxidizable sugars. In this case, wells containing immobilized mucus are treated with 0.4 ml of 10 m*M* sodium metaperiodate in 0.2 *M* sodium acetate buffer (pH 4.5) for 2 hr in the dark at 37°, and are then washed twice with HEPES–Hanks' buffer (pH 7.4). As a control, wells containing immobilized mucus are treated with 10 m*M* sodium iodate, the reduction product of sodium metaperiodate, as described above.

In the event that the receptor is suspected to be a protein or glycoprotein, ^{35}S-labeled bacteria can be used to probe mucus proteins that have been Western blotted following separation by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). In this way, it was shown that *E. coli* F-18, a normal human fecal strain, binds specifically to only two glycoproteins in mouse colonic mucus (50.5 and 66 kDa) and to only three glycoproteins present in mouse cecal mucus (94, 73, and 66 kDa).[13]

As stated above, intestinal mucus contains many lipids. Lipids can also be immobilized in polystyrene wells. In this case, the lipids are dissolved in methanol (500 μg/ml), 0.2-ml aliquots are placed in the wells, the methanol is allowed to evaporate, 0.2-ml aliquots of BSA (1 mg/ml) are added to the wells to fill in any exposed plastic, and the plates are incubated overnight at 4°. Methanol-treated wells containing immobilized BSA serve as controls. Adhesion assays are then performed as described above. In this way it was shown that porcine small intestine mucus contains a neutral lipid receptor (galactosylceramide), specific for K88 fimbriae.[14] Lipids are extracted from intestinal mucus either in chloroform–methanol–water (4:8:3, v/v)[15] or in chloroform–methanol (2:1, v/v)[14] and then separated into neutral and acidic fractions by high-performance liquid chromatography on silica Iatrobeads[16] and by anion-exchange chromatography on DEAE-cellulose,[17] respectively.

Penetration of Mucus Layer

To reach the underlying epithelial cells, bacteria must first traverse the mucus layer. In general, we have found that strains of *E. coli* and *S. typhimurium* which bind to intestinal mucus far better than their parents are poor colonizers of the intestinal tract and have a difficult time traversing a layer of mucus *in vitro*.[9,11,18] The penetration assay can be broken down

[13] E. A. Wadolkowski, D. C. Laux, and P. S. Cohen, *Infect. Immun.* **56,** 1036 (1988).
[14] L. Blomberg, H. C. Krivan, P. S. Cohen, and P. L. Conway, *Infect. Immun.* **61,** 2526 (1993).
[15] L. Svennerholm and P. Fredman, *Biochim. Biophys. Acta* **617,** 97 (1980).
[16] S. Ando, M. Isobe, and Y. Nagai, *Biochim. Biophys. Acta* **424,** 98 (1976).
[17] G. Rouser, G. Kritchevsky, and A. Yamamoto, *in* "Lipid Chromatographic Analysis" (G. Rouser, ed.), Vol. 3, p. 713. Dekker, New York, 1976.
[18] B. A. McCormick, P. Klemm, K. A. Krogfelt, R. L. Burghoff, L. Pallesen, D. C. Laux, and P. S. Cohen, *Microb. Pathog.* **14,** 33 (1993).

into three parts. These are immobilization of intestinal epithelial cells, addition of a layer of mucus, and performance of the penetration assay.

Immobilization of Intestinal Epithelial Cells

Intestinal epithelial cells are prepared by the method of Weiser,[19] and 0.2-ml aliquots (4×10^6 cells/ml) in HEPES–Hanks' buffer (pH 7.4) are added to polystyrene wells. The wells are incubated overnight at 4° and then washed twice with HEPES–Hanks' buffer (pH 7.4).

Addition of Mucus Layer

Intestinal mucus is prepared as described above except that the concentration is adjusted to 4 mg/ml with respect to protein. Aliquots of 0.5 ml are then added to each well, just prior to performing the penetration assay.

Performance of Penetration Assay

Radioactive bacteria in HEPES–Hanks' buffer (pH 7.4) are prepared as described above, and 0.1 ml is gently layered on top of the mucus layer in each well. The plates are incubated at 37°. At desired intervals (e.g., every 2 hr for 8 hr), the mucus layer is gently aspirated from the top down to the epithelial cell–mucus interface, SDS (0.5%, 0.5 ml) is added to each well, the wells are incubated at 37° for 3 hr, and the SDS is then removed from each well. The level of radioactivity is determined by scintillation counting. At each time point, duplicate wells are assayed.

In addition to determining the rate of traversing the mucus layer, it is possible to determine the extent to which the cells that have reached the epithelial cell–mucus interface adhere to the epithelial cells. That is, at each time point, the mucus layer is aspirated from two additional wells, but, prior to adding the SDS, the cells are washed twice with HEPES–Hanks' buffer (pH 7.4) to remove nonadherent bacteria. In this way, we have been able to show that although rough, poor colonizing *S. typhimurium* strains bind to both intestinal epithelial cells and mucus far better than their smooth parent, they fail to traverse the mucus layer *in vitro* and therefore do not bind to the underlying epithelial cells.[9] In contrast, the smooth, good colonizing parent traverses the mucus layer rapidly and subsequently binds to the epithelial cells.[9]

[19] M. M. Weiser, *J. Biol. Chem.* **248,** 2536 (1973).

[25] Identification of Intestinal Receptors for Enterotoxigenic *Escherichia coli*

By EVELYN A. DEAN-NYSTROM

Introduction

The ability to colonize the small intestine is an important virulence attribute of enterotoxigenic *Escherichia coli* (ETEC), bacteria that cause diarrhea in humans and animals. Bacterial adhesins, known as fimbriae or pili, facilitate intestinal colonization by promoting attachment of ETEC to specific receptors on the villous epithelium; they are well-characterized and described in numerous reviews.[1-4] However, little is known about the molecular nature of intestinal receptors to which the fimbriae bind. Until relatively recently, receptors were thought to be glycoproteins or glycolipids because carbohydrate or lectin reagents block fimbriae-mediated adhesion to isolated intestinal epithelial cells, intestinal mucus, tissue or cell cultures, or red blood cells.[1,3] Since the development of immunoblot[5] and overlay techniques[6,7] for identifying fimbriae-specific receptors, there have been numerous descriptions of glycoproteins and glycolipids that bind ETEC fimbriae *in vitro*.[6,8-16] It is important to determine if the putative receptors are relevant in intestinal colonization. Determination of such relevance is

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[2] P. Klemm, *Rev. Infect. Dis.* **7,** 321 (1985).
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[5] E. A. Dean and R. E. Isaacson, *Infect. Immun.* **47,** 98 (1985).
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[7] K.-A. Karlsson and N. Strömberg, this series, Vol. 138, p. 220.
[8] D. C. Laux, E. F. McSweegan, T. J. Williams, E. A. Wadolkowski, and P. S. Cohen, *Infect. Immun.* **52,** 18 (1986).
[9] M. Kyogashima, V. Ginsburg, and H. C. Krivan, *Arch. Biochem. Biophys.* **270,** 391 (1989).
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[12] D. Seignole, M. Mouricout, Y. Dubal-Iflah, B. Quintard, and R. Julien, *J. Gen. Microbiol.* **137,** 1591 (1991).
[13] P. T. J. Willemsen and F. K. de Graaf, *Microb. Pathog.* **12,** 367 (1992).
[14] A. K. Erickson, J. A. Willgohs, S. Y. McFarland, D. A. Benfield, and D. H. Francis, *Infect. Immun.* **60,** 983 (1992).
[15] S. Teneberg, P. T. J. Willemsen, F. K. de Graaf, and K.-A. Karlsson, *FEMS Microbiol. Lett.* **109,** 107 (1993).
[16] D. Payne, M. O'Reilly, and D. Williamson, *Infect. Immun.* **61,** 3673 (1993).

facilitated by the availability of animals that are known to be susceptible to and animals that are known to be resistant to fimbriae-mediated ETEC diarrhea.[17–19] Such animals permit identification of relevant fimbriae-specific receptors that are present and functional in susceptible animals, but absent or nonfunctional in resistant animals.

This chapter summarizes the immunoblot techniques used to identify receptors for 987P fimbriae in porcine small intestines and to demonstrate a correlation between the distribution of intestinal receptors for 987P and age-related susceptibility to 987P-mediated ETEC diarrhea. With some modifications, the methods are applicable for studying other intestinal receptors for other ligands. For example, Erickson *et al.*[14] used similar techniques to identify two porcine intestinal receptors for K88 fimbriae of ETEC and demonstrated that pigs genetically resistant to K88-mediated ETEC diarrhea lack the receptors. Thin-layer chromatogram overlay assays are the subject of another review,[7] and their use for identifying glycolipid receptors for 987P[20] is not discussed here.

Methods

Animals

Neonatal pigs (<3 days old) are susceptible to 987P-mediated ETEC diarrhea, but older pigs (>3 weeks old) are resistant.[19,21,22] Relevant 987P receptors are identified by comparing intestinal receptors for 987P in susceptible and resistant pigs.

Neonatal pigs (<8 hr old) are obtained by cesarean section from crossbred swine, deprived of colostrum, not fed, and euthanized using sodium pentobarbital.[23] Pigs are deprived of colostrum because the presence of maternal antibodies against ETEC 987P fimbriae in the small intestines of suckling pigs interferes with 987P receptor assays. Older pigs are either 3- to 6-week-old piglets or adult pigs (>6 months old). The former are farrowed naturally out of crossbred gilts, weaned at 3 weeks of age, fed a postweaning diet,[24] and euthanized using sodium pentobarbital 24 hr to 3 weeks postweaning. Adult pigs are slaughtered at a local abattoir.

[17] M. J. Kearns and R. A. Gibbons, *FEMS Microbiol. Lett.* **6,** 165 (1979).
[18] R. Sellwood, R. A. Gibbons, G. W. Jones, and J. M. Rutter, *J. Med. Microbiol.* **8,** 405 (1975).
[19] E. A. Dean, S. C. Whipp, and H. W. Moon, *Infect. Immun.* **57,** 82 (1989).
[20] E. A. Dean-Nystrom and J. E. Samuel, *Infect. Immun.* **62,** 4789 (1994).
[21] O. Soderlind, B. Thafvelin, and R. Mollby, *J. Clin. Microbiol.* **26,** 879 (1988).
[22] R. A. Wilson and D. H. Francis, *Am. J. Vet. Res.* **47,** 213 (1986).
[23] H. U. Bertschinger, H. W. Moon, and S. C. Whipp, *Infect. Immun.* **5,** 595 (1972).
[24] J. I. Sarmiento, T. A. Casey, and H. W. Moon, *Am. J. Vet. Res.* **49,** 1154 (1988).

Purification of 987P Fimbriae and Preparation of Anti-987P Serum

Purify 987P fimbriae from 987P$^+$ *E. coli* strain 987[25] by homogenization and selective precipitation with $MgCl_2$, as described by Isaacson and Richter.[26] Determine fimbriae concentration by absorption spectroscopy at 280 nm, using a molar extinction coefficient of 0.337. Prepare antiserum against 987P fimbriae and the immunoglobulin G (IgG) fraction as previously described.[11,19]

Electrophoresis

Separate molecular components of intestinal brush borders, washes, or fractions by electrophoresing through 10 to 20% gradient sodium dodecyl sulfate (SDS)–polyacrylamide minigels (Enprotech, Hyde Park, MA) using the buffer system of Laemmli.[27] Prepare samples for electrophoresis by boiling for 3 min in sample buffer [1:2 with 2× buffer for final concentrations of 62.5 m*M* Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol] prior to electrophoresis. Omit 2-mercaptoethanol from sample treatment buffers because it affects 987P binding to some receptors. Visualize the components by staining with Coomassie blue,[28] PAS (periodic acid–Schiff),[28] or silver[29] (Gelcode; Pierce, Rockford, IL), or electroblot to a solid support for Western blot assay for 987P receptors (see below).

Isolation of Small Intestine Brush Borders

The method for isolating "intact" brush borders from intestinal scrapings is based on the original separation strategy used by Miller and Crane[30] and includes several previously described modifications.[18,24,31] It takes advantage of the relative resistance of brush borders to both osmotic and mechanical forces and consistently yields highly purified preparations of porcine intestinal epithelial cell brush borders.

Reagents

Phosphate-buffered saline (PBS): 8 m*M* Na_2HPO_4, 15 m*M* KH_2PO_4, pH 7.5, 2.7 m*M* KCl, 137 m*M* NaCl

PBS–EDTA: 5.6 m*M* Na_2HPO_4, 8 m*M* KH_2PO_4, pH 6.8, 1.5 m*M* KCl, 96 m*M* NaCl, 10 m*M* EDTA (disodium salt, dihydrate)

[25] B. Nagy, H. W. Moon, and R. E. Isaacson, *Infect. Immun.* **16,** 344 (1977).
[26] R. E. Isaacson and P. Richter, *J. Bacteriol.* **146,** 784 (1981).
[27] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).
[28] G. Fairbanks, T. L. Steck, and D. F. H. Wallach, *Biochemistry* **10,** 2606 (1971).
[29] D. W. Sammons, L. D. Adams, and E. E. Nishizawa, *Electrophoresis* **2,** 135 (1981).
[30] D. Miller and R. K. Crane, *Anal. Biochem.* **2,** 284 (1961).
[31] E. A. Dean and R. E. Isaacson, *Infect. Immun.* **36,** 1192 (1982).

Hypotonic EDTA: 5 m*M* EDTA, pH 7.4 (adjust pH with 500 m*M* Na_2CO_3)

Procedure. Obtain desired segments of small intestines (ileal segments for 987P studies) from pigs at necropsy or immediately after slaughter, then rinse inside and out with cold (4°) PBS–EDTA. Use at least 50 ml per meter intestine to remove contents and debris and discard the rinse. Place rinsed segments in cold PBS–EDTA (segments should be covered by buffer) and keep on ice for 30 to 60 min. Throughout the separation, keep buffers and samples on ice and perform all centrifugations at 4°. Keep segments cold during scraping by working with only about 30 cm (three 10-cm segments) of intestine at a time on a shallow plastic tray on ice. Slit intestines open and wipe the mucosal surface gently with gauze moistened with PBS–EDTA to remove excreta and mucus. Pour 5 to 10 ml of PBS–EDTA over the exposed mucosal surface and scrape the surface gently with the edge of a clean glass slide to remove the villous epithelium. Collect scrapings in PBS–EDTA and pool in centrifuge bottles. Use a starting volume of approximately 500 ml buffer for scrapings from approximately 60 cm of 3-week-old pig intestines or 250 ml buffer for a similar length of neonatal pig intestines. At this point, preparations should contain mostly sheets of columnar shaped epithelial cells with identifiable brush borders (they appear to have halos) when observed by phase-contrast microscopy.

Pellet epithelial cells by centrifugation at 400 *g* (r_{max}) for 10 min. Discard the supernatant. Wash the pelleted cells 1 or 2 times by suspending pellets in the starting volume of cold PBS–EDTA, shaking well (by hand), centrifuging as above, and then decanting the supernatant. The last wash will have a clear supernatant.

Lyse cells by suspending the pellets in the starting volume of hypotonic EDTA and placing on ice for approximately 30 min. Centrifuge at 1000 *g* for 10 min and discard the supernatant. Suspend each pellet in approximately 35 ml of hypotonic EDTA and homogenize in Potter–Elvehjem tissue grinder, 5 strokes at approximately 5000 to 6000 rpm. Dilute the homogenate to the starting volume with hypotonic EDTA, and immediately filter through hard glass wool (Pyrex 8 μm fiber glass, Corning Glass Works, Corning, NY) loosely packed in a 4-inch glass funnel. Monitor the purity of brush borders in the filtrate by phase-contrast microscopy. Brush borders have a distinctive crescent shape and are easily recognized. Repeat filtrations through fresh glass wool until nuclei and cellular debris are no longer seen.

Pellet brush borders by centrifuging the filtrate at 1800 *g* for 10 min. Discard the supernatant. Suspend the pellet in starting volume of PBS, repeat the centrifugation, and resuspend the final pellet to the desired brush border density (typically 10^6 to 10^7 brush borders/ml) in PBS with NaN_3 added to 0.01% (w/v). Quantitate brush borders by phase-contrast micros-

copy using a hemocytometer. For microscopic adhesion assays, store brush borders up to 3 days at 4°; alternatively, add glycerol to a final concentration of 20% (v/v), divide into aliquots, and store longer at −80°. For receptor assays, divide brush borders into aliquots and store at −80°. Stored brush borders have been used up to 5 years after isolation.

Collection of Small Intestine Washes and Isolation of Intestinal Mucus

Small intestine luminal contents may bind 987P fimbriae and prevent attachment of 987P$^+$ bacteria to 987P-specific receptors on intestinal epithelial cells. Intestinal washes are collected according to the method of Elson *et al.*[32] This method is also used in our laboratory for collecting intestinal secretions for antibody determinations.[33] Intestinal mucus is separated from other small intestine contents by size-exclusion chromatography.

Reagents

Soybean trypsin inhibitor solution: 50 μg/ml soybean trypsin inhibitor in 25 m*M* EDTA

Phenylmethylsulfonyl fluoride (PMSF) solution: 100 m*M* PMSF in 95% (v/v) ethanol, prepared fresh on day of use and kept on ice

Phosphate buffer: 100 m*M* sodium phosphate, pH 7.0

10% BSA: 10% (w/v) bovine serum albumin (Sigma, St. Louis, MO) in distilled water

10% NaN_3: 10% (w/v) sodium azide (Sigma) in distilled water

Procedure. At necropsy, ligate one end of a 1- to 2-m segment from the distal half of the small intestine and fill with 50 to 100 ml (>3-week-old pigs) or 10 ml (<1-day-old pigs) of soybean trypsin inhibitor solution, ligate other end of segment, and place on ice for transport to the laboratory. Begin processing within 2 hr of collection. Place the segments at room temperature for 20 min. Collect the intestinal contents and centrifuge at 900 *g* at 4° for 10 min. Harvest the supernatants, note volumes, add 10 μl of PMSF solution per milliliter of supernatant, vortex, and further clarify by centrifugation at 39,000 *g* for 20 min at 4°. Harvest the supernatants, note volumes, and add PMSF (10 μl PMSF solution per milliliter of supernatant) and NaN_3 to 0.01% final concentration (1 μl of 10% NaN_3 per milliliter of supernatant). Let stand at room temperature for 15 min, add bovine serum albumin (BSA) to 0.1% final concentration [10 μl of 10% (w/v) BSA per milliliter of supernatant], divide into aliquots, and store at −80°.

Separate intestinal washes into mucus and nonmucus fractions by chromatography in 0.1 *M* sodium phosphate buffer, pH 7.0, on a Sepharose

[32] C. O. Elson, W. Ealding, and J. Lefkowitz, *J. Immunol. Methods* **67,** 101 (1984).

[33] E. A. Dean-Nystrom, J. I. Sarmiento, and P. L. Runnels, *Immunol. Infect. Dis.* **2,** 263 (1992).

CL-4B column (90 × 2.6 cm, Pharmacia Biotech, Piscataway, NJ)[11,34] at room temperature (10 ml intestinal wash, flow rate of 140 ml/hr, 5-ml fractions). The void volume (first peak) contains intestinal mucus. Pool the void volume fractions and dialyze extensively (50 volumes, 3 changes in 18 hr) against distilled water at 4°. Concentrate the intestinal mucus by drying in a SpeedVac centrifugal vacuum evaporator (Savant Instruments, Farmingdale, NY). Suspend dried mucus in distilled water (usually 1/10 of original mucus pool volume) and dialyze extensively against PBS.

Microscopic Adhesion Assay

Epithelial cells that have receptors for given fimbriae specifically bind bacteria bearing homologous fimbriae; conversely, fimbriate bacteria bind only to specific receptor-bearing cells. This is the basis of the microscopic adhesion assay that is used to differentiate between susceptibility and resistance to K88-mediated ETEC diarrhea in pigs.[18] The assay is also used to identify epithelial cells that have receptors for other ETEC fimbriae and for studying mechanisms of fimbria–receptor interactions.

Reagents

PBS: 8 m*M* Na_2HPO_4, 15 m*M* KH_2PO_4, pH 7.5, 2.7 m*M* KCl, 137 m*M* NaCl

PBS–mannoside: 1% (w/v) α-D-methylmannoside (Sigma) in PBS

Procedure. Perform bacterial adhesion assays as previously described[35] by mixing 987P$^+$ *E. coli* strain 987[25] or 987P$^-$ *E. coli* strain I36[36] [4×10^8 colony-forming units (cfu) in 0.1 ml PBS–mannoside] with small intestine epithelial cell brush borders (2×10^5 brush borders in 0.1 ml PBS–mannoside) and incubating with gentle shaking for 30 min at 37°. Remove nonadherent bacteria by washing 3 times by centrifugation (1000 *g* for 3 min, 5 ml PBS per wash). Remove supernatants carefully by aspiration to avoid loss of brush borders from the pellet and shake tubes gently to suspend pellets. Suspend the final pellet in a drop of PBS and examine by phase-contrast microscopy. Quantitate bacterial adhesion to pelleted brush borders either as the average number of bacteria per brush border (count at least 20) or as the percentage of brush borders that have adherent bacteria.

Assays for 987P Receptor Activity

Materials and Reagents

Nitrocellulose, pore size 0.1 or 0.45 μm (Schleicher and Schuell, Keene, NH)

[34] P. M. Sherman and E. C. Boedeker, *Gastroenterology* **93,** 734 (1987).
[35] E. A. Dean and R. E. Isaacson, *Infect. Immun.* **36,** 1192 (1982).
[36] R. E. Isaacson, B. Nagy, and H. W. Moon, *J. Infect. Dis.* **135,** 531 (1977).

Polyvinylidene difluoride (PVDF), pore size 0.1 or 0.45 μm (Immobilon PVDF transfer membrane, Millipore, Bedford, MA)
TBS (Tris-buffered saline): 10 m*M* Tris, 154 m*M* NaCl, pH 7.4
BSA–TBS: 1% (w/v) bovine serum albumin (A7030, Sigma) in TBS
4-Chloro-1-naphthol substrate: 0.5 mg/ml 4-chloro-1-naphthol, 0.015% (w/v) H_2O_2, and 16.7% (v/v) methanol in TBS, prepared just before use

Slide Agglutination Assay. Multivalent receptors for 987P cause visible agglutination of 987$^+$ bacteria and can be detected by slide agglutination.[35] This provides a convenient assay for following 987P receptor activity during purification procedures and monitoring effects of various treatments on 987P receptor activity. However, it is important to remember that loss of agglutinating activity can result either from destruction of receptors or from conversion of multivalent receptors to a monovalent state. It is possible to distinguish the two effects by additional assays for 987P receptor activity, such as the filter blot assay described below.

Mix one drop of the intestinal material being tested for 987P receptors with one drop of 987P$^+$ *E. coli* strain 987[25] or 987P$^-$ *E. coli* strain I36[36] (10^8 to 10^9 cfu/ml in PBS) on a glass slide. Agglutination is visible within 30 to 60 sec. Always include a diluent control because 987P$^+$ bacteria are agglutinated by extreme pH or salt concentrations.[35]

Filter Spot Assay and Western Blot Assay for 987P Receptors. Receptors specific for 987P fimbriae retain binding activity when immobilized by spotting or electroblotting onto solid supports such as nitrocellulose or PVDF membranes. The blots can be assayed for ligand binding by incubation with purified 987P fimbriae followed by detection of bound fimbriae using enzyme-linked immunoassays.[5,11] The filter spot assay is used to determine optimal binding conditions, identify fractions that contain 987P receptors, and monitor effects of various treatments on 987P receptor activity. The Western blot assay is used to identify specific intestinal components that bind 987P and to monitor receptor purification.

The first step is sample application. For filter spot assays, spot 10 to 20 μl of test material directly onto nitrocellulose or PVDF membranes, or apply larger volumes of dilute samples onto membranes using a filtration blotting system (e.g., dot blotter or slot blotter) and remove membranes from the apparatus before further use. For Western blot assays, first separate test material by SDS–polyacrylamide gel electrophoresis and then electroblot onto nitrocellulose or PVDF membranes by the method of Towbin.[37] Prepare at least two sets of test samples: one for 987P binding (test assay) and one for nonspecific binding of immunoreagents (control assay). Prepare additional sets of samples as needed to titrate reagents

[37] H. Towbin, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350 (1979).

or test other variables. To avoid denaturation of receptors, do not allow membranes to dry between sample application and the blocking step. If blots cannot be tested within 24 hr, wash membranes in TBS, sandwich wet membranes between blotting filters (Gel Blot Paper, GB 003, Schleicher and Schuell, Keene, NH) soaked in TBS, and store at −20° (2 weeks is the longest we have stored filters).

For blocking, incubate membranes containing samples in BSA–TBS for 16–18 hr at 4° or for 1 hr at room temperature to saturate unoccupied protein binding sites on membranes. Wash membranes 3 times for 10 min each with BSA–TBS between all subsequent incubations, except where noted.

The next step is 987P binding. Perform subsequent steps at room temperature. Cut membranes into strips or sections for test and control assays. Incubate test membranes with purified 987P (100 μg/ml in BSA–TBS) and control membranes with BSA–TBS for 1 hr. Mix incubation chambers on a rocking or rotary mixer at room temperature with gentle agitation during all incubations and use moderate agitation during washing steps.

Evaluate anti-987P binding. Treat test and control membranes identically for the remainder of the assay. Incubate membranes for 1 hr with rabbit anti-987P IgG (2.5 μg/ml in BSA–TBS).

For antibody localization, incubate membranes for 30 min with a 1:200 dilution of biotinylated affinity-purified anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in BSA–TBS. Wash, as above, with BSA–TBS. Next, incubate membranes for 30 min with a solution of avidin and biotinylated horseradish peroxidase (Vectastain ABC reagent, Vector Laboratories) in BSA–TBS. Wash membranes three times for 10 min each with TBS. Incubate for 1 hr with 4-chloro-1-naphthol substrate. Decant the substrate, immerse the membrane in 100 ml distilled water for 5 min to stop the reaction, and blot dry. The 987P-specific binding is identified by the appearance of purple bands that are seen on the test filter incubated with 987P fimbriae but not on the control filter, for which that incubation is omitted.

Results and Discussion

Identification of Porcine Intestinal Receptors for 987P

Figure 1 shows 987R and 987M, two porcine small intestine receptors for 987P, identified by Western blot assay.[11] Brush borders from both susceptible (lane A, Fig. 1) and resistant pigs (lane B, Fig. 1) contain 987R, a complex of 987P-binding components with molecular masses ranging from 33 to 40 kDa. Intestinal washes (lane D, Fig. 1) and intestinal mucus from

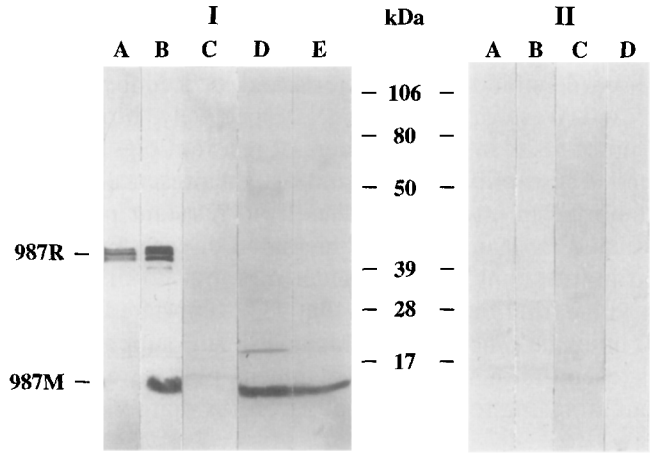

FIG. 1. Demonstration of porcine small intestine receptors for 987P fimbriae by Western blot assay. (I) Western blot on Immobilon membrane showing the 987P receptors (987R, 30–40 kDa) in small intestine epithelial brush borders isolated from 987P-susceptible (lane A) and 987P-resistant pigs (lane B) and the 987P receptors (987M, <17 kDa) in small intestine brush borders (lane B), washes (lane D), and mucus (lane E) from resistant pigs, but not in brush borders (lane A) or intestinal washes (lanes C) from susceptible pigs. (II) The 987P receptors were not detected when incubations with 987P were omitted. Assays were performed as described in text. Wells contained approximately 15 (lane A), 25 (lane B), 20 (lane C), 3 (lane D), or 0.4 (lane E) μg protein.

resistant pigs (lane E, Fig. 1) contain 987M, a less than 17-kDa receptor for 987P. However, 987M is not detected (lane C, Fig. 1) or occurs only in trace amounts (not shown) in intestinal washes from susceptible pigs. Variable amounts of 987M are also found in brush border preparations from resistant pigs (lane B) but not susceptible pigs (lane A).

It is difficult to determine the best way to standardize intestinal preparations from pigs for comparison of 987P receptors. Intestinal washes from resistant (older) pigs contain more carbohydrate relative to the amount of protein than do washes from susceptible (neonatal) pigs. However, the age-related differences in the distribution of 987P receptors are similar to those shown in Fig. 1 whether samples are adjusted on the basis of protein or carbohydrate concentrations.[11]

Correlation of 987P Receptors with Susceptibility to 987P-Mediated Adhesion

Neonatal pigs are susceptible to 987P-mediated ETEC diarrhea, but older pigs are resistant.[18,21,22] The 987P$^+$ *E. coli* strains closely associate with intestinal epithelium when inoculated into ligated loops in susceptible pigs. When similarly inoculated into resistant pigs, 987P$^+$ *E. coli* associate

with intestinal mucus and debris, but not with the epithelium.[19] Resistant pigs clearly have functional 987P receptors in their brush borders because their brush borders bind 987P$^+$ bacteria and 987P fimbriae in microscopic adhesion[19] and Western blot assays,[11] respectively. Moreover, the brush border receptor 987R in brush borders of resistant pigs has a similar mass as that found in susceptible pigs. In contrast, the presence of 987M receptors for 987P fimbriae in intestinal washes from resistant pigs does correlate with age-related resistance to 987P-mediated disease. Furthermore, 987M is localized in intestinal mucus of older pigs, but not in neonates. This is consistent with earlier observations that 987P-fimbriated bacteria associate with small intestine mucus in resistant pigs. This supports the hypothesis that 987P receptors in the intestinal mucus bind to and prevent 987P$^+$ ETEC from attaching to 987P receptors on the epithelial cell surface.

[26] Methods for Studying Adhesion of Diarrheagenic *Escherichia coli*

By MICHAEL S. DONNENBERG and JAMES P. NATARO

Introduction

Although predominantly a commensal species, *Escherichia coli* includes certain strains endowed with virulence factors that allow them to cause diarrhea. There are several distinct mechanisms by which pathogenic *E. coli* may induce enteric disease. Thus, various categories of diarrheagenic *E. coli* are distinguished (summarized in Table I). Strains from several categories adhere to tissue culture cells in distinctive patterns that can be considered pathognomonic of the class to which they belong. However, it should be noted that definitive assignment of pathogenetic class is best achieved by examination for virulence phenotypes. For example, enterotoxigenic and enterohemorrhagic strains are identified by assays for toxin production, and electron microscopy is used to identify the ultrastructural markers on cells characteristic of enteropathogenic and enterohemorrhagic strains. For many of the categories, virulence genes have been cloned and DNA probes are available for accurate diagnosis.[1] It should also be noted that rare strains may possess more than one virulence phenotype and therefore resist categorical assignment. The purpose of this chapter, however,

[1] S. M. Faruque, K. Haider, M. J. Albert, Q. S. Ahmad, A. N. Alam, S. Nahar, and S. Tzipori, *J. Med. Microbiol.* **36,** 37 (1992).

with intestinal mucus and debris, but not with the epithelium.[19] Resistant pigs clearly have functional 987P receptors in their brush borders because their brush borders bind 987P$^+$ bacteria and 987P fimbriae in microscopic adhesion[19] and Western blot assays,[11] respectively. Moreover, the brush border receptor 987R in brush borders of resistant pigs has a similar mass as that found in susceptible pigs. In contrast, the presence of 987M receptors for 987P fimbriae in intestinal washes from resistant pigs does correlate with age-related resistance to 987P-mediated disease. Furthermore, 987M is localized in intestinal mucus of older pigs, but not in neonates. This is consistent with earlier observations that 987P-fimbriated bacteria associate with small intestine mucus in resistant pigs. This supports the hypothesis that 987P receptors in the intestinal mucus bind to and prevent 987P$^+$ ETEC from attaching to 987P receptors on the epithelial cell surface.

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TABLE I
CATEGORIES AND STRAINS OF *Escherichia coli* USED TO TEST ADHESION

Category	Clinical syndrome	Representative strain[a]	Adhesion phenotype
Enteropathogenic (EPEC)	Infantile diarrhea in developing countries	E2348/69	Localized adhesion, attaching and effacing
Enterohemorrhagic (EHEC)	Hemorrhagic colitis and hemolytic–uremic syndrome	EDL933	Poor adhesion, attaching and effacing
Enterotoxigenic (ETEC)	Endemic and travelers' diarrhea	H10407	Inconsistent
Enteroinvasive (EIEC)	Diarrhea and dysentery	E134	Cellular invasion without preliminary adhesion
Enteroaggregative (EAggEC)	Acute and persistent diarrhea in developing countries	JM221	Aggregative adhesion
Diffuse adherent	Childhood diarrhea in developing countries?	C1845	Diffuse adhesion
Normal microbiota	None	HS	Nonadherent

[a] Strains are available by request from Dr. James P. Nataro, Center for Vaccine Development, University of Maryland School of Medicine, 10 S. Pine Street, Baltimore, MD 21201.

is to provide methods by which many diarrheagenic *E. coli* can be categorized by adhesion patterns. It is first necessary, however, to define briefly the categories of diarrheagenic *E. coli* currently recognized.

Categories of Diarrheagenic Escherichia coli

Strains in the first category of *E. coli* to be linked with diarrhea were designated enteropathogenic (EPEC).[2] These strains were the etiologic agents of devastating nosocomial and community-acquired outbreaks of diarrhea among neonates and young infants. Although no longer a common cause of diarrhea in developed countries, EPEC strains remain a leading cause of infantile diarrhea in developing nations worldwide. EPEC strains are associated with two distinct and well-recognized effects on tissue culture cells. They adhere to the cells via a plasmid-encoded bundle-forming pilus in densely packed, circumscribed microcolonies, forming a pattern termed "localized adhesion." In addition, chromosomal factors enable the bacteria to become intimately attached to cells, efface microvilli, and activate a

[2] M. S. Donnenberg and J. B. Kaper, *Infect. Immun.* **60,** 3953 (1992).

signal transduction cascade that results in the deposition of cytoskeletal elements, including filamentous actin, directly beneath the adhering organisms. This second step is known as the "attaching and effacing" effect. Assays for both localized adhesion and attaching and effacing are described in this chapter. Adhesion to HEp-2 cells (human epidermoid carcinoma cells) in a localized pattern is both sensitive and specific for EPEC strains.

The enterohemorrhagic *E. coli* (EHEC) cause outbreaks and sporadic cases of hemorrhagic colitis and the hemolytic–uremic syndrome.[3] Although they do not adhere in a localized pattern to tissue culture cells and do not produce the EPEC fimbriae, they share with EPEC the genes and capacity to induce the attaching and effacing effects in tissue culture. The most significant difference between EHEC and EPEC is the production by the former of high levels of Shiga-like toxins, which are presumed to be instrumental in causing the clinical syndromes associated with EHEC infection.[4]

Enterotoxigenic *E. coli* (ETEC) strains cause watery diarrhea in developing countries and are among the principal agents of travelers' diarrhea.[5] These strains produce a variety of fimbrial and nonfimbrial adhesins known as colonization factor antigens. Although some of the adhesins are associated with hemagglutination reactions, adhesion to tissue culture cells is variable and thus is not a reliable method of identifying ETEC. Diarrhea results from the production of heat-labile or heat-stable enterotoxins that cause elevations in cyclic nucleotide levels leading to net electrolyte and fluid secretion by enterocytes.

The enteroinvasive *E. coli* (EIEC) are close relatives of *Shigella* spp. and share with them many phenotypic and genotypic virulence traits.[6] Like shigella, the strains often cause watery diarrhea, but they have the capacity to cause dysentery. They invade cells in tissue culture with high efficiency and escape the phagosomal vacuole to spread within and between cells without leaving the intracellular environment. Invasion proceeds almost immediately on contact with cells, without an easily recognized preliminary adhesion phase.

Enteroaggregative *E. coli* (EAggEC) strains are defined solely by the HEp-2 cell adhesion pattern, which consists of a distinctive "stacked brick" mosaic of bacteria often adhering to the glass as well as the target cells.[7] Work is shedding light on both the epidemiology and pathogenesis of the

[3] P. M. Griffin and R. V. Tauxe, *Epidemiol. Rev.* **13,** 60 (1991).
[4] V. L. Tesh and A. D. O'Brien, *Mol. Microbiol.* **5,** 1817 (1991).
[5] M. M. Levine, *J. Infect. Dis.* **155,** 377 (1987).
[6] R.-C. Hsia, P. L. C. Small, and P. M. Bavoil, *J. Bacteriol.* **175,** 4817 (1993).
[7] J. P. Nataro, J. B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M. M. Levine, *Pediatr. Infect. Dis. J.* **6,** 829 (1987).

infection. The presence of EAggEC is associated with prolonged diarrhea in children,[8] which in turn is an important factor in malnutrition and mortality. Adhesion is associated with plasmid-encoded fimbriae, which are reminiscent of but distinct from those produced by EPEC.[9] In addition, a heat-stable enterotoxin has been described in some strains.[10] It is not yet clear whether the strains represent a unique category of diarrheagenic agent, or whether a heterogeneous group of organisms share a common adhesion phenotype.

The final category of diarrheagenic *E. coli* considered here are those exhibiting diffuse adhesion to epithelial cells (DAEC). It is almost certain that this category comprises a heterogeneous collection of strains that do not belong to any of the other categories. Indeed, at least two distinct factors conferring the diffuse adhesion phenotype have been cloned,[11,12] and many strains that exhibit the pattern possess neither. Thus the strains are also defined by their HEp-2 cell adhesion pattern. Several epidemiological studies have failed to show an association between identification of strains exhibiting diffuse adhesion and diarrhea, and volunteer studies have not verified their virulence.[13] However, a few reports, especially those in which adhesion patterns of *E. coli* from older children with and without diarrhea were examined, have described a significant association with disease.[14]

The term "enteroadherent *E. coli*" was coined to describe strains that attach to tissue culture cells but do not belong to serotypes often associated with EPEC. We do not favor this term, as it embraces strains that exhibit localized, aggregative, as well as diffuse adhesion. Such strains are more accurately placed in the categories associated with these phenotypes.

HEp-2 Cell Adhesion Assay

The assay that follows is based on that originally reported by Cravioto *et al.*[15] A large number of epidemiological studies have used this assay or

[8] M. K. Bhan, P. Raj, M. M. Levine, J. B. Kaper, N. Bhandari, R. Srivastava, R. Kumar, and S. Sazawal, *J. Infect. Dis.* **159,** 1061 (1989).

[9] J. P. Nataro, D. Yikang, J. A. Giron, S. J. Savarino, M. H. Kothary, and R. Hall, *Infect. Immun.* **61,** 1126 (1993).

[10] S. J. Savarino, A. Fasano, J. Watson, B. M. Martin, M. M. Levine, S. Guandalini, and P. Guerry, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 3093 (1993).

[11] I. Benz and M. A. Schmidt, *Infect. Immun.* **57,** 1506 (1989).

[12] S. S. Bilge, C. R. Clausen, W. Lau, and S. L. Moseley, *J. Bacteriol.* **171,** 4281 (1989).

[13] C. O. Tacket, S. L. Moseley, B. Kay, G. Losonsky, and M. M. Levine, *J. Infect. Dis.* **162,** 550 (1990).

[14] C. Jallat, V. Livrelli, A. Darfeuille-Michaud, C. Rich, and B. Joly, *J. Clin. Microbiol.* **32,** 2031 (1993).

[15] A. Cravioto, R. J. Gross, S. M. Scotland, and B. Rowe, *Curr. Microbiol.* **3,** 95 (1979).

modifications of it to examine the role of *E. coli* in diarrhea. Among the variations on the assay reported are the substitution of HeLa or other cells for HEp-2, modifications in the tissue culture medium or wash buffers, the use of chamber slides in lieu of coverslips, and variations in the incubation periods. Of these, we have found only the incubation time to have a substantial effect on results (see below). The procedure in use for many years at the Center for Vaccine Development is described.

Procedure

The adhesion assay is performed by incubating fresh bacterial cultures with a monolayer of HEp-2 cells (ATCC, Rockville, MD, CCL 23), then fixing and staining the monolayer and observing the pattern of bacterial adhesion.

1. HEp-2 cells are grown in Eagle's minimal essential medium according to standard tissue culture methods. Cells are grown in 24-well tissue culture dishes containing 15-mm glass coverslips. We use cells at approximately 50% confluence. This may be achieved by inoculating $1–2 \times 10^2$ cells per well and incubating for 48 hr.
2. Bacteria are grown in Luria–Bertani (LB) broth without shaking at 37° for 12–18 hr.
3. Spent tissue culture medium is aspirated aseptically from the wells, and cells are washed three times with 1 ml sterile Hanks' balanced salt solution.
4. One milliliter of fresh Eagle's minimal essential medium with 1% (w/v) mannose (or, alternatively, 1% α-methylmannoside) is added to each well. Then 20 μl of bacterial culture is added to each well, and plates are incubated at 37° under 95% air, 5% CO_2 (v/v) for 3 hr.
5. After incubation, the medium is aspirated from each well and coverslips are washed four times with phosphate-buffered saline (PBS) (pH 7.3).
6. Cells are fixed by incubation with 1 ml of 70% (v/v) methanol for 5 min at room temperature.
7. Methanol is aspirated, and cells are stained by the addition of 1 ml of 10% Giemsa stain for 15 minutes. Results are improved if the stain is passed through filter paper prior to use.
8. After staining, the wells are rinsed with distilled water until washes are colorless. Coverslips are removed from the wells and air dried.
9. Coverslips are mounted on glass slides using a permanent mounting resin (e.g., Cytoseal 60, Stephens Scientific, Riverdale, NJ) and examined under oil immersion.

As an alternative to using coverslips, the protocol can be performed with 8-well chamber slides (Nunc, Naperville, IL), which can be examined directly under light microscopy after staining and mounting. For the chamber slide technique, all volumes are 0.3 ml instead of 1 ml, and the bacterial inoculum is 10 μl.

Interpretation

Interpretation of the assay requires some experience and the inclusion of controls with each series of experiments (Table I). Nonadherent bacteria are occasionally found as individual organisms, with typically fewer than three bacteria per cell; few bacteria adhere to the glass coverslip. In localized adhesion, large microcolonies are seen on the surface of the HEp-2 cells (Fig. 1A). The appearance of the microcolonies is quite characteristic, as they project a three-dimensional structure that can be appreciated by changing focus. It is generally difficult to discern single bacteria in the center of the mass. Few bacteria are seen on the glass coverslip free from the HEp-2 cell. Diffuse adhesion is characterized by dispersion of bacteria over the surface of the HEp-2 cell; little aggregation is seen, and there are essentially no microcolonies (Fig. 1B). Few if any bacteria are seen adhering to the glass coverslip. Aggregative adhesion is the most difficult and heterogeneous phenotype. The *sine qua non* of the pattern is the "stacked brick" autoagglutination of the bacteria (Fig. 1C). The organisms can be seen adhering on the surface of the cell, on the glass coverslip, or both. Regardless of the site of adhesion of the aggregate, however, the same stacked pattern is seen.

The optimal time for coincubation of bacteria and HEp-2 cells has been a matter of some controversy. Several investigators have recommended lengthening the incubation to 6 hr or performing a 3-hr incubation but with a change of the medium after the fiirst hour. Vial *et al.* compared the 3-hr incubation protocols with and without the change of medium after 1 hr. These investigators determined that changing the medium after 1 hr of incubation resulted in an inability to discern the aggregative phenotype.[16] The 6-hr incubation protocol has been shown to permit detection of EHEC and attaching and effacing bacteria which do not display typical localized adhesion.[17] The epidemiological significance of such bacteria, however, has not been established.

[16] P. A. Vial, J. J. Mathewson, H. L. DuPont, L. Guers, and M. M. Levine, *J. Clin. Microbiol.* **28,** 882 (1990).

[17] S. Knutton, A. D. Phillips, H. R. Smith, R. J. Gross, R. Shaw, P. Watson, and E. Price, *Infect. Immun.* **59,** 365 (1991).

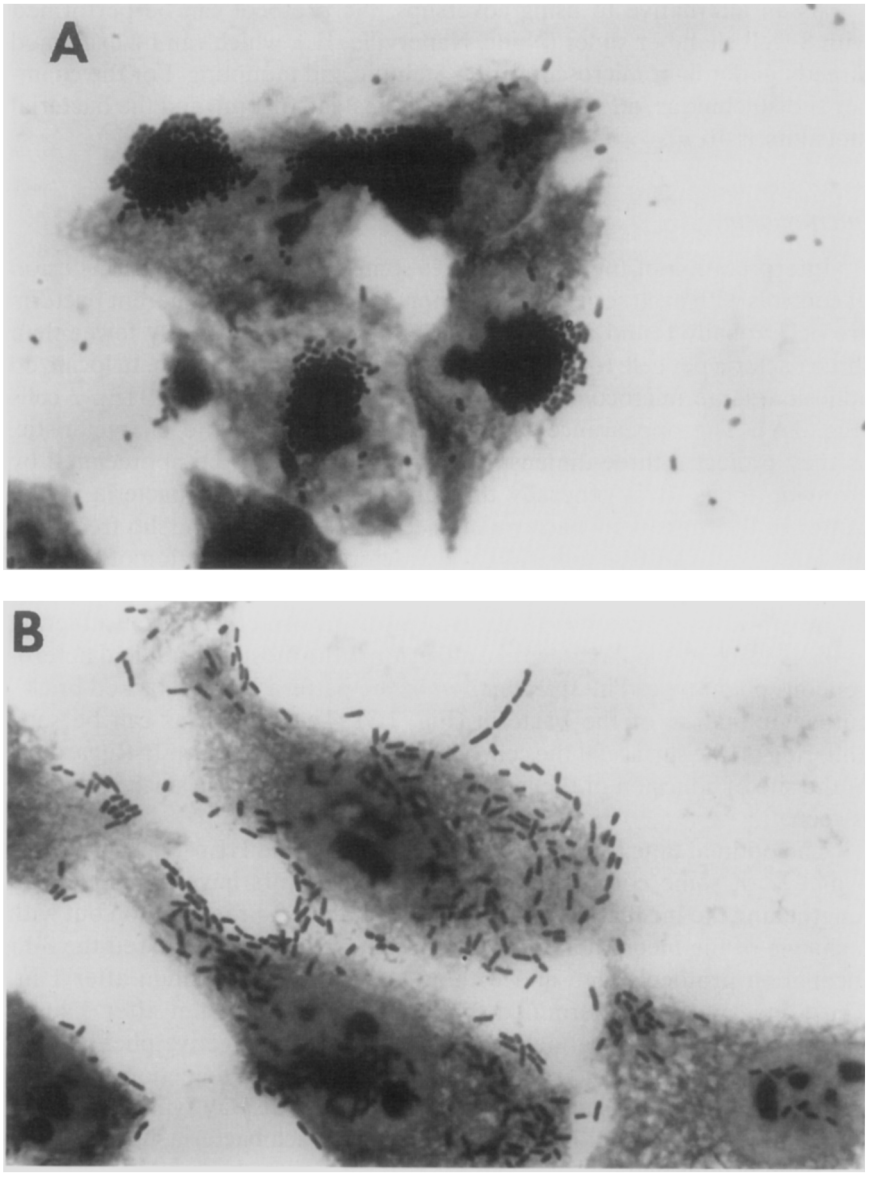

FIG. 1. Representative results obtained in the HEp-2 cell adhesion assay. In localized adhesion by enteropathogenic strains (A), bacteria adhere as discrete three-dimensional microcolonies. Diffuse adherent strains (B) cover the surface of the HEp-2 cells randomly. Aggregative strains (C) form characteristic two-dimensional arrays on cells, on slides, or both. Although the bacteria may form patterns resembling microcolonies, the "stacked brick" appearance is still evident.

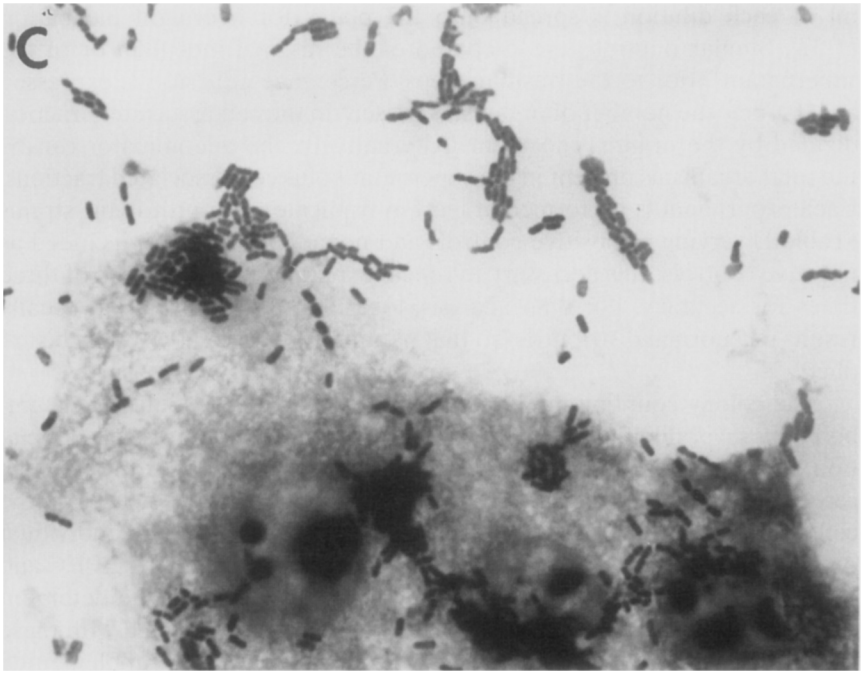

FIG. 1. (*continued*)

Quantitating HEp-2 Cell Adhesion

The adhesion assay described above was designed for categorization of *E. coli* into different classes and hence is, by definition, qualitative in nature. Nevertheless, it may be useful in certain circumstances to determine the amount as well as the type of adherence demonstrated by particular isolates. This may be accomplished by several methods, which are outlined below.

Colony Counting

The basic adhesion assay may be modified such that adherent organisms are recovered for enumeration by plate dilution. In this modification, the fixation and staining steps are replaced by serial dilutions for colony counting. Following the incubation and washing steps, 0.200 ml of 1% (v/v) Triton X-100 is added to each well or chamber to lyse epithelial cells and release adherent and intracellular bacteria. After incubation at room temperature for 20 min, the samples are aspirated into 10 × 75 mm test tubes and subjected to vigorous agitation with a vortex mixer to break up aggregates. Serial 10-fold dilutions of 0.100 ml in 0.900 ml PBS are made, and 0.100

ml of each dilution is spread onto LB plates for overnight incubation at 37°. Similar dilutions are prepared of the original inoculum or of the supernatant prior to the washing steps. Percentage adhesion is expressed as 100 times the number of organisms present in the cell-associated fraction divided by the original inoculum. Alternatively, the denominator can be the total organisms present in the supernatant plus cell-associated fractions. Each experiment is performed at least in triplicate, with prototypic strains (Table I) serving as positive controls and nonadherent strains included as negative controls. It is necessary to repeat experiments a minimum of three times for accuracy. For data analysis, logarithmic transformation usually results in a normal distribution so that parametric statistical tests can be applied.

The colony counting method gives reproducible data, but it is encumbered by several limitations. First, the assay actually measures cell association, not adhesion. For organisms such as the EIEC, most of the bacteria recovered may be intracellular rather than adherent to the surface of the cells. Second, the assay quantifies colony-forming units (cfu), not individual organisms. Thus it is especially problematic for assays involving EPEC and EAggEC, which form clusters that may not dissociate with the detergent treatment, and therefore spuriously low values may be expected with these organisms. Thus the colony count method should always be supplemented by direct counting by visual inspection, as follows.

Visual Inspection

In this application, the specimens are prepared exactly as for the HEp-2 cell adhesion assay, but adherent bacteria are counted under the microscope.[18] For each specimen, one to two hundred consecutive epithelial cells are examined under oil immersion (100× objective) and the following data are recorded: the number of epithelial cells with adherent bacteria, the number of clusters present on each cell (for EPEC and EAggEC), and either the numbers of bacteria within each cluster (for EPEC and EAggEC) or the numbers adherent to each cell (for other categories). For large or densely packed clusters, it is sometimes impossible to obtain an accurate number; a maximum value, such as greater than 50 organisms, may be employed. In these cases, it may be preferable to repeat the assay with a lower inoculum or a shorter incubation period. Data can be expressed three ways: as percentage of epithelial cells with bacteria, as total number of bacteria per 100 epithelial cells, and (for EPEC and EAggEC) as mean bacteria per cluster. Use of all three analyses yields the most information. Experiments are repeated several times.

[18] M. S. Donnenberg, J. A. Girón, J. P. Nataro, and J. B. Kaper, *Mol. Microbiol.* **6,** 3427 (1992).

This method has the advantage of reducing underestimation of adhesion caused by cluster formation, although this problem persists because of the inherent difficulty of accurately counting large clusters. An additional disadvantage of the method is the tendency for the eye to be drawn toward cells with more bacteria. Constant vigilance must be maintained to examine every cell within a field to reduce this bias.

Other Methods

Additional methods may be employed to quantify *E. coli* adhesion. Methods employing metabolic labeling with radioisotopes may be adapted from other systems (detailed elsewhere in this volume). In addition, quantification based on β-galactosidase activity of the adherent organisms has been reported.[19] Although the assay is appealing because of the expected direct correlation between enzyme activity and number of organisms present (regardless of whether the organisms are present in clusters), we have found that it is not sufficiently sensitive to quantify poorly adherent isolates (M. S. Donnenberg and J. P. Nataro, unpublished data).

Assays for Attaching and Effacing Phenomenon

The definitive assay for the ability of EPEC and EHEC strains to destroy microvilli, become intimately attached to the cell membrane, and induce the characteristic cytoskeletal reorganization recognized as the attaching and effacing effect involves incubation of the bacteria with human intestinal tissue explants and examination by transmission electron microscopy. Fortunately, Knutton *et al.* have described an alternative procedure that seems to correlate perfectly with the results obtained by electron microscopy, and which is much more accessible to most laboratories.[20] The assay, termed the fluorescence actin staining (FAS) test, takes advantage of the fact that filamentous actin is intensely concentrated directly subjacent to bacteria causing the attaching and effacing effect. Fluorescein isothiocyanate (FITC)–phalloidin, a conjugate of a toxin isolated from the poisonous mushroom *Amanita phalloides,* specifically binds filamentous actin and is used as a fluorescent probe to detect the lesions. Although many bacteria cause transient accumulations of filamentous actin on entry into epithelial cells, the pattern produced by EPEC and EHEC is quite characteristic and far brighter than that seen with other bacteria. Furthermore, the fluorescence seen with EPEC and EHEC is so tightly focused as to give the impression that the bacteria themselves are stained. The assay requires

[19] J. Minami, A. Okabe, and H. Hayashi, *Microbiol. Immunol.* **31,** 851 (1987).
[20] S. Knutton, T. Baldwin, P. H. Williams, and A. S. McNeish, *Infect. Immun.* **57,** 1290 (1989).

access to a microscope equipped with epifluorescence and phase-contrast optics. Care must be taken in handling the FITC–phalloidin, as it is a potent toxin. Gloves should be worn when handling the specimens.

The tissue culture cells to be used may be HeLa, HEp-2, or human embryonic lung (HEL 229) cells (ATCC CCL 137). We have also observed a positive response in a variety of other cell lines. HEL cells seem to give the most dramatic results because they spread thinly, reducing depth-of-field problems. Growth of the cells, growth of the bacteria, inoculation, coincubation, and washing are performed exactly as for the adhesion assay, except that mannose should not be added to the tissue culture medium. Positive and negative controls are included in each assay. Either coverslips or chamber slides may be employed, although the latter are easier to use. Incubation may be for 3 or 6 hr with a change in medium after 3 hr. The longer time is essential for poorly adherent organisms such as EHEC. After incubation and three washes with PBS, the cells are fixed for 20 min at room temperature with 3% (v/v) formalin, washed three times with PBS, and permeabilized by incubation for 4 min at room temperature with 0.1% (v/v) Triton X-100 in PBS. Following three washes with PBS to remove the detergent, the cells are covered with FITC–phalloidin (Sigma, St. Louis, MO) at a concentration of 0.5 μg/ml in PBS. The FITC–phalloidin may be diluted from a 50 μg/ml stock solution, which remains stable at $-20°$ for several months. Care should be taken to minimize exposure to light for subsequent steps to prevent loss of fluorescence intensity. After a 20-min incubation in the dark at room temperature, the cells are washed three times with PBS. At this point, if chamber slides are employed, they are disassembled. Coverslips are then mounted with 90% (v/v) glycerol. It is helpful to seal the edges of the coverslips with transparent nail polish to minimize exposure to air and to reduce the risk of transferring glycerol to the microscope objective. Slides may be examined immediately or stored for up to 2 weeks at $-20°$.

Identical fields are examined both by phase-contrast microscopy to identify bacteria and by fluorescent microscopy using a blue excitation filter to examine filamentous actin staining. Because the fluorescence signal rapidly diminishes with light exposure, it is unwise to dwell on a particular field for greater than 30 sec. At lower depths of focus, stress fibers are observed. In specimens containing EPEC or EHEC viewed at higher depths of focus, spots of intense fluorescence corresponding to the size, shape, and location of adherent bacteria are apparent (Fig. 2). Often the pattern of fluorescence outlines the longitudinal borders of the adhering bacteria like parallel railroad tracks. Adherent bacteria from the ETEC, EAggEC, and diffuse adherent categories are visualized by phase-contrast microscopy, but no corresponding alteration in filamentous actin staining is ob-

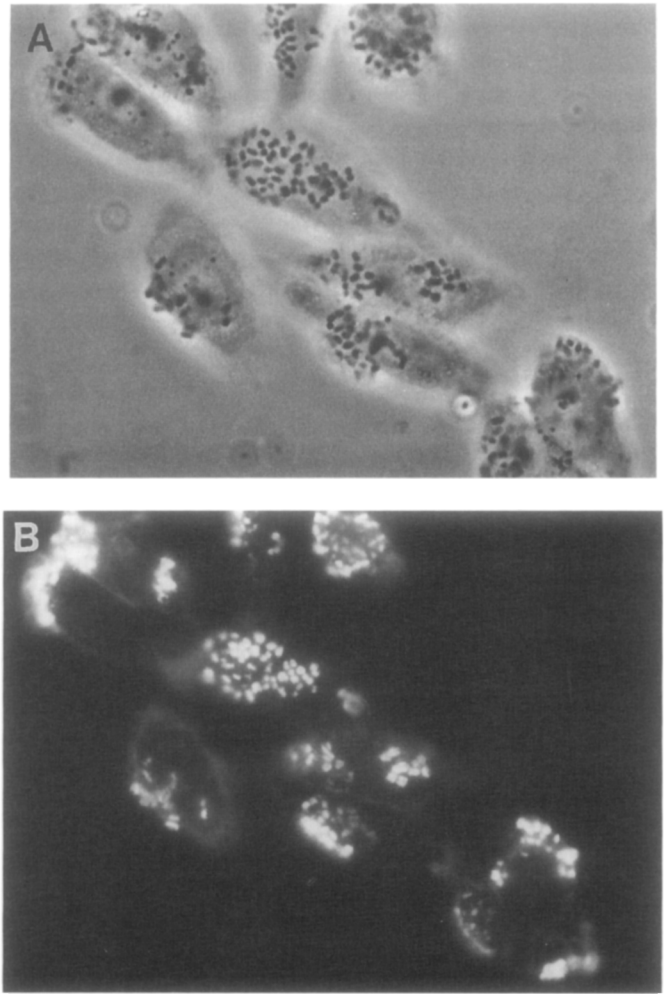

FIG. 2. Phase-contrast (A) and fluorescent (B) views of HEp-2 cells infected with an enteropathogenic strain in the fluorescent actin staining test. Bright spots of fluorescence correspond to the shape and location of adherent bacteria. Photomicrograph kindly provided by S. Knutton. (Reprinted from Ref. 20 with permission.)

served. *Shigella* (and presumably EIEC) may cause a diffuse increase in actin staining early in infection as they invade the cell,[21] but this effect is transient, is weak in comparison to EPEC or EHEC, and is diffuse, rather than outlining the bacteria.

[21] P. Clerc and P. J. Sansonetti, *Infect. Immun.* **55,** 2681 (1987).

Acknowledgments

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[27] Adhesion Properties of *Helicobacter pylori*

By DOLORES G. EVANS and DOYLE J. EVANS, JR.

General Considerations

Biological Significance of Bacterial Adhesion in Disease

Disease caused by bacterial pathogens is the result of the outcome of host–pathogen interactions. The first step in an infectious disease, whether colonization only or entry into deeper host tissues, requires specialized protein factors synthesized by the bacterium which allow its binding to the host cells. Such colonization factors, or adhesins, may not induce tissue damage but do determine the severity of the disease and thus the outcome of the host–parasite interaction, the target organ specificity, and the animal species in which the bacteria may produce disease. In bacterial infections, adhesins of pathogenic bacteria are involved in sequential recognition of cognate epithelial cell receptors. Adhesins often act as biological effectors; they may initiate bacterial intracellular invasion alone or in association with secondary proteins. They have the ability to initiate, subvert, or coopt the host defense systems in favor of the pathogen.

Once a pathogen encounters a susceptible host it may colonize a particular host tissue surface or go down deeper into host tissues before it multiplies. Host tissue damage may accrue at any time during the process. Overt disease may result from products synthesized by the bacterial pathogen (e.g., toxins or enzymes) or from harmful responses of host defense mechanisms (i.e., the inflammatory response), or both.

Biochemical Nature of Bacterial Colonization Factors

Adhesins are proteins with fimbrial, fibrillar, or nonfimbrial structures which usually recognize carbohydrates on eukaryotic cells.[1] Most bacteria can display more than one adhesin; for example, in *Escherichia coli* at least 10 different adhesins have been characterized, including colonization factor

[1] K.-A. Karlsson, *Annu. Rev. Biochem.* **58,** 309 (1989).

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[1] K.-A. Karlsson, *Annu. Rev. Biochem.* **58,** 309 (1989).

antigens I and II, K88, K99, O75X, 987P, F41, P fimbriae, type 1 fimbriae, and S fimbriae; *Bordetella pertussis* encodes 7 potential adhesins.[2] Many bacterial adhesins have lectinlike properties, the cognate carbohydrate receptor sequences being on glycoprotein and/or glycolipoprotein components of various types of epithelial cells and often on erythrocyte membranes.[2–5] Adhesins can be classified by hemagglutinating patterns obtained by testing for hemagglutination with erythrocytes of different animal species or the same species with known antigenic differences.[5–7]

Chemical Nature of Bacterial Adhesin Receptors

Bacterial pathogens usually adhere to epithelial cell surfaces, particularly when the portal of entry is the mucosal membranes of the respiratory, gastrointestinal, or urogenital tracts. Bacterial adhesion may result from direct binding of colonization factors to receptors on the surface of epithelial cells or to host-encoded, secreted polysaccharides or proteins that bind to receptors on the surface of epithelial cells.[8–10] Although for many adhesins the cognate carbohydrate structure is known, the identity of the native molecules carrying the receptor carbohydrate sequences are mostly unknown.

It has become evident that the carbohydrate recognition domains for some colonization factors (adhesins) have structural features in common with eukaryotic lectin molecules, for instance, ELAM and GMP-140, which promote leukocyte adhesion.[2,9,11] Other bacteria, for example, *Legionella pneumophila,* adhere by adsorbing the natural ligand, C3bi, of another eukaryotic cell adhesin, integrin CR3, onto the bacterial surface, thus using a natural pathway to gain access to the eukaryotic cell.[12,13] Other bacterial adhesins bind to the carbohydrate of glycosidic integrins instead of binding to the recognition motif Arg-Gly-Asp.[10] In a third adhesion process, the

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[3] K. A. Krogfelt, *Rev. Infect. Dis.* **13,** 721 (1991).
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[8] S. N. Abraham, E. H. Beachy, and W. A. Simpson, *Infect. Immun.* **41,** 1261 (1983).
[9] D. Relman, E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright, *Cell (Cambridge, Mass.)* **61,** 1375 (1990).
[10] N. Sharon, A. Gbarah, C. G. Gahmberg, I. Ofek, and U. Jacobi, *Infect. Immun.* **59,** 4524 (1991).
[11] A. I. M. Hoepelman and E. I. Tuomanen, *Infect. Immun.* **60,** 1729 (1992).
[12] H. C. Krivan, D. D. Roberts, and V. Ginsburg, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6157 (1988).
[13] N. R. Payne and M. A. Horwitz, *J. Exp. Med.* **166,** 1377 (1987).

pathogen encodes for an analog of a eukaryotic adhesin or a protein which contains the Arg-Gly-Asp carbohydrate recognition motif of an integrin.[9] The receptor that the pathogen uses to interact with the eukaryotic cell determines the fate of the bacterium because each receptor induces in the epithelial cell a limited type of response; however, as most pathogens have more than one type of adhesin and each adhesin binds to a different receptor, this allows the pathogen to interact with eukaryotic cells by more than one pathway. Apparently, the outcome of the interaction of an adhesin with its receptor will depend not only on the type of receptor involved but on the sequential order in which the receptors are engaged. For instance, in *Shigella,* the eukaryotic transmembrane signaling that determines the ultimate fate of the bacteria, invasion and intracellular multiplication, requires a series of events in which more than one bacterial product acts sequentially.[14,15]

Helicobacter pylori is the etiological agent of chronic type B gastritis. Mounting epidemiological evidence and clinical studies implicate chronic *H. pylori* infection in the development of gastric and duodenal ulcers and gastric carcinoma.[16–22] The spiral-shaped, highly motile bacterium is well adapted for residence on the gastric mucosa. Electron micrographs of antral biopsies show *H. pylori* adhered to the apical membrane of mucus-secreting gastric epithelial cells; adhesion is characterized by intimate contact between the bacterial cell and the host cell membrane, often in the form of adhesin pedestals, and rearrangement of microskeletal cell proteins.[23–26] Studies on adhesion properties of *H. pylori* at present are for the most part

[14] I. Rosenshine, M. S. Donnenberg, J. B. Kaper, and B. B. Finlay, *EMBO J.* **11,** 3551 (1992).
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[18] M. J. Blaser, *Gastroenterology* **102,** 720 (1992).
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[21] L.-E. Hansson, L. Engstrand, O. Nyren, D. J. Evans, Jr., A. Lindgren, R. Bergstrom, B. Andersson, L. Athlin, O. Bendtsen, and P. Tracz, *Gastroenterology* **105,** 1098 (1993).
[22] L. Emody, A. Carlsson, A. Ljungh, and T. Wadstrom, *Scand. J. Infect. Dis.* **20,** 53 (1988).
[23] C. S. Goodwin, J. A. Armstrong, and B. J. Marshall, *J. Clin. Pathol.* **39,** 353 (1986).
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[26] F. A. Wyle, A. Tarnawski, D. Shulman, and W. Debros, *J. Clin. Gastroenterol.* **12**(Suppl. 1), 92 (1990).

descriptive, referring to phenotypic adhesion properties of intact bacteria, and only in few instances has a putative adhesin molecule been described.

Different *in vitro* adhesion assays which employ cell types of human origin or from other animal species have been used to study *H. pylori* adhesin phenotypes. Other *in situ* adhesion methods use human gastric antral biopsies in the form of tissue explants or thin sections of either frozen or paraffin-embedded tissue. Studies of *H. pylori* adhesin phenotypes after infection of experimental animals have also been used.[27,28] *In vitro H. pylori* adhesion model systems are emphasized here.

Helicobacter pylori–Erythrocyte Interaction

Erythrocytes are a convenient source of mammalian cells which have a myriad of exposed complex carbohydrates representing potential cognate carbohydrate sequences for bacterial adhesins. Most bacterial adhesins are detectable as hemagglutinins; a positive hemagglutination (HA) test performed with intact bacterial cells is presumed to indicate the presence of an adhesin. With many gram-negative enteric pathogens, adhesins that recognize different receptors can be detected by both quantitative and qualitative differences in HA test results when erythrocytes obtained from a variety of animals are compared; HA-typing schemes have been derived from this type of information.[5,29] With *H. pylori* this approach to adhesin characterization has not been successful, although differences in results obtained with different species of erythrocytes have been noted.[22,30–33] Another approach is to test a variety of antigenically characterized human erythrocytes to discover if a particular blood-type antigen can be identified as the receptor; however, this approach has not been productive with *H. pylori* because no HA-negative human blood types have been found.[7]

Adhesin receptor specificity can also be characterized by demonstrating inhibition of HA by either small molecules with specific carbohydrate structures or by sialic acids, glycolipids, or large, complex molecules such as

[27] L. Engstrand, S. Gustavsson, A. Jorgensen, A. Schwan, and A. Scheynius, *Infect. Immun.* **59,** 1763 (1990).

[28] D. G. Rudmann, K. A. Eaton, and S. Krakowka, *Infect. Immun.* **60,** 2121 (1992).

[29] D. J. Evans, Jr., D. G. Evans, and H. L. DuPont, *Infect. Immun.* **23,** 336 (1979).

[30] L. Emody, J. Heesemann, H. Wolf-Watz, M. Skurnik, G. Kapperud, P. O'Toole, and T. Wadstrom, *J. Bacteriol.* **171,** 6674 (1989).

[31] J. Huang, C. J. Smyth, N. P. Kennedy, J. P. Arbuthnott, and P. W. N. Keeling, *FEMS Microbiol. Lett.* **56,** 109 (1988).

[32] T. Nakazawa, M. Ishibashi, H. Konishi, T. Takemoto, M. Shigeeda, and T. Kochiyama, *Infect. Immun.* **57,** 989 (1989).

[33] J. Robinson, C. S. Goodwin, M. Cooper, V. Burke, and B. J. Mee, *J. Med. Microbiol.* **33,** 277 (1990).

glycoproteins and sialoglycoproteins. It has been shown that *H. pylori* hemagglutinin is blocked by preexposure to a specific small molecule, the *N*-acetylneuraminyl-α-2,3-galactose isomer of *N*-acetylneuraminyl-lactose (NL), and also by glycoproteins such as fetuin, α_2-macroglobulin, and erythrocyte glycophorin A which contain these moieties. NL is a component of ganglioside G_{M3}-containing glycoconjugates which have been shown to occur on human gastric epithelial cells.[34,35]

It is interesting that adhesion of the *H. pylori* hemagglutinin to erythrocyte membranes has characteristics similar to that seen when the bacteria attach to epithelial cells, namely, intimate contact and distortion of cell morphology.[7]

Molecular characterization of specific adhesin–erythrocyte interactions can also be achieved by testing for destruction of either the adhesin or its receptor by enzymes of known specificity. The *H. pylori* hemagglutinin is a protein, as suggested by the fact that its activity is destroyed by pretreatment with Pronase and papain, although not by pepsin or trypsin. Erythrocytes pretreated with *Clostridium perfringens* sialidase, which specifically cleaves *N*-acetylneuraminyl-2,3-lactose, do not hemagglutinate with *H. pylori,* which is in agreement with the HA-inhibition results described above.

Method for Testing Adhesion of Helicobacter pylori to Erythrocytes

Helicobacter pylori strains isolated from antral biopsies are expanded from a single colony by two transfers on blood agar plates, incubated for 48 hr at 37°, with 12% (v/v) CO_2 and 98% relative humidity. Stock cultures are prepared by suspending the growth from one blood agar plate in 0.5 ml of Albimi cysteine medium (Remel, Lexena, KS) containing 20% (v/v) glycerol and stored at −70°. Blood agar plates consist of brain–heart infusion (Difco Laboratories, Detroit, MI), 1.5% agar, and 7% (v/v) horse blood no older than 8 days.

Hemagglutination. For consistent results, fresh erythrocytes from a particular animal species are washed three times by centrifugation with phosphate-buffered saline [PBS; 0.15 *M* NaCl in 0.1 *M* sodium phosphate buffer, pH 7.3 (hemagglutination buffer, Difco)]; the blood of some animal species, such as the horse, is much less stable, and on occasion, if this step is not performed or if the blood is used after extensive lysis, false-negative results may be obtained. The assay is performed at room temperature on clean

[34] B. L. Slomiany, J. Pitrowski, A. Samanta, K. Vanhorn, V. L. N. Murty, and A. Slomiany, *Biochem. Int.* **19,** 929 (1989).

[35] T. Saitoh, H. Natomi, W. Zhao, K. Okuzumi, K. Sugano, M. Iwamori, and Y. Nagai, *FEBS Lett.* **282,** 385 (1991).

glass slides or in wells of a microtiter plate by mixing 20 μl of bacterial suspension in PBS containing approximately 10^8 colony-forming units (cfu)/ml with 20 μl of 2% erythrocyte suspension in PBS. Mixtures on glass slides are rotated and read within 1 to 2 min. Microtiter plates are sealed, mixed, and read after 1 hr of incubation at room temperature. A positive control includes a positive hemagglutinating *H. pylori* strain; as a negative control, the bacterial suspension is substituted by 20 μl of PBS. The HA reactions are recorded as 4(+) if all the erythrocytes are agglutinated, 3(+) if more than 50% agglutinated, 2(+) if less than 50%, and 1(+) if only a few clumps or strings are evident. It is important to consider that the test is influenced by the densities of both reactants since it depends on lattice formation. Excess bacteria may produce a false-negative result.

Hemagglutination Inhibition. The hemagglutination inhibition (HAI) assay is basically the same as HA tests except that the bacterial suspension is first titrated to determine the minimal dilution of the bacteria that gives a 3(+) reaction; then 10 μl of double the concentration of the dilution that gave a 3(+) reaction is mixed with 10 μl of various concentrations of the test substances; the mixtures are incubated at room temperature prior to the addition of 20 μl of 2% erythrocytes. In the control test the 10 μl of test substance is replaced by 10 μl PBS. When testing serum for HAI, it is important first to determine whether the serum from the animal used to prepare the hyperimmune serum agglutinates the erythrocytes to be used in the HAI assay; such cross-species hemagglutination is not uncommon.

Hemagglutination Assay to Assist in Purification of Helicobacter pylori Adhesins

Proteins contained in column fractions in a purification scheme can be used to coat latex beads. Protein-coated latex beads, instead of whole bacteria, can then be used in HA and/or HAI assays as described above.[36] Latex beads with an average diameter of 0.81 μm (Difco) are washed three times by centrifugation at 7000 *g* with 0.1 *M* glycine–saline, pH 8.2, and diluted in the same buffer to approximately 10^8 beads/ml. Column fractions adjusted to a protein concentration of 20 μg/ml in the same buffer are mixed with equal volumes of washed latex beads and incubated at 37° for 1 hr with gentle shaking. The unbound protein is then washed out by centrifugation and finally suspended in the pH 8.2 buffer containing 0.2% (w/v) bovine serum albumin (BSA). This procedure, in conjunction with HAI tests using the receptor or receptor analog proven to inhibit HA when using intact bacteria, is a fast and reliable method to determine whether

[36] D. G. Evans, D. J. Evans, Jr., S. Clegg, and J. A. Pauley, *Infect. Immun.* **25,** 738 (1979).

the material purified from cell-free homogenates, or from bacterial extracts obtained with nonchaotropic detergents at nonlytic concentrations, is the same as the hemagglutinin present on the bacterial surface. If antibody against the adhesin is available, the protein-coated beads should agglutinate with this antibody.[36]

Hemagglutination properties of *H. pylori* can also be used to isolate putative adhesive proteins. Formalinized erythrocytes can be exposed to cell-free homogenates or extracts of *H. pylori,* the proteins which are not bound diluted out by washing with PBS, and adsorbed proteins then eluted with 1.5 *M* NaCl. This method has been used to detect 16K, 25K, and 59K *H. pylori* proteins which adhere to dog erythrocytes.[37] The 25K protein has the characteristics of the N-acetylneuraminyllactose-binding adhesin (NLBH).[37]

Because of the ready availability of erythrocytes and the large variety of potential receptors represented by various animal species, many bacterial adhesins have been initially detected as hemagglutinins.[5,29] The NLBH of *H. pylori* was detected as a hemagglutinin which could be inhibited by preexposure of the bacterial cells to soluble NL and by NL-containing proteins such as fetuin and α-macroglobulin.[7,30]

Adhesion of *Helicobacter pylori* to Continuous Tissue Culture Cell Lines

Continuous cell lines in tissue culture have been used with great success to study the phenotypic adhesion properties of bacteria and to identify and characterize adhesin gene(s) systems of many gram-negative bacterial pathogens.[38,39]

Several continuous epithelial cell lines have been used to assess the adhesin phenotypes of *H. pylori* strains, namely, human esophageal epithelial cell line HEp-2 (ATCC CCL-23), human gastric carcinoma KATO III, (ATCC HTB 103), human gastric adenocarcinoma AGS (ATCC 1739), HeLa cells, and human embryonic intestine 407 cells (ATCC CCL6).[40–44]

[37] J. Huang, P. W. N. Keeling, and C. J. Smyth, *J. Gen. Microbiol.* **138,** 1503 (1992).
[38] T. L. Hale, *Microbiol. Rev.* **55,** 206 (1991).
[39] S. Knutton, P. H. Williams, D. R. Lloyd, D. C. A. Candy, and A. S. McNeish, *Infect. Immun.* **44,** 599 (1984).
[40] J. L. Fauchere and M. J. Blaser, *Microb. Pathog.* **9,** 427 (1990).
[41] B. E. Dunn, M. Altmann, and G. P. Campbell, *Rev. Infect. Dis.* **13S8,** 657 (1991).
[42] M. Clyne and B. Drumm, *Infect. Immun.* **61,** 4051 (1993).
[43] D. G. Evans, D. J. Evans, Jr., and D. Y. Graham, *Gastroenterology* **102,** 1557 (1992).
[44] M. Dytoc, B. Gold, M. Louie, M. Huesca, L. Fedorko, S. Crowe, C. Lingwood, J. Brunton, and P. Sherman, *Infect. Immun.* **61,** 448 (1993).

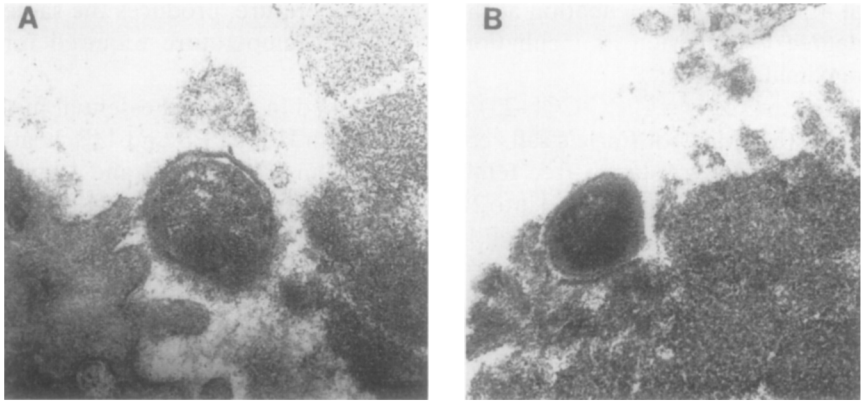

FIG. 1. Transmission electron micrographs of a gastric mucosal biopsy obtained from a 14-year-old boy colonized with *H. pylori*. Approximate magnification: ×30,000. Similar to the findings *in vitro*, *H. pylori* is seen both attached directly to microvilli (A) and attached to the host plasma membrane (B).[44]

Nonepithelial continuous cell lines have also been used, for instance, human embryonic lung fibroblasts, hamster ovary cells, and mouse Y-1 adrenal cells (ATCC CCL 79).[44,45] *Helicobacter pylori* strains adhere to all these tissue culture cell lines, independently of the disease type from which they were isolated. Adhesion of *H. pylori* in all the continuous cell lines so far tested is characterized by intimate contact of bacteria with the eukaryotic cell membrane, a characteristic electron-dense eukaryotic cell membrane, and rearrangement of cytoskeletal proteins at the site of bacterial contact.[40–43,45,46] These adhesion characteristics closely resemble those seen with *H. pylori* adhered to gastric epithelial cells *in vivo* (Fig. 1).

Helicobacter pylori Adhesion Assay Using HEp-2 Cells

Human epithelial cell line HEp-2 was originally derived from a carcinoma of the human larnyx and is widely employed as a model system for characterizing eukaryotic cell infection by bacterial pathogens. In general, adhesion of *H. pylori* strains to HEp-2 cells is fast, maximal within 15 min of incubation, and independent of pH in the range pH 5 to 8 and temperature. Reduced adhesion seen at 4° is primarily due to decreased motility. Increasing bacteria–cell contact by centrifugation of cell monolayers and bacteria

[45] D. G. Evans, D. J. Evans, Jr., and D. Y. Graham, *Infect. Immun.* **57,** 2272 (1989).

[46] D. T. Smoot, J. H. Resau, T. Naab, B. C. Desbordes, T. Gilliam, K. Bull-Henry, S. B. Curry, J. Nidiry, J. Sewchand, K. Mills-Robertson, K. Frontin, E. Abebe, M. Dillon, G. R. Chippendale, P. C. Phelps, V. F. Scott, and H. L. T. Mobley, *Infect. Immun.* **61,** 350 (1993).

at 4°, followed by incubation at the same temperature, produces the same degree of adhesion as incubation at 37°, the temperature required for maximal motility.[43]

HEp-2 cells (ATCC CCL-23) are maintained in minimal essential medium (MEM) with Earle's salts containing 25 m*M* HEPES and 14% heat-inactivated mycoplasma-free fetal bovine serum (GIBCO, Grand Island, NY). HEp-2 cells are seeded into 24-well tissue culture plates (Flow Laboratories, McLean, VA), containing 12-mm coverslips (Fisher Scientific, Pittsburgh, PA) with 2×10^5 cells in 0.5 ml/well (counted with a hemocytometer under phase contrast) and allowed to attach overnight by incubation at 37° under 12% (v/v) CO_2 and 98% relative humidity. A HEp-2 monolayer of no more than 60% confluency is recommended to facilitate detection of adherent bacteria and to assess the efficiency of the washing procedure. In an optimal washing procedure no bacteria should be seen in cell-free spaces. The next day, cell monolayers are washed twice with MEM containing 0.1% bovine serum albumin (BSA) in PBS but no serum or antibiotics (MEM–BSA). The *H. pylori* strains to be tested for adhesion are grown for 24 hr on blood agar plates and suspended in MEM–BSA, adjusting the cell density to 2.5×10^9 cfu/ml. Triplicate wells receive 20 μl of the bacterial suspension; plates are incubated at 37° under 12% (v/v) CO_2 for 1 hr. Bacterial adhesion under these conditions is fast; within 15 min maximal numbers of bacteria per cell and a maximal number of cells with adherent bacteria can be achieved. Controls include a *H. pylori* strain known to adhere to HEp-2 cells and HEp-2 cell monolayer with no bacteria added.

The adhesion assay described here can be used to determine the optimal conditions for adhesion in terms of time, temperature, pH, and bacteria-to-cell ratio. Cell viability is assessed by trypan blue exclusion at the end of each incubation time and after testing the various incubation conditions.[47] Typical results, documented by transmission electron microscopy, are shown below.

Methods to Quantitate Adherent Bacteria

Visual Counting. Several different methods can be used to determine the number of *H. pylori* adherent to HEp-2 cell monolayers. One is based on visual counts of adherent bacteria after immunostaining. In this method the culture medium is discarded after incubation of bacteria with HEp-2 cell monolayers, and the monolayers are washed three times with MEM–BSA and three times with Hanks' balanced salt solution (HBSS, GIBCO), with 3-min shaking intervals to dislodge loosely bound bacteria. Cell mono-

[47] J. H. Hanks and J. H. Wallace, *Proc. Soc. Exp. Biol. Med.* **98,** 188 (1958).

layers are then fixed with 95% ethanol for 15 min and stained for immunoassay using polyclonal rabbit antiserum against whole *H. pylori* and goat anti-rabbit immunoglobulin (IgG), conjugated with horseradish peroxidase (Fisher) at a dilution predetermined to prevent nonspecific binding of reagents. In our hands the best results are obtained using as substrate 0.06% 3,3′-diaminobenzidine (Sigma, St. Louis, MO) in 50 m*M* Tris-HCl buffer, pH 7.6, containing 3% hydrogen peroxide. At this point only the bacteria are stained deep dark brown; monolayers are counterstained for 1 min with carbol-fuchsin (Difco), which stains the cells light pink, facilitating counting the number of bacteria per HEp-2 cell. A total of 50 cells are counted in each of triplicate coverslips and scored for having 0–4, 5–20, or over 20 bacteria/cell. Typical results are shown in Fig. 2.

Viable Cell Counts. Another method for detecting *H. pylori* adhesion to tissue culture cells is by direct counting. The experimental conditions are essentially as described above except that for each incubation interval five wells are set up; two will be used to determine the number of viable HEp-2 cells after each incubation time, and three wells will be used to determine the number of adherent bacteria per 10^6 viable HEp-2 cells. After various incubation periods with bacteria, HEp-2 cell monolayers in wells are washed eight times with 0.5 ml/well of MEM–BSA; each washing is discarded by complete aspiration by vacuum. Cells and adherent bacteria are then treated with 0.25% (w/v) trypsin and 0.1 m*M* EDTA (GIBCO) for 4 min, at which time 2.0 ml of MEM with 12% fetal bovine serum is added to stop trypsin action. Cells and bacteria are then centrifuged at 7000 *g* for 10 min, suspended in sterile distilled water, vortexed vigorously to disperse the bacteria, and 10-fold serially diluted in sterile distilled water. In general duplicate 100-μl aliquots of each dilution are spread on a blood agar plate. Plates are incubated at 37° under 12 % (v/v) CO_2 and 98% relative humidity for 4–5 days prior to colony counting. Adherent bacteria are scored as cfu per 10^6 cells. The results of a typical experiment are shown in Table I.

Immunofluorescence Methods and Use of Radiolabeled Bacteria. Human gastrocarcinoma KATO III cells which grow primarily in suspension are best suited for assessing adhesion of *H. pylori* strains by indirect immunofluorescence staining monitored by flow cytometry as described by Dunn *et al.*[41] KATO III cells are maintained in RPMI 1640 containing 10% (v/v) heat-inactivated fetal bovine serum and antibiotics (GIBCO). Adhesion assays are performed in 10-mm culture dishes (Falcon Plastics, Oxnard, CA) containing 10 ml of culture medium and 10^6 KATO III cells/ml. *Helicobacter pylori* cells grown for 48 hr as indicated above are added to a bacteria-to-cell multiplicity of 100:1. After incubation at 37° for 30 min, unbound bacteria are separated by centrifugation at 200 *g* for 5 min after the addition of 14 ml of 15% sucrose. KATO cells with and without adherent

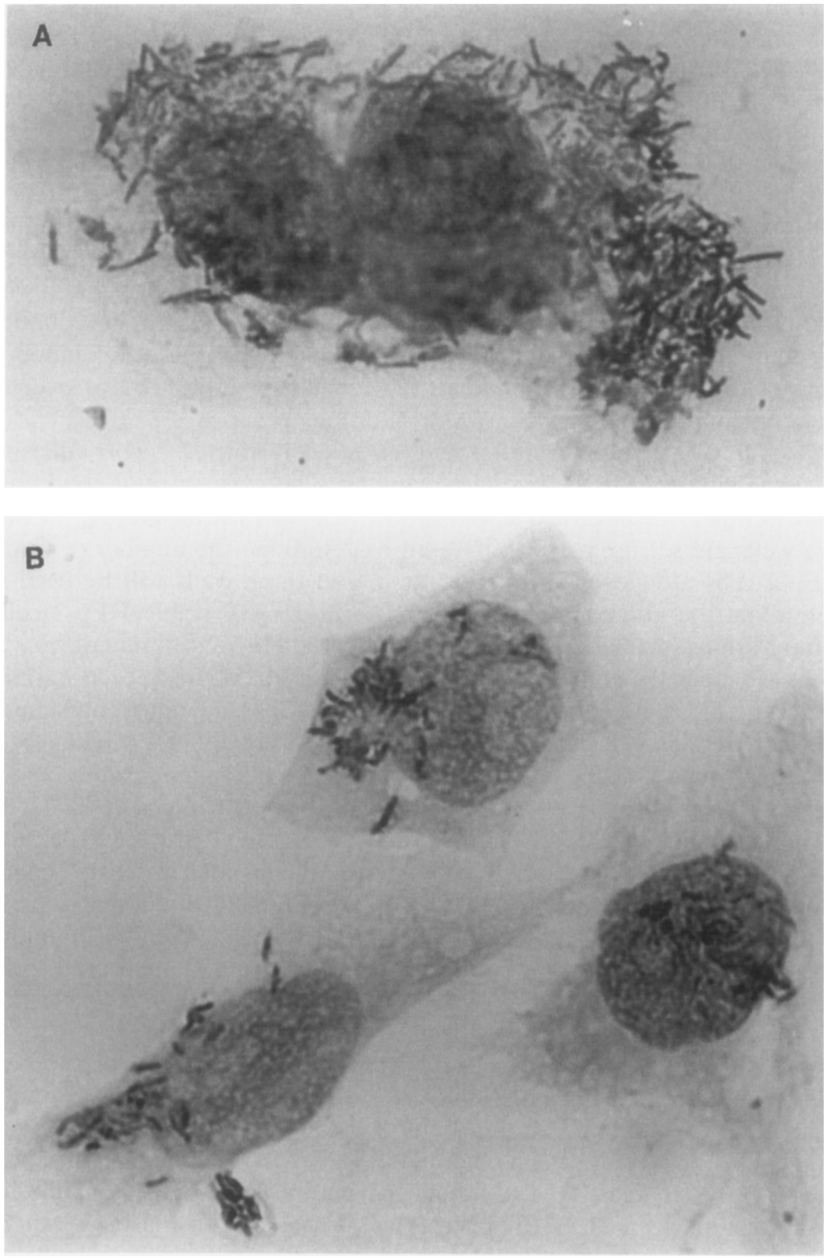

FIG. 2. (A) HEp-2 cells with adherent *H. pylori*. Note the diffuse pattern of adhesion. Approximately 5×10^7 cfu *H. pylori* cells were added to a HEp-2 monolayer and incubated for 1 hr at 37° under microaerophilic conditions. Approximate magnification: ×2400.[43] (B) Y-1 cells with adherent *H. pylori*. Note nondiffuse, polar pattern of adhesion. Around 2×10^8 cfu *H. pylori* were added to a Y-1 cell monolayer and incubated for 30 min at 37° under microaerophilic conditions. Approximate magnification: ×2400.[45]

TABLE I
ADHESION OF *Helicobacter pylori* TO TISSUE CULTURE CELLS, DETERMINED BY COUNTING VIABLE BACTERIA

Incubation time (min)	*N*	*H. pylori*, cfu/10^6 HEp-2 cells
15	6	$8.70 \pm 0.85 \times 10^6$
30	6	$1.00 \pm 0.32 \times 10^7$
60	6	$8.17 \pm 0.92 \times 10^8$
120	6	$1.25 \pm 0.32 \times 10^7$

[a] D. G. Evans, unpublished results, 1992.

bacteria are stained by indirect immunofluorescence using rabbit serum against whole bacteria and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Fisher) as second antibody. It is advisable to determine the optimal dilution of the two reagents first to prevent high backgrounds.

Analysis of the fluorescent bacteria adherent to KATO cells is performed with the aid of a flow cytometer (Coulter Electronics, Hialeah, FL). Green fluorescence is analyzed with a 488-nm dichroic mirror, 515-nm long-pass and 530-nm short-pass interference filters, and a log-linear amplifier.[41] Data are obtained with a 256-channel scale with 70 channels equivalent to 1 log decade. Cellular debris, unbound bacteria, and cell clumps are excluded from the analysis by volume gating. The peaks representing the mean number fluorescence channel is calculated by an analysis of more than 2000 cells per determination, consisting of KATO cells without adherent bacteria and KATO cells with adhered *H. pylori.* Data are integrated by means of an Integra program on a Coulter Easy 88 microcomputer system. The data are expressed in terms of mean fluorescence intensity (*MFI*) which is calculated from the product of the natural log of the mean fluorescence channel number given by the Integra program times a constant, 0.0271. Using this technique, the relative *MFI* of intestinal-407 (ATCC CCL6), KATO III (ATCC HBT103), Hs 746T (ATCC HBT135), embryonic carcinoma (EC), and yolk sac-like epithelial cells has been calculated using a single *H. pylori* strain 87-263.[41] Typical results are shown in Fig. 3.

Adhesion of *H. pylori* to epithelial cells can also be assessed with radiolabeled bacteria or bacteria stained by indirect immunofluorescence.[41,48] Although with these methods the number of adherent bacteria per cell cannot be quantitated, a relative estimate of the degree of adhesion under various conditions can be determined. For optimal results, the techniques require

[48] S. G. Hemalatha, B. Drumm, and P. Sherman, *J. Med. Microbiol.* **35,** 197 (1991).

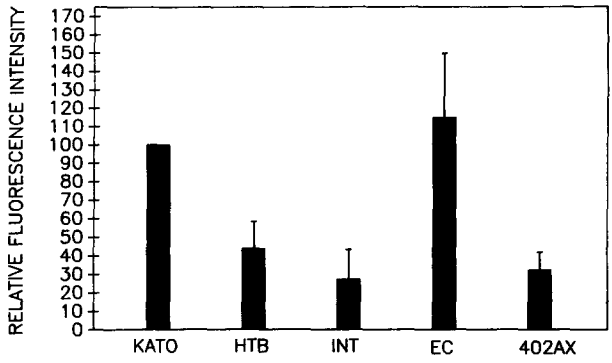

FIG. 3. Relative fluorescence intensity of five epithelial cell lines exposed in suspension to *H. pylori* strain 87-263 at a bacteria-to-cell ratio of about 100 : 1. The *MFI* was normalized to that of KATO cells. Each value represents the mean of three or four determinations (±SEM).[41]

the use of cells that primarily grow in suspension; this facilitates washing of excess reagents and the quantitation of adherent *H. pylori,* although cells grown in monolayers have also been used for this purpose. In this case, cell monolayers are lifted with a rubber policeman and dispersed by vigorous shaking with a capillary pipette.[41]

Adhesion of Helicobacter pylori to Primary Cultured Gastric Epithelial Cells of Humans and Other Animal Species

Primary gastric epithelial cells have been isolated from humans[46] and from the stomach of several different animal species.[49] Gastric epithelial cells are prepared from gastric biopsies obtained by endoscopy. The biopsy tissue is minced with a scapel and then incubated with collagenase–dispase for 60 minutes at 37°. The gastric cells, pelleted by centrifugation, are cultured in Ham's F12 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and 0.25 mg/ml gentamicin (GIBCO); 60-mm-diameter tissue culture dishes (Costar, Cambridge, MA) are used for plating the cells. After 48 hr cells are exposed to *H. pylori* (10^7 cfu/ml) suspended in Ham's F12 medium without antibiotics. After 1 to 3 hr at 37° nonadherent bacteria are removed by three washes in PBS, and the gastric cells are fixed with glutaraldehyde for electron microscopy.[46] Adhesion of *H. pylori* to cultured primary human gastric cells shows characteristic intimate contact with the gastric cell membrane and changes in the epithelial cell cytoskeleton.[46]

[49] Y. Kobayashi, K.-I. Okazaki, and K. Murakami, *Infect. Immun.* **61,** 4058 (1993).

A similar approach was used to isolate antral gastric epithelial cells by collagenase digestion of the stomach from several animal species. Kobayashi *et al.*[49] demonstrated a characteristic adhesion of *H. pylori* to monkey and pig primary gastric epithelial cells which was inhibited by fetuin.[49] Organ-culture techniques can be successfully applied to demonstrate adhesion of *H. pylori* to human gastric epithelium of surgically removed stomach mucosa and pig stomach biopsies.[50,51]

Adhesion of Helicobacter pylori to Tissue Sections of Human Antral Biopsies, Employing Histochemical Methods

Immunomicroscopic techniques can be used to detect adhesion of cell-free *H. pylori* proteins with epithelial cells. Serial frozen thin sections of gastric biopsies, obtained from *H. pylori*-negative individuals, are exposed to *H. pylori* cell-free putative adhesins. Adherent antigen is detected with monospecific antibody and a second antibody conjugated with alkaline phosphatase and an appropriate substrate[52] (see Tables II and III). Adventitious binding can be discouraged by using a high salt concentration in the buffer, PBS, in the washing steps, and the specificity of the interaction can be tested using a known specific inhibitor. In this method controls include a primary antibody against antigen(s) not including the adhesin, antigen proteins not including the adhesin, and deletions of either the primary or secondary antibody.

The gene *hpaA,* encoding the NL-binding adhesin, has been cloned and sequenced. The above method was used to show that the product of *hpaA* (ORF2 in pHPA24), expressed in *E. coli,* binds to gastric epithelial cell membranes and that the binding is blocked by *N*-acetylneuraminyllactose and by monospecific anti-NLBH (Fig. 4).[52]

A second method involves formalinized paraffin-embedded tissue sections from human stomach. Tissue sections are deparaffinized in xylene and 2-propanol, rinsed with water and PBS, and blocked against nonspecific binding using 0.2% BSA/0.05% Tween 20 in PBS. Fluorescein isothiocyanate-labeled bacteria, in blocking buffer, are added, and nonadherent bacteria are removed by washing in PBS after incubating for 1 hr at room

[50] K. Rosberg, R. Hubinette, G. Nygard, T. Berglindh, and W. Rolfsen, *Scand. J. Gastroenterol.* **26,** 43 (1991).

[51] K. Rosberg, T. Berglindh, S. Gustavsson, R. Hubinette, and W. Rolfsen, *Scand. J. Gastroenterol.* **26,** 1179 (1991).

[52] D. G. Evans, T. L. Karjalainen, D. J. Evans, Jr., D. Y. Graham, and C. H. Lee, *J. Bacteriol.* **175,** 674 (1993).

TABLE II
CONCENTRATION OF GLYCOSPHINGOLIPIDS IN FUNDIC AND ANTRAL MUCOSA OF HUMAN STOMACH[a]

Glycosphingolipid	Concentration (nmol/g, dry weight)	
	Fundus	Antrum
GlcCer	284	316
GalCer	570	775
LacCer	716	420
Gb_3Cer	882	551
Gb_4Cer	767	376
II^3NeuAc-LacCer	92	122
$II^3NeuAc2$-LacCer	106	141
I^3SO_3-GalCer	416	934
Sulfatides/gangliosides	2.1	3.5

[a] Carbohydrate composition of CMH (GlcCer or GalCer) was determined by gas–liquid chromatography (GLC) on 3% OV-101 coated on Chrolite with a programmed temperature rise of 2°/min from 150° to 200° as the *N*-acetyl-*O*-trimethylsilyl derivatives.[35]

temperature.[53] Binding of *H. pylori* cells to gastric epithelial cells via the NL-binding adhesin could not be demonstrated using these methods; however, binding to a fucosylated blood group-type antigen on gastric mucous cells was observed.[53]

Identification of Receptors for Bacterial Adhesins

General Considerations

In general, adhesin receptors are carbohydrate moieties on surface-exposed glycoproteins or glycosphingolipids, often attached covalently to membrane proteins of epithelial cells which reside in the target tissue or organ. These same carbohydrate receptor moieties are often found on the same glycoconjugates, proteins, or sialoglycoproteins of cell membranes at sites other than the target tissue or organ. For instance, the erythrocyte displays an enormous variety of complex glycoproteins, glycosphingolipids, and gangliosides which are identical to or closely mimic epithelial cell adhesin receptors.

[53] P. Falk, K. A. Roth, T. Boren, T. U. Westblom, J. I. Gordon, and S. Normark, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 2035 (1993).

TABLE III
BINDING OF *Helicobacter pylori* TO GLYCOSPHINGOLIPIDS PURIFIED FROM ANTRAL MUCOSA[a]

Mucosa	Peak area		Ratio
	I^3SO_3-GalCer (*a*)	II^3-NeuAc-LacCer (*b*)	(*a*/*b*)
Viable *H. pylori*			
Antral mucosa 1	27,861	3438	8.1
Antral mucosa 2	27,862	5199	5.4
Sonicated organisms			
Antral mucosa 1	5797	6426	0.9
Antral mucosa 2	3015	5796	0.5

[a] Peak areas (*a*) and (*b*) represent values obtained from the same TLC plate by electric integrator (μV). Antral mucosa 1 and 2 were from patients 1 and 2, respectively.[35]

Cognate carbohydrate can also be present on different complex proteins and sometimes occur in soluble form, that is, in serum or external secretions. Colostrum and milk contain many complex proteins, some of which are adsorbed onto fat globules, which bind to and neutralize a variety of bacterial toxins and adhesins.[53,54,55]

Enzyme-Linked Immunosorbent Assay Techniques for Detecting and Quantitating Adhesin–Receptor Interactions

Enzyme-linked immunosorbent assay (ELISA) techniques provide convenient and sensitive methods for adhesin detection when a pure protein bearing the specific receptor moiety is available. Because ELISAs are sensitive to nonspecific interactions, they are best used for detecting cell-free adhesins, as in the purification of an adhesin. A fetuin ELISA has been described for detecting the NL-binding adhesin of *H. pylori*.[7] In this assay, 96-well microtiter plates are coated with 1.0 μg/ml of fetuin (Sigma) in 50 m*M* Tris-HCl (pH 8.0) for 18 hr at 37°. The wells are then blocked with 1.0% bovine serum albumin in PBS before the addition of test samples; standardized procedures are subsequently followed, including as reagents an adhesin-specific primary antibody and enzyme-labeled secondary antibody and the appropriate substrate.

[54] A. Laegreid, A.-B. K. Otnaess, and J. Fuglesang, *Pediatr. Res.* **20,** 416 (1986).
[55] J. Holmgren, A.-M. Svennerholm, and M. Lindblad, *Infect. Immun.* **39,** 147 (1983).

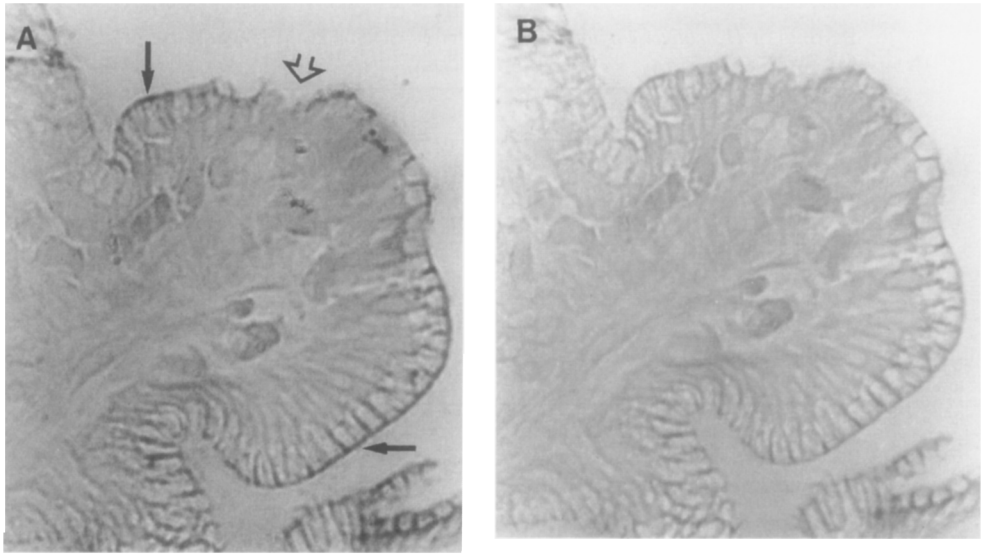

FIG. 4. Binding of the pHPA24-expressed product (ORF2) to human gastric epithelial cells. (A) Representative tissue section exhibiting on adhesion-positive reaction obtained with the 20K protein expressed by pHPA24. A similar reaction was obtained with partially purified 20K *H. pylori* adhesin and with total *H. pylori* protein. Note that the ORF2 product (the 20K adhesin) bound only to intact epithelial cells (solid arrows) and not to cells damaged during tissue sectioning (open arrow). (B) Serial tissue section showing a representative adhesion-negative reaction obtained with the pHPA23-encoded product. A similar negative reaction was obtained with purified *H. pylori* urease and tissue sections exposed to only anti-NLBH or secondary antibody.[52] Approximate magnification: ×500.

Solid-Phase Overlay Method

Several methods can be utilized to identify potential target eukaryotic cell receptors. The solid-phase overlay method has been used with success to determine glycolipid receptors for several pathogens.[56–58] A solid-phase overlay immunostaining method has been utilized to detect binding of *H. pylori* to sulfated alkylacylglycerolipid from pig stomach and to identify two *H. pylori* receptors II^3NeuAc-LacCer and I^3SO_3-GalCer which were extracted and purified from human antral mucosa.[35,59] Glycosphingolipids with the same structure have also been identified as potential *H. pylori*

[56] G. C. Hansson, K.-A. Karlsson, G. Larson, N. Stromberg, and J. Thurin, *Anal. Biochem.* **146,** 158 (1985).
[57] K.-A. Karlsson and N. Stromberg, this series, Vol. 138, p. 220.
[58] K.-A. Karlsson and N. Stromberg, *J. Biol. Chem.* **265,** 11244 (1990).
[59] C. A. Lingwood, H. Law, A. Pellizzari, and P. Sherman, *Lancet* **29,** 238 (1989).

receptors, by strong inhibition of *H. pylori* hemagglutination of human group A, B, and O erythrocytes with II^{3}NeuAc-LacCer and sulfated lactosylceramide purified from dog gastric mucosa.[34] The solid-phase overlay immunostaining method detailed here is similar to that reported by Saitoh *et al.*[35]

Preparation of Glycolipids. Glycolipids are extracted from human gastric antrum obtained at autopsy following scheme II as described by Hakomori and Siddiqui[60] and fractionated into neutral and acidic glycolipids by DEAE-Sephadex A-25 (acetate form, Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography and then purified into neutral and acidic glycosphingolipids as described by Iwamori *et al.*[60,61]

Thin-Layer Chromatography Technique. Glycosphingolipids are resolved by ascending thin-layer chromatography (TLC) on silica gel-coated plates. High-performance gel 60 gives high resolution (Eastman Kodak, Rochester, NY). Plates are activated at 110° for 30–45 min and cooled in a vacuum desiccator prior to sample application. Approximately 30–60 μg of a mixture of neutral or acidic glycolipids in 30–50 μl of chloroform–methanol, 1:1 (v/v), are applied with a Hamilton syringe dropwise to form a 1.5-cm band at a distance of 2 cm from the bottom of the plate. Individual components, 5–15 μg in 30–60 μl of the same solvent, are spotted. After sample application the plates are dried under vacuum for 20–30 min for reactivation. Plates are developed using chloroform–methanol–water, 65:35:8 (v/v), as the solvent system.[35,62] It is recommended to add the solvent into the development tank 2–3 hr prior to development and to line the tank with filter paper to prevent alterations in sample migration. When the solvent has ascended to within 2.5–3.0 cm of the top, plates are removed and completely dried using a stream of warm air supplied by a hair dryer.

Development of Chromatograms. Gangliosphingolipids are visualized by spraying with orcinol–H_2SO_4, or in parallel plates by immunostaining after overlay with either intact *H. pylori* or *H. pylori* sonicates. At the completion of the TLC, plates are dried and then treated with blocking buffer consisting of 1% polyvinylpyrrolidone (PVP), 1% ovalbumin, and 0.02% NaN_3 in PBS at 37° for 1 hr. This step prevents nonspecific binding of bacteria during the overlay step. Plates are then overlaid with a solution containing approximately 10^8 cells/ml of intact or sonicated *H. pylori* and incubated for 1 hr at 37°. Plates are then washed three

[60] S.-I. Hakomori and B. Siddiqui, this series, Vol. 32, p. 345.
[61] M. Iwamori, J. Shimomura, S. Tsuyuhara, M. Mogi, S. Ishizaki, and Y. Nagai, *J. Biochem.* (*Tokyo*) **94,** 1 (1983).
[62] S. K. Kundu, this series, Vol. 72, p. 185.

times with PBS containing 0.1% Tween 20, incubated with the blocking buffer at 37° for at least 15 min, and washed twice with PBS. Plates are then treated for 1 hr at 37° with an optimal dilution (1:200) of hyperimmune rabbit anti-*H. pylori* antibody diluted in PBS containing 3% PVP. This is followed by incubation for 1 hr at 37° with the second antibody consisting of an optimal dilution (1:500) of peroxidase-conjugated anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg MD) and then a 15-min incubation at 37° with the peroxidase substrate consisting of 0.1% H_2O_2 and 3.4 m*M* 4-chloro-1-naphthol in 50 m*M* Tris-HCl, pH 7.4, plus 200 m*M* NaCl. Prior to the addition of immunostaining reagents, plates are submitted to the washing steps as indicated after plates were treated with rabbit anti-*H. pylori* antibody. It is recommended to determine the optimal dilutions of primary and secondary antibodies to assure maximal detection of *H. pylori* with minimal or no nonspecific background. Controls include TLC plates developed wherein *H. pylori* or either the primary or secondary antibodies are omitted. The intensity of the staining of the spots can be quantitated using a dual-wavelength TLC densitometer.[35] Figure 5 depicts binding of *H. pylori* to gangliosphingolipids as demonstrated by this method.

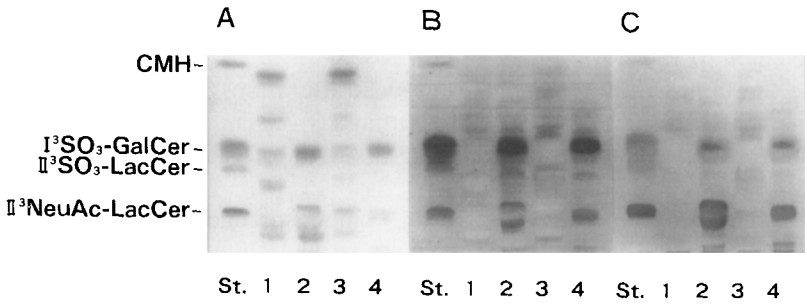

FIG. 5. Binding of *H. pylori* to glycosphingolipids purified from the human gastric antral mucosa on a TLC plate. The amount of glycosphingolipids applied to each lane corresponds to about 5 mg dry weight of tissue, and the TLC plate was developed with chloroform–methanol–water (65:35:6, v/v). (A) Glycosphingolipids visualized by orcinol–H_2SO_4 reagent. (B, C) The plates were incubated with cultured *H. pylori* (B) or sonicated organism (C) and then stained by TLC immunostaining. Lanes 1 and 2 contain neutral and acidic glycosphingolipids, respectively, from the antral mucosa of patient 1. Lanes 3 and 4 contain neutral and acidic glycosphingolipids, respectively, from the antral mucosa of patient 2. Lane St contains standard glycosphingolipids [1 μg each of GalCer (CMH) from human brain, I^3SO_3-GalCer from human brain, II^3SO_3-LacCer from SNG II cells derived from human uterine cancer, and II^3NeuAc-LacCer from bovine brain].[35]

Assessment of *Helicobacter pylori* Adhesion by Electron Microscopy

Electron microscopic observation of bacteria–eukaryotic cell interaction can assist in determining the type of adhesion; for instance, *H. pylori* adhesion *in vivo* to gastric epithelial cells shows intimate contact and rearrangement of eukaryotic cell microskeletal proteins at the site of contact with the bacteria. To validate hemagglutination assays or adhesion tests with tissue culture cell lines as methods for identifying the adhesin(s) of *H. pylori* involved in this type of adhesion, it is necessary to confirm that the *in vitro* model actually mimics the type of adhesion seen with *H. pylori* attached to gastric epithelial cells *in vivo* in human disease. Electron microscopy provides powerful techniques for characterizing the exact nature and consequences of bacteria–eukaryotic cell binding.

Transmission Electron Microscopy Method for Examining Erythrocytes Agglutinated with Helicobacter pylori

Freshly isolated *H. pylori* strains are grown for 24 hr on blood agar plates as indicated above, and the cells are adjusted to 10^8 cfu/ml with PBS

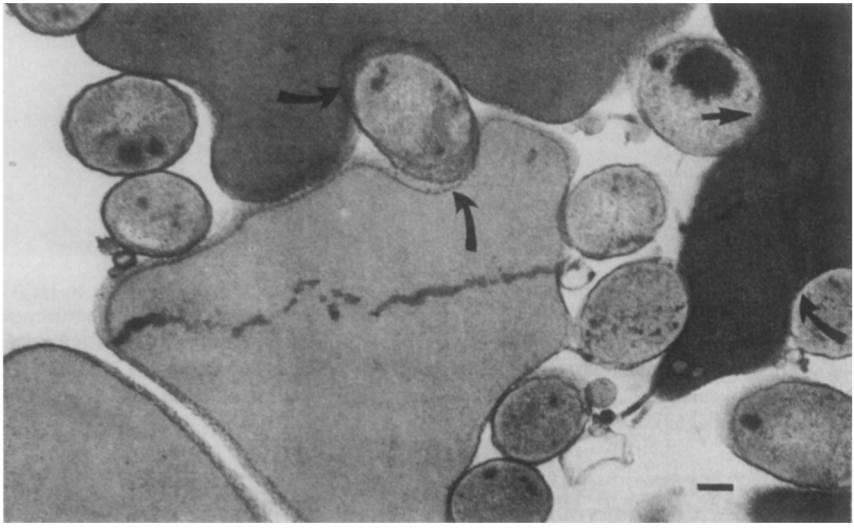

FIG. 6. Thin-section view of *H. pylori* cells interacting with human erythrocytes. Note arrows indicating intimate contact with indentation of the erythrocytes at the site of contact with bacteria. Bar: 0.1 μm.[7]

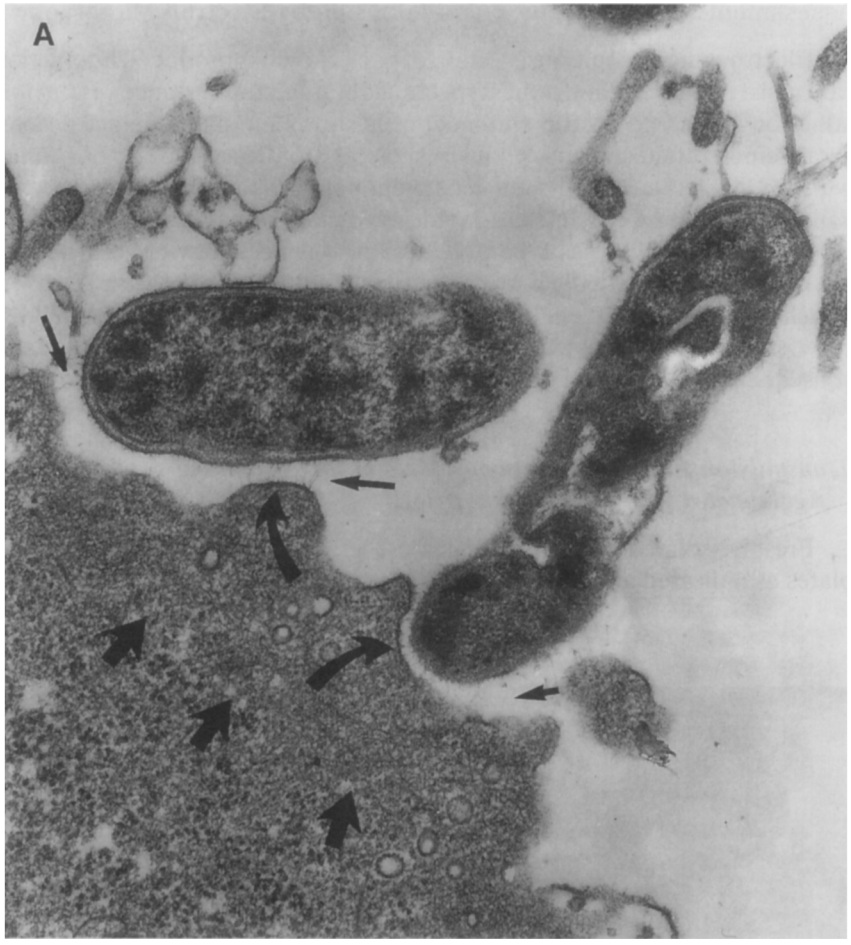

FIG. 7. Electron micrograph showing thin sections of *H. pylori* cells adherent to HEp-2 cells. (A) Note the bacterial fibrillar contacts with HEp-2 cells (small arrows), the thickness of the cell membrane (curved arrows), and the change in ultrastructure of the HEp-2 cell at the site of bacterial contact (large arrows). Approximate magnification: ×36,580. (D. G. Evans, unpublished results, 1992.) (B) Note the intimate contact between *H. pylori* and HEp-2 cells. Approximate magnification: ×51,350.[43]

and mixed with equal volumes of 2% washed erythrocytes on a clean glass slide. The agglutinated erythrocytes are then fixed at 4° overnight by treatment with 3% (v/v) glutaraldehyde (EM grade, Sigma), buffered with 0.1 *M* PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] and 0.05% (w/v) ruthenium red, pH 6.5 (Sigma). The pellet is then washed three times with

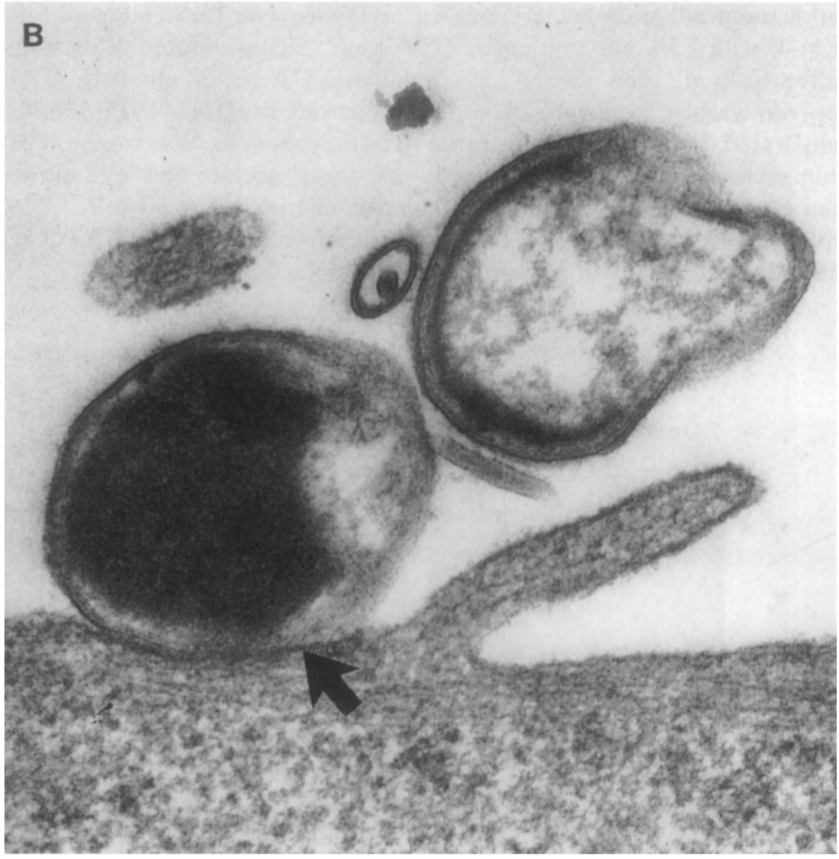

FIG. 7. (*continued*)

0.1 *M* PIPES buffer, suspended, and kept for 40 min in 3% osmium tetroxide plus 75 mg ruthenium red per dl. Samples embedded in SPI-PON 812 (Sigma) resin and dehydrated with a graded series of ethanol are cut with glass knives to select suitable 1-μm sections to prepare 100-Å sections using a diamond knife. Sections stained with uranyl acetate and lead citrate are then examined with a transmission electron microscope at 80 keV (Fig. 6).

Transmission Electron Microscopy of Helicobacter pylori Adherent to Tissue Culture Cells

The adhesion assay is performed as indicated above except that glass coverslips are omitted. Wells containing tissue culture cell monolayers with

and without adherent bacteria are washed twice with PBS and fixed for 1 hr at 4° with 2.5% glutaraldehyde (EM grade; Sigma) diluted in the same buffer. Cells are then washed three times with HA buffer, allowing 30 min between washes, fixed with 2% osmium tetroxide in 50 m*M* PIPES buffer, dehydrated, and embedded in Epon 812 (Polysciences, Warrington, PA). Thin sections of 70 nm are stained with uranyl acetate and lead citrate, then examined with a transmission electron microscope at 80 keV.[7,43,45] A typical result obtained with HEp-2 cells is shown in Fig. 7. A KATO cell with adherent *H. pylori* is shown in Fig. 8.

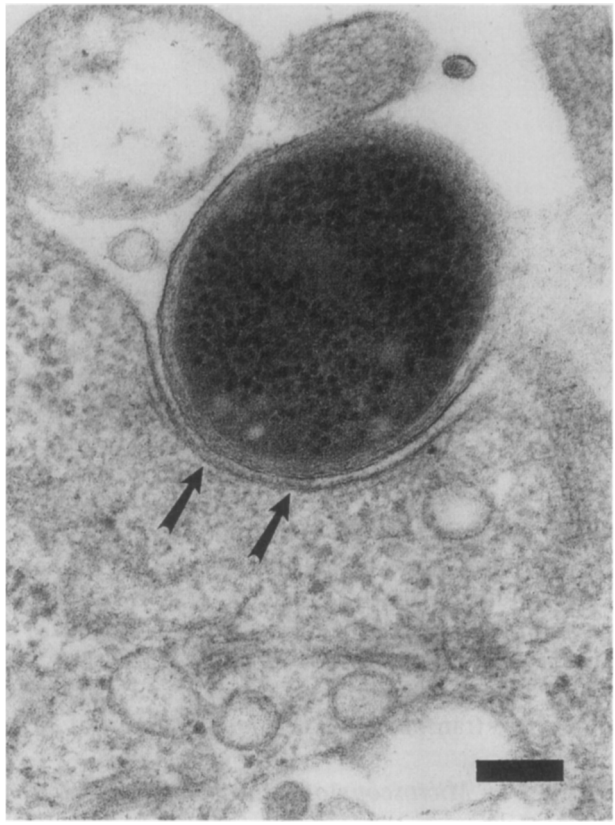

FIG. 8. Electron micrograph of a single cell of *H. pylori* (strain 87-263) associated with the membrane of a KATO cell. The KATO cell membrane is indented and surrounds the associated bacterium in a manner resembling an "adhesin pedestal" (arrows).[41] Bar: 0.1 μm.

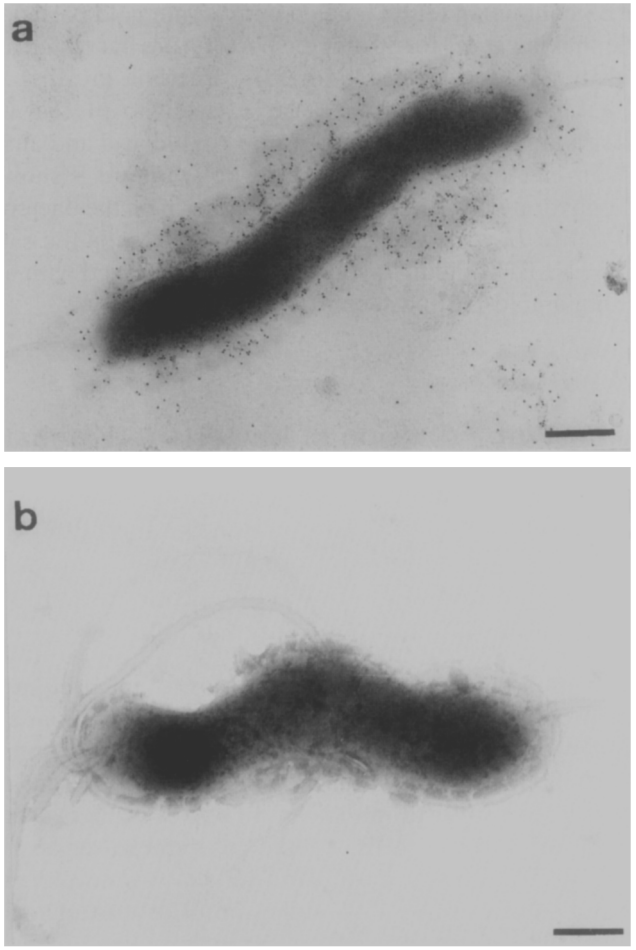

FIG. 9. Electron micrographs of negatively stained *H. pylori* incubated with gold-labeled fetuin. (a) Adhesive strain SR7791; (b) poorly adhesive strain SR6371.[49] Bar: 0.1 μm.

Use of Glycoprotein Carrying Cognate Carbohydrate to Detect Putative Adhesin on Bacterial Surface

Another approach to detect the *H. pylori* adhesin on the surface of the bacteria is based on the use of a glycoprotein containing the cognate carbohydrate which has been labeled with gold particles to permit visualization of the receptor–ligand interaction by electron microscopy.[49] A 24-hr culture is conducted on blood agar plates, as indicated above, at about 10^8

cfu/ml in PBS containing fetuin labeled with 10-nm gold particles (Sigma). After gentle shaking at 37° for 30 min the bacteria are harvested and washed by centrifugation, at least twice, with PBS. Bacteria are then negatively stained with 1.5% phosphotungstic acid, adjusted to pH 6.8 with 0.1 *M* KOH. Bacteria are then applied to a carbon-coated grid and analyzed with a transmission electron microscope at 80 keV. Figure 9 shows that the cognate carbohydrate on fetuin binds to the adhesin on the bacterial surface, coating the entire *H. pylori* cell. This binding pattern is the same as that seen when the NLBH-binding hemagglutinin is visualized with anti-NLBH antibody using a gold-labeled second antibody.[7]

[28] *In Vitro* Adhesion of Bacteria to Exfoliated Uroepithelial Cells: Criteria for Quantitative Analysis

By HARRY L. T. MOBLEY, GWYNN R. CHIPPENDALE, and JOHN W. WARREN

Introduction

The first step in bacterial colonization of a host may require adhesion to receptors on epithelial cells. Many bacteria–host interactions are dictated by the specificity of a bacterial adhesin for defined molecules on the host cell surface. Other interactions may, on the other hand, represent nonspecific adhesion associated with hydrophobicity. In any event, it is useful to measure the ability of bacterial cells to bind to epithelial cells whether the interaction is specific or nonspecific. A technique was developed by Svanborg-Eden and colleagues[1] to study adhesion of uropathogenic strains of *Escherichia coli* to exfoliated uroepithelial cells isolated from urine. Although the assay was quite useful, it has now been found that a number of desirable features of other reported techniques could be incorporated into the method to simplify preparation and gain additional information. Adaptations to the assay[2] used to measure bacterial adhesion combine features from several previous reports[1,3–5] and provide ease of sample preparation and accurate evaluation of adherent bacteria.

[1] C. Svanborg-Eden, B. Eriksson, and L. A. Hanson, *Infect. Immun.* **18,** 767 (1977).
[2] H. L. T. Mobley, G. R. Chippendale, J. H. Tenney, and J. W. Warren, *J. Gen. Microbiol.* **132,** 2863 (1986).
[3] S. Chick, M. J. Harber, R. Mackenzie, and A. W. Asscher, *Infect. Immun.* **34,** 256 (1981).
[4] G. Reid, H. J. L. Brooks, and D. F. Bacon, *J. Infect. Dis.* **138,** 412 (1983).
[5] A. J. Schaeffer, S. K. Amundsen, and L. N. Schmidt, *Infect. Immun.* **24,** 753 (1979).

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Bacterial Growth

Expression of adhesion factors may be favored by a particular medium, growth temperature, pH, or degree of aeration. Conditions that favor expression of adhesins by a specific species must be determined empirically. These assays were developed for enteric bacteria but can be adapted to other bacterial genera.

Bacteria are recovered from storage by growth on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, MD), or any general nutrient agar, and can then be subcultured on a variety of media, including nutrient broth, brain–heart infusion broth (Difco, Detroit, MI), Luria broth, minimal salts medium,[6] or even urine. The pH of the medium may be adjusted to suit the needs of the investigator. Liquid cultures are grown with aeration (200 rpm) for 18 hr or statically for 24 or 48 hr; agar (1.5%, w/v) plates are incubated for 18 hr. Urine agar is prepared by mixing three parts filter-sterilized human urine with one part 6% (w/v) sterilized agar (Difco) at 45°. Sealing the plates before incubation is recommended. All cultures are incubated at 37°; however, temperature can also be varied.

For enteric bacteria, aerated nutrient broth and statically grown urine cultures give the highest mean adhesion values. Statically grown brain–heart infusion broth cultures yield the lowest values.

Bacteria grown in liquid medium are harvested by centrifugation (6000 *g*, 10 min, 4°). Supernatants are aspirated, cell pellets are well drained, and bacteria are gently suspended in phosphate-buffered saline (PBS; 0.145 *M* sodium chloride, 50 m*M* sodium phosphate, pH 7.2) with a Pasteur pipette to an OD_{550} of 0.1 (1 cm light path) ($\sim 2 \times 10^8$ bacteria/ml as determined by colony counts) unless otherwise indicated. Bacteria from agar plates are gently taken from the surface with a sterile swab and suspended in PBS in a similar fashion.

Repeated washing of bacteria by centrifugation before assay incubation diminishes adhesion.[1,2] Therefore, for the adhesion assay, bacteria should be centrifuged once, the pellets well drained, and the bacteria suspended to the desired density and assayed with no additional centrifugations. Adhesion assays are done using either PBS, pH 7.2, or filter-sterilized urine.

Uroepithelial Cells

First morning urine (~100 ml), collected aseptically in a sterile urine specimen cup by a cooperative individual who has had no urinary tract infection (UTI) or antibiotics in the last 6 months, is transported on ice

[6] J. H. Miller, "Experiments in Molecular Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1992.

to the laboratory within 2 hr. Uroepithelial cells (UEC) are isolated by centrifugation (4000 *g*, 10 min, 4°), washed three times with cold PBS, pH 7.2, suspended in 1 ml PBS, quantified in a hemocytometer (Fig. 1a), and adjusted to a density of 2×10^5 cells/ml.

Immediately following the collection, harvesting, and preparation of the UEC suspension, the optimal viability of donor cells is assessed by exclusion of the viability stain erythrosin B (Sigma, St. Louis, MO) (see below). Extensive experience has shown that, after 30 min of incubation, 50% (100/200) of the UEC are viable; after 60 min, 21% (41/200) exclude stain. Viability remains at that level (21%; 42/200) in samples taken at 180 min. Of viable cells obtained from urine of a single donor, 98% (2463/2515) are of squamous morphology and 2% (52/2515) are of transitional morphology.

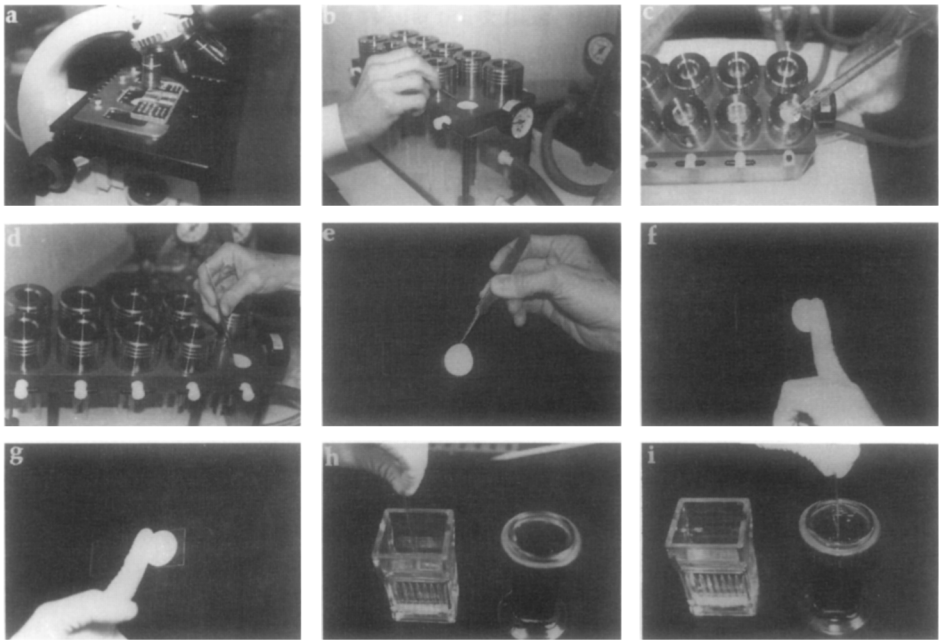

FIG. 1. Selected steps in the adhesion assay. (a) Quantification of UEC using a hemocytometer. (b) Prewet filter (12-μm pore size) is placed on filtration apparatus. (c) After addition of UEC–bacteria suspension, filters are washed with PBS. (d) Filter is removed with a pair of forceps and (3) placed upside down on microscope slide. (f, g) Finger is rolled across the entire filter to transfer UEC to the surface of the slide. (h) Sample is fixed in ethanol. (i) Sample is stained with Giemsa.

Selection of Cell Donor

For continuity, a single donor may be preferred over a pool of donors. To select a single donor of UEC for the assay, individual adhesion assays can be done using the UEC obtained from first morning urines from several healthy individuals who have not had a UTI or taken antibiotics in the last 6 months. Day-to-day reproducibility of mean adherent endogenous bacteria (bacteria adherent to UEC at the time of collection) per individual is determined. A donor whose cells have provided consistent adhesion values and levels of endogenous bacteria no greater than 5 bacteria per UEC is suitable. Also note that when female donor(s) are used, levels of adhering bacteria may be influenced by the time within the menstrual cycle when UEC are isolated, for example, with *Proteus mirabilis*.[7]

Assay Method

Equal volumes (0.2–0.5 ml) of bacterial suspension (2×10^8 cells/ml) and UEC suspension (2×10^5 cells/ml) are mixed in glass tubes (10×75 mm) in the presence of 50 m*M* mannose (Sigma) to prevent mannose-sensitive adhesion[8] to uromucoid,[3] sealed with cork or rubber stoppers, and incubated at 37° on a test-tube rotator at 8.5 rpm (Roto-Mix, Fisher Scientific, Pittsburgh, PA). After 20 min, 0.1 ml erythrosin B (0.8%, w/v, in PBS) is added, and incubation is continued for 10 min. The suspension is then filtered through nitrocellulose filters (8-μm pore diameter; Millipore, Bedford, MA) under 20 psi (103.5 kPa) vacuum and washed three times with 5 ml ice-cold PBS (Fig. 1b,c). The filters are removed (Fig. 1d) and placed upside down on a carefully cleaned glass microscope slide (Fig. 1e). The UEC are transferred to the microscope slide by gentle rolling finger pressure (Fig. 1f,g); the filters are peeled from the slide and discarded. The slides are air dried, fixed for 5 min in 70% (v/v) ethanol (Fig. 1h), stained with freshly diluted Giemsa stain (1:50, v/v) for 10 min (Fig. 1i), rinsed with distilled water, and air dried. Control slides of (1) endogenous bacteria of UEC (no added bacteria), (2) a known adherent strain, for example, P-fimbriated *E. coli* SH1,[9] and (3) a known nonadherent strain, for example, *E. coli* HB101, are included with each run. Selection of appropriate control strains will, of course, depend on the desired application.

[7] C. Svanborg-Eden, P. Larsson, and H. Lomberg, *Infect. Immun.* **27,** 804 (1980).
[8] D. C. Old and S. S. Scott, *J. Bacteriol.* **146,** 404 (1981).
[9] R. A. Hull, R. E. Gill, P Hsu, B. H. Minshew, and S. Falkow, *Infect. Immun.* **33,** 933 (1981).

Quantification of Adhesion

Slides are observed under oil immersion (×1000, total magnification) (Fig. 2). Bacteria that touch the border of or rest within the boundary of the UEC are counted on each of the first 40 epithelial cells observed, starting at the top left of the cell imprint. An epithelial cell is counted if it (1) excludes viability stain completely; (2) has a well-defined border (no

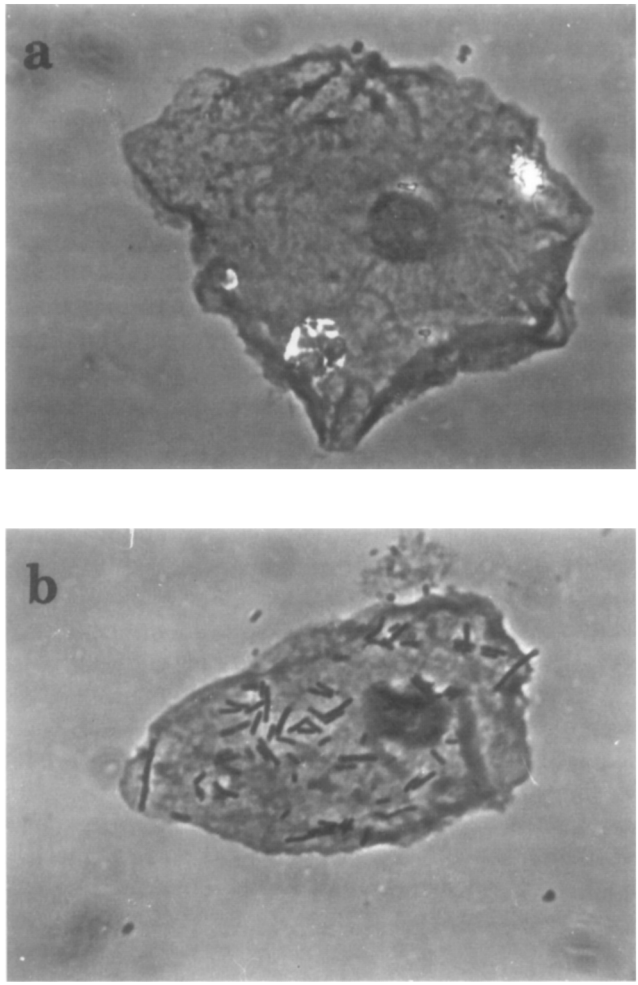

FIG. 2. Light microscopy of uroepithelial cells without (a) and with (b) adherent bacteria. Note that the UEC is intact, not folded back over itself, and does not have other cells overlapping the border. Total magnification is 1000×.

overlapping cells); (3) is not folded back onto itself; (4) is in a field free of background bacteria (patches of organisms found within a field not associated with UEC); and (5) is not visibly fragmented. Because of the inherent variability, assays should be done in triplicate.

Analysis of Data

The decision to quantify adherent bacteria by direct visualization rather than by addition of radiolabeled organisms is based on the types of information that can be obtained. (1) By observing cells directly, viable cells can be singled out for counting and nonviable cells excluded. In addition, the percentage of viable epithelial cells can be assessed at the end of each experiment. (2) Endogenously bound and background bacteria can be accurately assessed. A high endogenous microbiota has been shown to protect against adhesion of exogenously added bacteria.[10] The possibility of inhibition by endogenous bacteria cannot be detected in the routine assay quantifying radiolabeled bacteria. (3) Direct observation also allows the investigator to avoid areas of nonspecific background bacteria or adhesion of organisms to cellular and noncellular debris. (4) By direct visualization of cellular morphology, one can quantify adhesion to both squamous epithelial cells, which originate primarily from the lining of the urethra, and transitional epithelial cells that line the bladder wall. (5) The distribution of bacteria on epithelial cells can be examined; that is, the number of bacteria on each cell can be quantified, which is not possible when radiolabeled bacteria are used.

Statistical Analyses

In most reports using *in vitro* adhesion assays, values for adherent bacteria are reported as the mean number of bacteria per UEC. Many reports include adhesion values with rather narrow standard deviation ranges about the mean as a result of individual assays.[1,4,11,12] We have observerd a broad distribution pattern (Fig. 3), which is also reported for a limited number of observations by Mardh *et al.*[13] This may be due to the large range in size of UEC, the mean area of which was found by us to be $1820 \pm 915\ \mu m^2$. Moreover, the distributions of adherent bacteria are

[10] R. C. Y. Chan, A. W. Bruce, and G. Reid, *J. Urol.* **131,** 596 (1984).

[11] A. W. Bruce, R. C. Y. Chan, D. Pinkerton, A. Morales, and P. Chadwick, *J. Urol.* **130,** 293 (1983).

[12] G. Reid, M. L. Zorzitto, A. W. Bruce, M. A. S. Jewett, R. C. Y. Chan, and J. W. Costerton, *Curr. Microbiol.* **11,** 67 (1984).

[13] P. A. Mardh, S. Colleen, and B. Hovelius, *Invest. Urol.* **16,** 332 (1979).

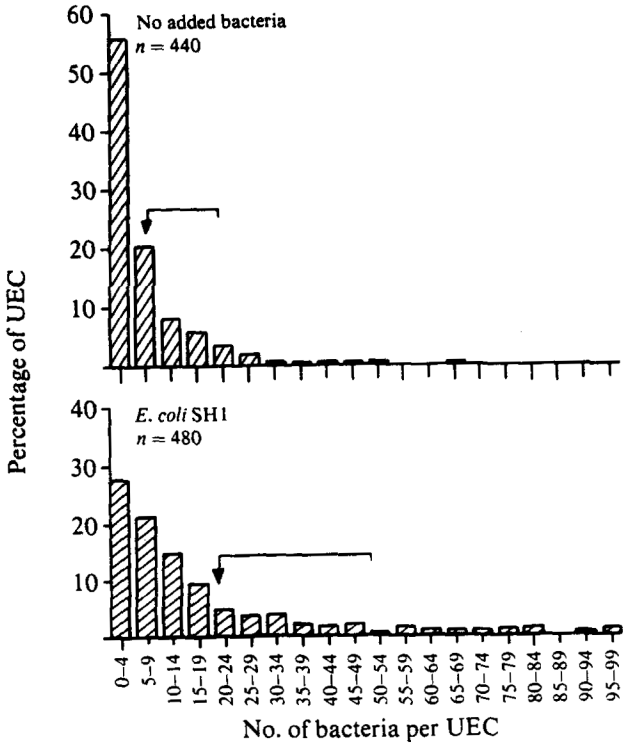

FIG. 3. Frequency distribution of numbers of bacteria per UEC. Results from a number of adhesion assays done under the optimal conditions were pooled. The percentage of UEC bearing a given number of bacteria is plotted for endogenous (no added) bacteria and *E. coli* SH1. *n* is the number of UEC for which adherent bacteria were quantified. The arrows indicate the mean; the bars span one standard deviation. *Escherichia coli* SH1 was grown on Trypticase soy agar. [Adapted with permission from H. L. T. Mobley, G. R. Chippendale, J. H. Tenney, and J. W. Warren, *J. Gen. Microbiol.* **132,** 2863 (1986).]

neither normal nor Poissonian, but are skewed, suggesting that the mean and standard deviation are not the ideal measure of adhesion (Fig. 3). It is suggested that the value "percentage of UEC with over 10 adherent bacteria" may prove valuable in assessing adhesion. This value does not assume a normal distribution, can be compared with other values by the chi-squared (χ^2) test, and does not give undue weight to a few cells with large numbers of bacteria per UEC.

An example is seen in Fig. 3 where the mean number of bacteria per UEC for "endogenous" (no added) bacteria is 8.2 ± 14.1 and that for *E. coli* SH1, a P-fimbriated strain, is 20.9 ± 29.4. Although the values are significantly different ($p < 0.0001$ by the *t*-test), comparison of these

nonnormally distributed values by the *t*-test may not be appropriate. If, however, the number of UEC with over 10 bacteria per UEC [values for endogenous bacteria of 20.0% (88/440)] are compared with *E. coli* SH1 of 46.0% (221/480), the same level of significance ($p < 0.0001$) is found using the χ^2 test. This latter test does not assume a normal distribution. By adopting a significance level of $p = 0.01$ and using values determined experimentally for a nonadherent bacterial strain and using mean adhesion values (±standard deviation) for triplicate assays (3×40 UEC), a cutoff point for statistically significant adhesion can be determined for the specific bacterial strain under investigation.

[29] Adhesion of Mycoplasmas

By ITZHAK KAHANE

Introduction

Mycoplasma is the trivial name of the class Mollicutes, a group of minute wall-less bacteria now comprising more than 120 species.[1] They are characterized by their small genomic size, which is in the range of 5×10^8 to 2×10^9 Da—the smallest of bacteria. They are also the smallest self-replicating organisms but, in order to replicate, require a complex medium. Most mycoplasmas are parasites, and many are pathogens of a vast variety of hosts, spanning the entire spectrum of flora and fauna, including humans. In addition, they are considered to be pests of cells in culture.

Evidence has been provided for at least several pathogenic mycoplasmas, including *Mycoplasma pneumoniae, M. pulmonis,* and *M. gallisepticum,* that adhesion is a prerequisite for their pathogenicity. It is not surprising, therefore, that adhesion to the host cells has been studied extensively over the past three decades. The thrust was fueled by the rationale that by revealing the nature of the adhesion, better ways would be found for protection from infection by inhibition of adhesion and also by the need to develop effective vaccines. This aim is still ahead of us. In addition, studies conducted primarily on six mycoplasmas, namely, *M. pneumoniae, M. genitalium, M. hominis, Ureaplasma urealyticum, M. gallisepticum,* and *M. pulmonis,* indicate that it is not an easy goal to reach. This is due to the fact that the adhesion process in mycoplasmas is complex, and, in

[1] J. G. Tully, *in* "Rapid Diagnosis of Mycoplasmas" (I. Kahane and A. Adoni, eds.), p. 3. Plenum, London, 1993.

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addition to specific adhesins, other membrane components and auxiliary proteins are involved. These aspects have been reviewed by Kahane and Horowitz[2] and Baseman.[3] The possibility that some mycoplasmas may fuse with host cells[4] or penetrate into the cells[2] adds to the complexity of the adhesion process.

Obviously, the morphological elements of adhesion can be studied primarily using electron microscopic techniques. These are summarized elsewhere in this volume.[13] Methods and details specifically related to studying mycoplasmas have been reviewed by Kahane and Horowitz[2] and Saada *et al.*[5] Such studies have revealed that: (1) many nonsymmetrical mycoplasmas (e.g., *M. pneumoniae, M. genitalium,* and *M. mobile*) adhere via their tips; (2) the adhesion is an intimate event occurring between the membranes of the mycoplasmas and those of the host cell. This led to the assumption that mycoplasmas are membrane parasites; (3) some mycoplasmas (e.g., *Ureaplasma urealyticum* and *M. equigenitalium*) also adhere to cilia of the host epithelial cells; and (4) some mycoplasmas penetrate the host cells (e.g., *M. penetrans*).

Details of the adhesion process can only be characterized using quantitative assays. Those in use are detailed here and include (1) mycoplasma adhesion to ligand adsorbed to a solid surface matrix, (2) cells–enzyme-linked immunosorbent assay (ELISA) for detection of mycoplasmas adhering to cells in culture, and (3) adhesion-blocking antibody assay. The reader is also referred to an assay for adhesion of mycoplasmas to cells in suspension, as detailed by Kahane and Bredt.[6] That assay is now used less frequently because it involves many centrifugations; therefore, it is not detailed here. However, it proved very useful in many studies, among them those of the group of T. Feizi, regarding the sialic acid linkage of the sialoglycoprotein receptor to which *M. pneumoniae* adheres (see review by Kahane and Horowitz[2]).

Biosafety

It should be mentioned that some mycoplasmas are human pathogens, and therefore all necessary precautions should be taken before conducting the assays.

[2] I. Kahane and S. Horowitz, *in* "Subcellular Biochemistry: Mycoplasma Membranes" (S. Rottem and I. Kahane, eds.), Vol. 20, p. 225. Plenum, London, 1993.

[3] J. B. Baseman, *in* "Subcellular Biochemistry: Mycoplasma Membranes" (S. Rottem and I. Kahane, eds.), Vol. 20, p. 243. Plenum, London, 1993.

[4] M. Tarshis, R. Salman, and S. Rottem, *FEMS Microbiol. Lett.* **82,** 67 (1991).

[5] A. Saada, E. Rahamim, I. Kahane, and Y. Beyth, *in* "Rapid Diagnosis of Mycoplasmas" (I. Kahane and A. Adoni, eds.), p. 111. Plenum, London, 1993.

[6] I. Kahane and W. Bredt, *in* "Methods in Mycoplasmology" (J. G. Tully and S. Razin, eds.), Vol. 2, p. 345. Academic Press, New York, 1983.

1. Mycoplasma Adhesion to Ligands Adsorbed to Solid Surface Matrix

The assay is based on the studies of Krivan *et al.*[7] and Roberts *et al.*[8] and is useful in experiments aiming to identify purified host cell components (receptors) to which mycoplasmas adhere.

Procedure

Mycoplasmas are metabolically labeled by growth in [^{3}H]palmitate[6,7] or [^{35}S]methionine.[5] The organisms are harvested and washed by centrifugation and suspended in 50 m*M* Tris-HCl, pH 7.6, 150 m*M* NaCl, 1 m*M* $CaCl_2$, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF) buffer to 1 mg cell protein/ml.

Preparation of Immobilized Ligands. Preparation of immobilized ligands is described for glycolipids or glycoproteins, but the approach can be adapted to other ligands as well.

Glycolipids. Purified glycolipids are serially diluted in microtiter plate wells in 25 μl of methanol–lipid solution. The solvent is dried by evaporation, and the wells are filled with Tris–BSA buffer [50 m*M* Tris-HCl, pH 7.6, 110 m*M* NaCl, 5 m*M* $CaCl_2$, 0.2 m*M* PMSF, 1% (w/v) bovine serum albumin (BSA)], incubated for 1 hr at 37°, and rinsed with RPMI 1640 medium containing 1% (w/v) BSA (Sigma, St. Louis, MO, fatty acid free) and 25 m*M* HEPES, pH 7.3 (RPMI–BSA).

Glycoproteins. Glycoproteins, dissolved in phosphate-buffered saline (PBS; 10 m*M* sodium phosphate buffer, pH 7.4, containing 150 m*M* NaCl, 1 m*M* $CaCl_2$, and 0.01% NaN_3), are dispensed (100 μl) in the wells of microtiter plates and incubated for 16 hr at 4°. Unbound proteins are removed, and the wells are filled with Tris–BSA buffer, incubated for 30 min at room temperature, and rinsed with RPMI–BSA.

Assay. Labeled mycoplasma suspension (25 μl) is added to every well of the microplate. For inhibition studies, various ligands in RPMI–BSA are added to the appropriate wells. The plates are incubated for 2 hr at 37°. The wells are washed five times with PBS. The radioactivity of bound mycoplasmas in each well is quantified by scintillation counting.

Comments

The assay is reproducible, and one of its advantages is that the ligands are immobilized on the matrix to allow easy separation of bound and free

[7] H. C. Krivan, L. D. Olson, M. F. Barile, V. Ginsburg, and D. D. Roberts, *J. Biol. Chem.* **264,** 9283 (1989).

[8] D. D. Roberts, L. D. Olson, M. F. Barile, V. Ginsburg, and H. C. Krivan, *J. Biol. Chem.* **264,** 9289 (1989).

mycoplasmas. The optimal adhesion time should be determined for the mycoplasma tested. The assay can also be modified to characterize the adhesion of solubilized mycoplasma components. Evaluation of the adhering mycoplasma material can be assessed, not only by radioactivity, but also by specific antibodies which can be detected in an ELISA or by other means, as detailed in the following section.

A modification of the assay by which lipids to which mycoplasmas adhere can be distinguished from the total lipid fraction was described by Krivan *et al.*[7] In the modified assay, the glycolipids (or lipids) are first separated by thin-layer chromatography (TLC), which serves as the solid phase. The TLC plate is then overlaid with mycoplasmas, rinsed, dried, and subjected to autoradiography.

2. Cell–Enzyme-Linked Immunosorbent Assay for Detection of Mycoplasmas Adhering to Cells in Culture

The assay is based on that reported by Henrich *et al.*[9] for the detection of *M. hominis* adhering to cells in culture using specific antibodies and capture ELISA.

Procedure

The mycoplasmas are grown, harvested, and washed as in the previous section; however, no radioactive tracer is needed. The cell culture is grown to confluency in 96-well flat-bottom microplates in the appropriate culture medium, for example, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. The wells are washed twice with DMEM prewarmed to 37°. Mycoplasma suspension [100 μl, $\sim 10^7$ colony-forming units (cfu)] is added and incubated at 37°. Adhesion usually reaches a plateau after 2 hr; however, the incubation time should be tested and an appropriate number of plates prepared. Unbound mycoplasmas are washed away with DMEM. Cytadherent mycoplasmas are detected with mycoplasma-specific murine monoclonal antibodies incubated for 2 hr at 37° using a capture ELISA.[10]

Comments

The test is reproducible and relatively fast. The specificity of the antibodies for the mycoplasmas should be evaluated. Instead of using the ELISA,

[9] B. Henrich, R.-C. Feldmann, and U. Hadding, *Infect. Immun.* **61,** 2945 (1993).

[10] B. Gerstenecker and E. Jacobs, *in* "Rapid Diagnosis of Mycoplasmas" (I. Kahane and A. Adoni, eds.), p. 195. Plenum, London, 1993.

one can evaluate the quantity of adhering mycoplasmas employing radiolabeled mycoplasmas or by *in vitro* DNA amplification (e.g., polymerase chain reaction).[11]

3. Adhesion-Blocking Antibody Assay

The adhesion-blocking antibody assay was developed by Jacobs *et al.*[12] during studies of *M. pneumoniae* major adhesin P1, a 170-kDa membrane protein (reviewed by Razin and Jacobs[13]). In addition to being an important virulence factor, P1 is also a major protein antigen of this human pathogen. Using the Western immunoblot technique, it was found that, especially during the first contact with *M. pneumoniae* P1, early and prominent serum antibodies were directed to the P1 protein. To test them, the adhesion inhibition assay (AIA) was devised as a routine diagnostic approach.[12] Moreover, the AIA was found to be useful in screening supernatants of large libraries of monoclonal antibody-producing clones for those monoclonal antibodies with the capacity to inhibit adhesion of *M. pneumoniae*[14] or *M. genitalium*.[15]

Assay

Mycoplasma stock is grown in Roux bottles containing Hayflick's modified Edward's medium for about 48 hr at 37°. The supernatant is removed, and the glass-adherent mycoplasmas are washed with room temperature equilibrated PBS. Using a cell scraper, the mycoplasmas are harvested, suspended in 100 ml of fresh mycoplasma medium, and stored at −70° in 1-ml aliquots for further use. The stock suspension is thawed and sonicated by three short pulses to disrupt the microcolonies, diluted in 50 ml mycoplasma medium, and dispensed in 100-μl volumes into each well of sterile 96-well flat-bottom ELISA microtiter plates. The plates are sealed with polypropylene ELISA plate sealer and incubated overnight at 37°. The mycoplasma medium of each well is exchanged with fresh (100 μl) medium, and the plates are incubated for about 18–24 hr at 37°. Plates are fit for the AIA when there is only a slight indicator color change to orange, whereas plates with an indicator change to yellow are already overgrown with mycoplasmas and should be discarded.

[11] R. Harasawa, T. Uemori, K. Asada, and I. Kato, *in* "Rapid Diagnosis of Mycoplasmas" (I. Kahane and A. Adoni, eds.), p. 227. Plenum, London, 1993.
[12] E. Jacobs, K. Schöpperle, and W. Bredt, *Eur. J. Clin. Microbiol.* **4,** 113 (1985).
[13] S. Razin and E. Jacobs, *J. Gen. Microbiol.* **138,** 407 (1992).
[14] B. Gerstenecker and E. Jacobs, *J. Gen. Microbiol.* **136,** 471 (1990).
[15] O. Opitz and E. Jacobs, *J. Gen. Microbiol.* **138,** 1785 (1992).

To start the assay, discard the medium from the microtiter plates containing the mycoplasmas. The first row of the ELISA plate is filled only with PBS (without antibodies) to test the maximum adhesion of erythrocytes (positive control). The other wells receive 100-μl aliquots of serum (if human serum is used, heat-inactivate at 50° for 30 min) or monoclonal antibodies and two-fold dilutions prepared earlier in a separate microtiter plate. The plates are incubated for 1 hr at 37°. The microtiter plates are then washed gently twice with PBS using a microtiter pipette. To avoid washing off mycoplasmas, automatic ELISA plate washing machines should not be used. One hundred microliters of sheep erythrocyte suspension ($\sim 3 \times 10^8$ cells/ml), which has not been stored longer than 1 week in Alsever's solution, is added per well, and plates are incubated for an additional 1 hr at 37°. Each well is filled to the top with PBS and then sealed with a new strip of polypropylene sealing tape. The microtiter plate is inverted for 15 min to allow nonattached erythrocytes to sink down to the sticky tape, which is removed, and the plates are washed again with 100 μl PBS per well. Adherent erythrocytes are lysed with 100 μl of distilled water per well for 10 min. The light absorbance of hemoglobin from the lysed sheep erythrocytes is measured at 414 nm. The average absorbance of the first row (positive control) of each plate is taken as 100% (maximum adhesion). A serum dilution is considered to be positive if the antibodies in it inhibited at least 50% adhesion of erythrocytes.

Comments

The test is specific, rapid, and relatively inexpensive, and it does not demand the use of radioactive material. In addition, no false-positive reactions occur in sera from patients with autoantibodies. The latter can lead to false-positive results in the complement fixation test. However, its major disadvantage is that it is less sensitive than the complement fixation test or other serological tests used in serodiagnosis of *M. pneumoniae* diseases.[16] The limited sensitivity depends on the fact that, although during *M. pneumoniae* diseases prominent specific antibody responses are directed to the P1 protein, many of the antibodies are not directed to the regions of the P1 protein which support adhesion, but to other epitopes of the P1 protein with no function in adhesion.[17] On the other hand, the AIA is a useful tool in screening monoclonal antibody-secreting clones for antibodies with adhesion-inhibiting capacity or to follow up the production of adhesion-inhibiting antibodies in hybridoma cultures.[14]

[16] E. Jacobs, *Clin. Infect. Dis.* **17**(Suppl. 1), S79 (1993).
[17] E. Jacobs, *Rev. Med. Microbiol.* **2**, 83 (1991).

Conclusions

In employing the mycoplasma adhesion assays, one should bear in mind that, in addition to adhering to host cells, some mycoplasmas adhere to surfaces.[6] This should be minimized by using plastic or glass to which mycoplasmas adhere less, by treating the surfaces with silicones,[6] or by blocking the inert surface or minimizing its exposed part (e.g., by confluent growth of host cells).

The procedures detailed have potential use in current major studies of mycoplasma adhesion: (1) in studies of entire mycoplasmas, especially the newly described species (e.g., *M. penetrans*) and those that are incriminated in activating human immunodeficiency virus (HIV)[18–20]; (2) in studies of the identified adhesins (in this context, readers are referred to the concept of a superfamily of the adhesins so far suggested[3] to include *M. pneumoniae*, *M. genitalium*, and *M. gallisepticum*); and (3) in studies of antigenic variation which indicate variation of exposure of surface components.[21]

[18] S.-C. Lo, S. Tsai, J. R. Benish, J. W.-K. Shih, D. J. Wear, and D. M. Wong, *Science* **251,** 1074 (1991).

[19] L. Montagnier, D. Berneman, D. Guetard, A. Blanchard, S. Chamaret, V. Rame, J. Van Rietschoten, K. Mabrouk, and E. Bahraoui, *C.R. Acad. Sci. Ser. 3* **311,** 425 (1990).

[20] R. Nir-Paz, S. Israel, A. Honigman, and I. Kahane, *Proceedings of the 9th International Congress of IOM, Ames, Iowa,* August 1992.

[21] K. Wise, D. Yogev, and R. Rosengarten, *in* "Antigenic Variation in Mycoplasmas, Molecular Biology and Pathogenesis" (J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds.). p. 473. American Society for Microbiology, Washington, D.C., 1992.

[30] Adhesion of Oral Bacteria to Soft Tissue

By DIANE H. MEYER and PAULA M. FIVES-TAYLOR

Introduction

The oral cavity has a variety of features which make it ideal for the study of bacteria–host interactions. It contains several types of surfaces in a moist, warm, nutrient-rich environment, an environment ideal for bacterial colonization. Adhesion is a prerequisite for colonization of oral bacteria as the flow of saliva removes exposed bacteria which are not firmly bound.[1] Bacteria adhere to oral surfaces in a highly specific manner. The physical conditions in the mouth and components derived from the bacterium, from

[1] R. J. Gibbons, *J. Dent. Res.* **68,** 750 (1989).

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[21] K. Wise, D. Yogev, and R. Rosengarten, *in* "Antigenic Variation in Mycoplasmas, Molecular Biology and Pathogenesis" (J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds.). p. 473. American Society for Microbiology, Washington, D.C., 1992.

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the host, from the diet of the host, and from other bacteria are responsible for the specificity.[1]

The evidence is now overwhelming that certain oral bacterial species are etiologic agents or periodontal diseases and dental caries.[2] Whereas bacteria accumulate in large numbers on teeth (referred to as dental plaque), epithelial cell desquamation precludes large accumulations on mucosal surfaces such as gingival tissue.[1] Gram-positive organisms, such as the streptococci and to a lesser extent the actinomycetes, are associated with healthy gingival tissue.[1] In gingivitis the proportions and types of bacteria change, and the actinomycetes and gram-negative forms associated with periodontal disease predominate.[1] These include *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia* (*Bacteriodes intermedius*), *Eikenella corrodens,* and *Fusobacterium nucleatum.*[3] *Treponema denticola,* an oral spirochete, is also associated with periondontal disease.[1,2]

In this chapter procedures utilized to study adhesion of oral bacteria to soft tissue are described. The focus is on *A. actinomycetemcomitans* and *P. gingivalis*; this is not meant to lessen the importance of other oral pathogens. Many of the techniques and approaches are basically similar regardless of the bacterial strain and will be discussed in general terms.

Growth Conditions and Maintenance of Oral Bacteria

Most bacteria that colonize the soft tissues of the mouth are slow growing and fastidious. Many are anaerobes or capnophilic organisms that can be isolated and cultured only under very specific conditions. Periodontopathic organisms are isolated[4] by insertion of a medium or fine paper point (Johnson & Johnson, New Brunswick, NJ) for 10 sec to the depth of the periodontal pocket. The paper point is placed in a tube with 2 to 3 ml of prereduced anaerobically sterilized VMGA III [5.0% gelatin, 0.05% tryptose, 0.05% Thiotone E Peptone (Becton Dickinson, Cockeysville, MD), 0.2% washed agar, 0.05% thioglycolic acid, 0.05% L-cysteine hydrochloride, 1.0% sodium glycerophosphate, 0.0005% phenylmercuric acetate, 0.0003% methylene blue, 0.024% $CaCl_2$, 0.042% KCl, 0.1% NaCl, and 0.01% $MgSO_4$] and vortexed for 30 sec to elute the microorganisms. Serial dilutions (10- or 100-fold steps) are made in VMGA III and plated on selective medium. *Actinobacillus actinomycetemcomitans* selective medium is tryptic soy agar supple-

[2] F. L. Macrina, *Adv. Exp. Biol. Med.* **327,** 25 (1992).
[3] R. J. Gibbons, D. I. Hay, W. C. Childs III, and G. Davis, *Arch. Oral Biol.* **35,** 107S (1990).
[4] J. Slots, *Oral Microbiol. Immunol.* **1,** 48 (1986).

mented with 10% defibrinated horse serum, 0.1% yeast extract (YE), bacitracin (75 μg/ml), and vancomycin (5 μg/ml).[5] *Porphyromonas gingivalis* selective medium is tryptic soy agar supplemented with 5% defibrinated sheep blood, hemin (5 μg/ml), menadione (1 μg/ml), and kanamycin (40 μg/ml).[6]

Actinobacillus actinomycetemcomitans Growth and Maintenance Conditions

Capnophilic bacteria, such as *A. actinomycetemcomitans*, require CO_2 for growth. *Actinobacillus actinomycetemcomitans* is cultured[7,8] in tryptic soy broth or agar with 0.6% yeast extract (TSB–YE) at 37° in a humidified atmosphere of 10% (v/v) CO_2 in air or under anaerobic conditions. TSB (no YE) supplemented with 10 μ*M* cystine and nutrient-rich medium, such as brain–heart infusion broth, are also suitable. Special attention must be paid to culture conditions and phase of growth as both affect *A. actinomycetemcomitans* adhesion and cell surface ultrastructure.[9] A growth curve should be determined for each strain used. *Actinobacillus actinomycetemcomitans* strain SUNY 465 grown statically in TSB-YE in 10% CO_2 has a doubling time of 180 min[8] and enters stationary growth phase at an optical density (495 nm) of 0.25; other strains exhibit somewhat different growth characteristics.

Porphyromonas gingivalis Growth and Maintenance Conditions

Anaerobes, such as *P. gingivalis,* must be grown in an anaerobic chamber in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 (v/v).[10] Anaerobic chambers can be obtained from Coy Manufacturing (Ann Arbor, MI). An alternative (smaller scale) is to use a Gas Pak anaerobic system (Becton Dickinson). *Porphyromonas gingivalis* growth medium must be supplemented with hemin (0.5 μg/ml) and menadione (1 μg/ml). Blood agar [5% (v/v) blood] which contains hemin and menadione (BAPHK) is the preferred solid medium.

[5] J. Slots, *J. Clin. Microbiol.* **15,** 606 (1982).
[6] J. J. Zambon, H. S. Reynolds, and J. Slots, *Infect. Immun.* **32,** 198 (1981).
[7] D. H. Meyer, P. K. Sreenivasan, and P. M. Fives-Taylor, *Infect. Immun.* **59,** 2719 (1991).
[8] P. K. Sreenivasan, D. H. Meyer, and P. M. Fives-Taylor, *Oral Microbiol. Immunol.* **8,** 361 (1993).
[9] D. H. Meyer and P. M. Fives-Taylor, *Infect. Immun.* **62,** 928 (1994).
[10] M. J. Duncan, S. Nakao, Z. Skobe, and H. Xie, *Infect. Immun.* **61,** 2260 (1993).

In Vitro Adhesion Assays

Actinobacillus actinomycetemcomitans Assays

Viable Cell Assay. The KB cell line, derived from a human oral epidermoid carcinoma, is used for the viable cell assay.[7,9,11] Frozen stocks are maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS) and 8% dimethyl sulfoxide. Cells are cultured in RPMI 1640 supplemented with 5% FBS and 50 μg/ml gentamicin in 75-cm^2 flasks at 37° in a humidified atmosphere of 5% (v/v) CO_2 in air. Cultures are split twice weekly by treatment with 0.5 m*M* EDTA followed by 0.05% trypsin to detach cells. Approximately 6×10^5 KB cells in antibiotic-free RPMI 1640 are seeded onto sterile 12-mm-diameter glass coverslips in wells of 24-well (16 mm diameter) tissue culture plates and incubated for 18 hr. An inverted microscope is used to determine that the monolayer is confluent. It is absolutely necessary that no glass or plastic is exposed because *A. actinomycetemcomitans* adheres to those surfaces. *Actinobacillus actinomycetemcomitans* from overnight cultures are diluted in fresh TSB–YE and incubated until the desired optical density (495 nm) is reached. Bacterial number is determined from a standard curve which relates optical density of the suspended cells to colony counts. Bacteria are pelleted, either not washed or washed by suspension and centrifugation in phosphate-buffered saline (PBS), suspended in antibiotic-free RPMI 1640 at densities to obtain the desired multiplicity of infection (MOI), and added to wells which contain the KB monolayer. For standard assays an MOI of 100 bacteria to 1 KB cell is used. Washing with PBS will remove or alter the concentrations of certain surface components associated with *A. actinomycetemcomitans* adhesion.

Plates are incubated at 37° in an atmosphere of air containing 5% (v/v) CO_2 for 2 hr. The medium is removed from the infected monolayer by aspiration and washed three times with PBS which contains 0.5 m*M* $MgCl_2$ and 1.0 m*M* $CaCl_2$ (Mg^{2+}–Ca^{2+} PBS) to remove nonadherent bacteria. The Mg^{2+} and Ca^{2+} are present in PBS to maintain the integrity of the cell monolayer. Cells are released by the addition to each well of 0.1 ml of 0.5% Triton X-100 (in PBS) and agitation of the monolayer with the pipette tip, and 2.0 ml of PBS is added immediately to dilute the detergent. This step must be carried out rapidly, as prolonged exposure of *A. actinomycetemcomitans* to the detergent is bactericidal. Bacterial suspensions are diluted further (10^2), spread onto TSB–YE agar, and colony-forming units (cfu) are enumerated after 2 days of incubation. Triplicate

[11] D. H. Meyer and P. M. Fives-Taylor, *Infect. Immun.* **61,** 4933 (1993).

or quadruplicate measurements are carried out for each determination. KB monolayers are monitored throughout the course of assays to ensure that the monolayer remains intact.

The KB viable cell assay provides a good model of *A. actinomycetemcomitans* adhesion to epithelial cells, but it is restrictive in several ways. First, the number of fractions that can be assayed at one time is limited; second, it is based on the recovery and plating of viable *A. actinomycetemcomitans* which have a long generation time, and thus experimental results are not known for 2 days; and third, it necessitates the maintenance of both *A. actinomycetemcomitans* and KB cell viability. To circumvent the restrictions of the viable cell assay, two additional methods for detection of *A. actinomycetemcomitans* adhesion to KB cells have been developed.

Cell Enzyme-Linked Immunoadsorbent Assay. The enzyme-linked immunosorbent assay (ELISA),[12] a modification of a method[13] developed in our laboratory, uses a rabbit polyclonal antiserum to whole *A. actinomycetemcomitans.* KB cells (as above) are added to wells of 96-well (flat bottom) plates and incubated until monolayers reach confluency. The medium is removed, the monolayer is washed twice with Mg^{2+}–Ca^{2+} PBS, and cells are fixed in 0.25% glutaraldehyde (in PBS) at room temperature for 10 min. The glutaraldehyde is removed, the monolayer is washed twice with PBS, and 1% (w/v) bovine serum albumin (BSA) in PBS is added to block any exposed plastic and prevent nonspecific binding. The BSA is removed by rinsing each well twice with PBS. Cells of *A. actinomycetemcomitans* ($\sim 10^8$) are added to the cell monolayer, and the plate is incubated at 37° for 1 hr. The monolayer is washed with PBS, antiserum diluted in 1% BSA (in PBS) is added, and the plate is incubated for 1 hr at room temperature. Immune complexes are detected with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin diluted in 1% BSA. Conjugated antibody is removed by washing the monolayer with PBS which contains 0.05% (v/v) Tween 20, and enzymatic activity is determined by incubation with hydrogen peroxide in the presence of *o*-phenylenediamine in citrate buffer, pH 5.0. The reaction is stopped by the addition of 4 *N* H_2SO_4, and color development is quantitated by absorbance at 490 nm measured with a microtiter plate reader.

The ELISA can be used to screen nonadhesive mutants or to detect bacterial receptors on mammalian cells. The use of the assay to detect bacterial surface molecules involved in adhesion is limited because it uses antibodies directed toward these molecules.

[12] K. P. Mintz and P. M. Fives-Taylor, *Infect. Immun.* **62,** 3672 (1994).
[13] B. L. Elder, D. K. Boraker, and P. M. Fives-Taylor, *J. Clin. Microbiol.* **16,** 141 (1982).

[^{3}H]Thymidine-Labeled Bacterial Assay. KB monolayers in 96-well plates are fixed with glutaraldehyde and treated with BSA (as above).[12] Cells of *A. actinomycetemcomitans* are labeled by culture in TSB which contains 10 μM cystine and 5 μCi/ml [^{3}H]thymidine. Unincorporated label is removed by centrifugation and PBS washes. Labeled bacteria [~15,000 counts/min (cpm) or 10^7 bacteria] are added to monolayers and incubated at 37° for 1 hr. Monolayers are rinsed twice with PBS to remove nonadherent bacteria and solubilized by the addition of 0.5 *M* NaOH–0.1% sodium dodecyl sulfate (SDS). The solubilized cells are added to scintillation fluor, and the amount of radioactivity present is determined by scintillation spectroscopy. The radioassay can be used to study bacterial surface molecules involved in adhesion.

Porphyromonas gingivalis Assays

Viable Cell Assay. The *A. actinomycetemcomitans* viable cell assay has been modified to quantitate adhesion of *P. gingivalis* to KB epithelial cells.[10] KB cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum, 50 μg/ml gentamicin, 2 m*M* L-glutamine, and 10 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.3). For the assay, *P. gingivalis* cells are washed with PBS, diluted in DMEM which contains 1 m*M* $MgCl_2$ and 0.2 m*M* $CaCl_2$, added (10^8 cells or less) to PBS-washed confluent KB monolayers (~10^6 cells per well in a 24-well plate) and incubated in 5% (v/v) CO_2 at 37° for 2 hr. Assay medium is removed, and the monolayer is washed three times with agitation in PBS. KB cells are lysed by the addition of 1 ml of sterile distilled water per well and incubation at 37° for 10 min. Lysates are diluted, plated on BAPHK, incubated anaerobically at 37°, and colony-forming units are enumerated after 4 to 5 days of incubation.

Membrane Binding Assay. The membrane binding assay is a convenient procedure to screen *P. gingivalis* for the ability to bind to epithelial cells.[10,14] Bacteria are harvested from solid or liquid culture, washed twice with PBS, and suspended at 10^8 cells/ml in PBS. Bacteria (3.0 μl) are spotted onto Immobilon-P filters (Millipore, Bedford, MA) prewetted with methanol and then PBS. Filters are air-dried on 3MM paper (Whatman, Maidstone, U.K.) and then rewetted with methanol, then PBS. Filters bearing bacteria are transformed to 3MM paper soaked with 0.1% SDS in 10 m*M* Tris (pH 7.9) for 30 min at room temperature. This treatment intensifies binding of some bacteria to epithelial cells. Certain strains also require soaking with lysozyme (1 mg/ml in 10 m*M* Tris, pH 7.9–25 m*M* EDTA)

[14] J. M. Leong, L. Moitoso de Vargas, and R. R. Isberg, *Infect. Immun.* **60,** 683 (1992).

for 30 min at room temperature prior to SDS treatment to achieve consistent binding. It is presumed that SDS and lysozyme treatments enhance binding by unmasking or inducing changes in surface ligands.

Filters are incubated (colony side down) with shaking in 5% nonfat dry milk in PBS at room temperature for approximately 15 hr to block nonspecific binding. Filters are washed with PBS (3 times, 5 min with shaking at room temperature), blocked again for 1 hr, and washed. KB cells are washed with PBS twice and detached from flasks by treatment with 4.3 ml of 2 m*M* EDTA (in PBS) at 37° in 5% CO_2 for 20 min. $MgCl_2$ is added to a final concentration of 5 m*M*, and the KB cells are washed with 40 ml of 5 m*M* $MgCl_2$ (in PBS) and then with 1 m*M* $MgCl_2$–0.2 m*M* $CaCl_2$ (in PBS). The cells are suspended at approximately 10^6 cells/ml in DMEM (no serum) with 20 m*M* HEPES (pH 7.0), 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 0.4% BSA. KB cells are added to the bacteria-laden filters (2×10^5 cells/cm^2 or more are required), and the filters are incubated in 5% CO_2 at 37° for 90 min.

The filters are washed with PBS three times and fixed with 1% (v/v) glutaraldehyde (electron microscopy grade) in 100 m*M* PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.9]–50 m*M* KCl–4 m*M* $MgCl_2$ at room temperature for 22 min. One hundred fifty microliters of fixative is added per square centimeter of filter. Filters are washed three times with PBS, and KB cell alkaline phosphatase activity is assayed by the addition of nitro blue tetrazolium (100 μg/ml) and bromochloroindolyl phosphate (50 μg/ml) in 100 m*M* Tris (pH 9.5), 5 m*M* $MgCl_2$, and 100 m*M* NaCl at room temperature. The reaction is stopped after 30 min by submerging the filter in 2 m*M* EDTA in PBS. Dark spots on filters represent alkaline phosphatase activity and indicate that KB cells have bound to *P. gingivalis*. A uniform pale color is exhibited by filters on which no binding has occurred. Filter sections can be examined further with scanning electron microscopy.

Additional Cells and Transformed Cell Lines

Adhesion assays that use human buccal epithelial cells,[15] periodontal pocket epithelial cells,[16] and human gingival epithelium[17,18] have also been developed. At least one human gingival cell line, OEC-M1,[19] is also available.

[15] J. Slots and R. J. Gibbons, *Infect. Immun.* **19,** 254 (1978).

[16] R. J. Gibbons and J. van Houte, *Infect. Immun.* **3,** 567 (1971).

[17] M. J. Bobo, B. K. Saha, J. W. Dean, and J. P. Babu, *J. Dent. Res.* **72,** 412 (1993).

[18] R. J. Lamont, D. Oda, R. E. Persson, and G. R. Persson, *Oral Microbiol. Immunol.* **7,** 364 (1992).

[19] C.-L. Meng, C.-N. Tu, L.-C. Chang, S.-F. Hsu, and C.-H. Wu, *in* "Molecular Biology of Neoplasia" (E. H. Chang, J.-K. Lin, and P. C. Huang, eds.), p. 374. Academia Sinica, Taipei, 1985.

Factors Influencing Adhesion

Components on the surface of bacteria which are associated with adhesion include fimbriae, flagella, lipopolysaccharide, polysaccharide, microvesicles, and outer membranes.[20] Adhesion of oral bacteria to soft tissue is likely mediated by specific and nonspecific interactions of one or more of the adhesion determinants.

Actinobacillus actinomycetemcomitans Adhesion Components

Fimbriae. Most freshly isolated *A. actinomycetemcomitans* possess fimbriae and exhibit rough-surfaced colonies.[21,22,23] On subculture in the laboratory fimbriation is lost, and the colonial morphology changes from rough- to smooth-surfaced colonies. In liquid medium rough forms grow in clumps attached to the glass surface with no turbid growth. By contrast, smooth variants exhibit a uniformly turbid growth. Whereas it is likely that *A. actinomycetemcomitans* fimbriae function in adhesion, the smooth, nonfimbriated variants also exhibit adhesive properties,[9] which suggests that *A. actinomycetemcomitans* has multiple adhesins. The role of fimbriae in *A. actinomycetemcomitans* adhesion to epithelial cells has not been determined.

Fimbriae are isolated from *A. actinomycetemcomitans*[23] cultured aerobically in AA medium which contains, per liter, 0.5 g Na_2SO_4, 0.2 g KH_2PO_4, 0.4 g $MgCl_2$, 1.2 g NaCl, 0.3 g NH_4Cl, 0.3 g KCl, 0.11 g $CaCl_2$, 2.0 g yeast extract, 0.001 g resazurin, and 6.05 g Tris. Cells are scraped from the walls of the glass culture vessel with a policeman, suspended in PBS, and pelleted by centrifugation. Cells are suspended in 0.15 *M* ethanolamine hydrochloride buffer (pH 10.5) and homogenized while on ice with a Waring blendor at 15,000 rpm for 4 min. The homogenate is centrifuged at 10,000 *g* for 20 min and then at 25,000 *g* for 30 min to remove intact cells and cell debris. Saturated $(NH_4)_2SO_4$ solution is added dropwise until a final concentration of 10% is reached. The suspension is allowed to stand at room temperature for 18 hr, and the precipitate which contains crude fimbriae is collected by centrifugation. Further purification is achieved by suspension of the crude fimbriae in 0.5% deoxycholate in 50 m*M* Tris-HCl (pH 8.2) for 30 min at

[20] I. Ofek and N. Sharon, *Curr. Top. Microbiol. Immunol.* **151,** 91 (1990).

[21] F. A. Scannapieco, S. J. Millar, H. S. Reynolds, J. J. Zambon, and M. J. Levine, *Infect. Immun.* **55,** 2320 (1987).

[22] B. Rosan, J. Slots, R. J. Lamont, M. A. Listgarten, and G. M. Nelson, *Oral Microbiol. Immunol.* **3,** 58 (1988).

[23] T. Inouye, H. Ohta, S. Kokeguchi, K. Fukui, and K. Kato, *FEMS Microbiol. Lett.* **69,** 13 (1990).

room temperature. The precipitate is collected by centrifugation and treated in the same way with 0.7% *n*-octyl-β-D-glucopyranoside in the Tris buffer. This precipitate is composed solely of fimbriae. The fimbriae dissociate during SDS–polyacrylamide gel electrophoresis (SDS–PAGE) to form monomers with a molecular weight of 54,000.

Membrane Vesicles. A prominent feature of the surface of *A. actinomycetemcomitans* is vesicle material, which is present as fibrillar membranous extensions with knoblike ends, termed membrane vesicles (MemVes) or as small round vesicles or blebs, termed extracellular vesicles (ExVes).[9,11] *Actinobacillus actinomycetemcomitans* ExVes enhance its adhesion to epithelial cells. The *A. actinomycetemcomitans* cells release ExVes into the culture medium, and thus vesicles can be isolated from either the culture medium or from the cell surface.[11,24,25]

A 72 hr 1-liter culture is centrifuged at 10,000 *g* for 20 min at 4°, and the cellular pellet is suspended in 10 m*M* Tris-HCl buffered saline (pH 7.5). The suspension is subjected to 30 sec of light sonication to remove vesicles from the cell surfaces. The suspension is centrifuged at 10,000 *g* for 15 min. The supernatant, which contains the vesicles, is kept on ice while the cells are resuspended in buffered saline and the sonication procedure repeated (5 times). The combined supernatants or the original culture supernatants are centrifuged for 60 min at 100,000 *g* and 4°. (Sucrose density centrifugation may be used here.) The pellet, which is composed of vesicles, is washed and centrifuged at low speeds twice to remove residual cell debris.

An alternative method does not require an ultracentrifuge.[11,25] The cells are removed by centrifugation, solid $(NH_4)_2SO_4$ is added slowly to the culture supernatant to yield 40% saturation, and the mixture is centrifuged at 20,000 *g* for 30 min. The pellet is suspended in 30 ml of 50 m*M* Tris buffer (pH 9.5) and washed twice (by suspension in PBS and centrifugation at 27,000 *g* for 20 min) to dilute the $(NH_4)_2SO_4$. The final pellet containing the vesicles is suspended in a minimal volume of PBS and stored at −20°. Vesicles can be fractionated by conventional biochemical techniques provided they are pretreated with DNase/RNase to remove contaminating nucleic acid.[24]

Extracellular Amorphous Material. An extracellular amorphous material (ExAmMat) is associated with the surface of *A. actinomycetemcomitans* SUNY 75(S) after aerobic growth in broth.[9,11] ExAmMat enhances adhesion of SUNY 75(S) to KB epithelial cells. ExAmMat also increases the adhesion

[24] H. Ohta, K. Kato, S. Kokeguchi, H. Hara, K. Fukui, and Y. Murayama, *Infect. Immun.* **59,** 4599 (1991).

[25] D. Grenier and D. Mayrand, *Infect. Immun.* **55,** 111 (1987).

of adhesion-deficient *A. actinomycetemcomitans.* To date, the adhesins responsible have not been isolated, but ExAmMat is released from the surface of cells simply by washing the cells with PBS at 37°.

Outer Membranes. Outer membranes bear potential molecular adhesins which are likely to be proteinaceous. Preliminary studies suggest that certain *A. actinomycetemcomitans* strains may possess such adhesins. To identify this type of *A. actinomycetemcomitans* adhesin, whole bacteria are treated with proteolytic enzymes (such as trypsin), the bacteria are washed to remove the enzyme, and adhesion to epithelial cells is determined using one of the assays described above. Analysis of whole-cell extracts by SDS–PAGE is used to monitor proteins modified by the enzymatic treatment. Isolation of potential adhesins is carried out by the generation of outer membrane-enriched fractions.[26–29] Fractionation of outer membrane-enriched fractions can be achieved by standard biochemical procedures. Fractions are dialyzed to remove potential KB toxins and tested for the ability to inhibit adhesion of the oral bacterium from which they were isolated.

Carbohydrate-Specific Lectins. Lectins, such as fimbriae, mediate adhesion to mammalian cells by binding to specific surface sugars.[20] Approaches to determine the carbohydrate specificity of lectins involved in adhesion of oral bacteria to soft tissue are based on procedures routinely used in lectin research.[20] Initially, a panel of sugars (including monosaccharides, glycosides, and oligosaccharides) is tested to determine their effects on adhesion. The sugar which produces the greatest inhibitory effect defines the specificity of the lectin. To date, no carbohydrate-specific lectins which mediate adhesion of *A. actinomycetemcomitans* to KB cells have been identified.[9]

Porphyromonas gingivalis Adhesion Components

Fimbriae and Hemagglutinins. Porphyromonas gingivalis cells are capable of binding to human epithelial cells.[10,30,31] The cells exhibit fimbriae, and they also possess hemagglutinins which have adhesive properties.[32,33]

[26] S. Kokeguchi, K. Kato, F. Nishimura, H. Kurihara, and Y. Murayama, *FEMS Microbiol. Lett.* **77,** 85 (1991).
[27] J. M. Di Rienzo and E. L. Spieler, *Infect. Immun.* **39,** 253 (1983).
[28] C. Filip, G. Fletcher, J. L. Wulff, and C. F. Earhart, *J. Bacteriol.* **115,** 717 (1973).
[29] M. E. Wilson, *J. Periodontol.* **62,** 211 (1991).
[30] K. Okuda, J. Slots, and R. J. Genco, *Curr. Top. Microbiol.* **6,** 7 (1981).
[31] W. C. Childs III and R. J. Gibbons, *J. Periodontal Res.* **25,** 172 (1990).
[32] H. Isogai, E. Isogai, F. Yoshimura, T. Suzuki, W. Kagota, and K. Takano, *Arch. Oral Biol.* **33,** 479 (1988).
[33] E. Inoshita, A. Amano, T. Hanioka, H. Tamagawa, S. Shizukuishi, and A. Tsunemitsu, *Infect. Immun.* **52,** 421 (1986).

A role for fimbriae in adhesion to epithelial cells has been established, whereas a role for hemagglutinins remains unclear.[32,34–36] Hemagglutination is measured[33] by adjusting bacterial suspensions to an optical density of 1.0 (550 nm). Two-fold dilutions of bacterial suspensions are made in PBS, and 50 μl of suspensions and an equal volume of a 2% suspension of sheep erythrocytes are mixed in wells of 96-well round-bottomed microtiter plates and incubated for 90 min at room temperature. The hemagglutination titer is expressed as the last dilution showing complete agglutination of the erythrocytes.

Vesicles. Porphyromonas gingivalis extracellular vesicles exhibit a number of adhesive properties. They hemagglutinate erythrocytes, promote adhesion and coaggregation of other oral bacteria, and may bear fimbriae.[25,37] Trypsin-like proteases are associated with their ability to promote coadhesion of *P. gingivalis* and *Actinomyces viscosus*.[38] The proteases, which occur in *P. gingivalis* outer membranes as well, may be adhesins per se, or their role in adhesion may be to expose binding domains (see below). In this regard, they may function in adhesion to epithelial cells.[31] Procedures for isolation of *P. gingivalis* vesicles are basically the same as those for *A. actinomycetemcomitans.*

Other Considerations

Collagenous Proteins

Collagen is an important structural component of the periodontium.[39] Besides being the major protein in the matrix of alveolar bone and of dentin and cementum of teeth, collagen is found in basement membrane beneath the gingival epithelium[40] and in associated connective tissue. The interaction of *P. gingivalis* with basement membrane elements likely promotes their interaction with gingival tissue. Destruction of collagenous components is a prominent characteristic of destructive periodontal disease[39,40,41]; therefore, knowledge of the binding of oral bacteria to collagenous proteins is important. *Porphyromonas gingivalis* binds to both collagen[40] and fibrinogen.[42] The ability of *P. gingivalis* to bind collagenous

[34] J.-Y. Lee, H. T. Sojar, G. S. Bedi, and R. J. Genco, *Infect. Immun.* **60,** 1662 (1992).
[35] Y. Takahashi, F. Yoshimura, M. Kawanami, and H. Kato, *J. Periodontal Res.* **27,** 599 (1992).
[36] M. Deslauriers and C. Mouton, *Infect. Immun.* **60,** 2791 (1992).
[37] P. A. Goulbourne and R. P. Ellen, *J. Bacteriol.* **173,** 5266 (1991).
[38] J. Li, R. P. Ellen, C. I. Hoover, and J. R. Felton, *J. Dent. Res.* **70,** 82 (1991).
[39] L. Sandholm, *J. Clin. Periodontol.* **13,** 19 (1986).
[40] Y. Naito and R. J. Gibbons, *J. Dent. Res.* **67,** 1075 (1988).
[41] V. Uitto, *J. Periodontol.* **56,** 740 (1983).
[42] M. Lantz, R. W. Rowland, L. M. Switalski, and M. Hook, *Infect. Immun.* **54,** 654 (1986).

substrates may be significant, as *P. gingivalis* is highly proteolytic and one of the few oral bacteria which can degrade collagenous substrates.[41] Briefly, binding of bacteria to collagenous protein is carried out in an assay in which either the protein or the bacteria are labeled.[40,42] If appropriate antibodies are available, an ELISA may also be used. Incubations are carried out in the presence of 0.1% BSA to block any uncoated surfaces and minimize nonspecific binding.[42] Specificity of binding is determined by pretreatment of the bacteria with proteins such as egg albumin or the unlabeled protein of interest.[42] An alternative assay is to coat tissue cultureware with collagen proteins.

Epithelial Cell Receptors and Cryptitopes

Adhesion of oral bacteria to epithelial cells may involve hidden molecular segments, referred to as cryptitopes.[1] Cryptitopes are generated by conformational changes which occur as molecules are adsorbed to surfaces or by enzymatic modification.[3] To investigate epithelial cell receptors and cryptic receptors, epithelial cells (in monolayers or in suspension) are treated with various concentrations of enzymes[3] (such as sialidase, papain, trypsin, chymotrypsin, lysozyme, and phospholipase C) for various times. Enzymes with the highest degree of purity obtainable should be used. Control cells are incubated in buffer without enzyme. The cells are washed with PBS or cell culture medium prior to incubation with the bacteria. Epithelial cell viability and number are determined by staining with 0.16% trypan blue dye solution (monolayers are detached with EDTA–trypsin) and enumeration in a hemocytometer. Viable cells are recognized as those that do not take up stain. Receptors or cryptic receptors are suggested if adhesion is decreased or increased, respectively, by enzymatic treatment. Preliminary studies to identify cryptic receptors uncovered by conformational changes involve a comparison of components (cells and proteins) in solution and adsorbed to surfaces.[3]

Components of Secretions

Components of saliva have molecules which mimic those of receptors found on oral epithelial cells.[3] The interaction of bacterial adhesins with "receptors" in secretions would diminish or preclude interaction with receptors on oral epithelial cells. Saliva and serum of patients with periodontitis also exhibit specific antibodies to suspected periodontopathogens.[43] Furthermore, the attachment of oral bacteria to epithelial cells is inhibited by

[43] S. Hamada, T. Ogawa, H. Shimauchi, and Y. Kusumoto, *Adv. Exp. Biol. Med.* **327,** 71 (1992).

antibodies, including immunoglobulin A (IgA), which is elevated in the saliva of patients with periodontal disease.[44] The ability of saliva and antibodies to inhibit adhesion may represent important antiadhesion mechanisms for oral bacteria.

[44] R. C. Williams and R. J. Gibbons, *Science* **177,** 697 (1972).

[31] Coaggregations among Oral Bacteria

By PAUL E. KOLENBRANDER

Visual Assay

The characteristic observation of coaggregation occurs after simple mixing of dense suspensions of two coaggregation partner cell types (Fig. 1A, tube 3). Although nearly all human oral bacteria tested are known to coaggregate with at least one partner strain, the coaggregations are highly specific.[1] Each strain has its own set of partners and mechanisms of cell-to-cell recognition and may belong to a group consisting of strains that also coaggregate with the same set of partners. The clumps or flocs are called coaggregates because they are composed of an interacting network of both cell types, that is, co-aggregates.[2] The partners are genetically distinct cell types and are usually of different genera, such as *Streptococcus oralis* and *Actinomyces naeslundii* as shown, respectively, in tubes 1 and 2 in Fig. 1. The cell density of each suspension should be about 1×10^9 cells/ml, and the suspension should be evenly turbid. Clumping occurs immediately after mixing the two cell suspensions (Fig. 1A, tube 3). Verification of participation of both cell types in the coaggregates can be determined easily by inspection of a wet mount preparation of the coaggregation mixture with a standard phase-contrast microscope. Many of the interactions are completely reversed by adding 60 m*M* lactose, which is visualized as a change to an evenly turbid suspension (Fig. 1A, tube 4). The four tubes as they appear immediately after vortex mixing for 10 sec is shown in Fig. 1A. To indicate the rapid settling of coaggregates, the same set of four tubes is shown in Fig. 1B after standing 2 min.

[1] P. E. Kolenbrander, *in* "Microbial Cell–Cell Interactions" (M. Dworkin, ed.), p. 303. American Society for Microbiology, Washington, D.C., 1991.

[2] P. E. Kolenbrander and R. N. Andersen, *J. Bacteriol.* **168,** 851 (1986).

antibodies, including immunoglobulin A (IgA), which is elevated in the saliva of patients with periodontal disease.[44] The ability of saliva and antibodies to inhibit adhesion may represent important antiadhesion mechanisms for oral bacteria.

[44] R. C. Williams and R. J. Gibbons, *Science* **177,** 697 (1972).

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The characteristic observation of coaggregation occurs after simple mixing of dense suspensions of two coaggregation partner cell types (Fig. 1A, tube 3). Although nearly all human oral bacteria tested are known to coaggregate with at least one partner strain, the coaggregations are highly specific.[1] Each strain has its own set of partners and mechanisms of cell-to-cell recognition and may belong to a group consisting of strains that also coaggregate with the same set of partners. The clumps or flocs are called coaggregates because they are composed of an interacting network of both cell types, that is, co-aggregates.[2] The partners are genetically distinct cell types and are usually of different genera, such as *Streptococcus oralis* and *Actinomyces naeslundii* as shown, respectively, in tubes 1 and 2 in Fig. 1. The cell density of each suspension should be about 1×10^9 cells/ml, and the suspension should be evenly turbid. Clumping occurs immediately after mixing the two cell suspensions (Fig. 1A, tube 3). Verification of participation of both cell types in the coaggregates can be determined easily by inspection of a wet mount preparation of the coaggregation mixture with a standard phase-contrast microscope. Many of the interactions are completely reversed by adding 60 m*M* lactose, which is visualized as a change to an evenly turbid suspension (Fig. 1A, tube 4). The four tubes as they appear immediately after vortex mixing for 10 sec is shown in Fig. 1A. To indicate the rapid settling of coaggregates, the same set of four tubes is shown in Fig. 1B after standing 2 min.

[1] P. E. Kolenbrander, *in* "Microbial Cell–Cell Interactions" (M. Dworkin, ed.), p. 303. American Society for Microbiology, Washington, D.C., 1991.

[2] P. E. Kolenbrander and R. N. Andersen, *J. Bacteriol.* **168,** 851 (1986).

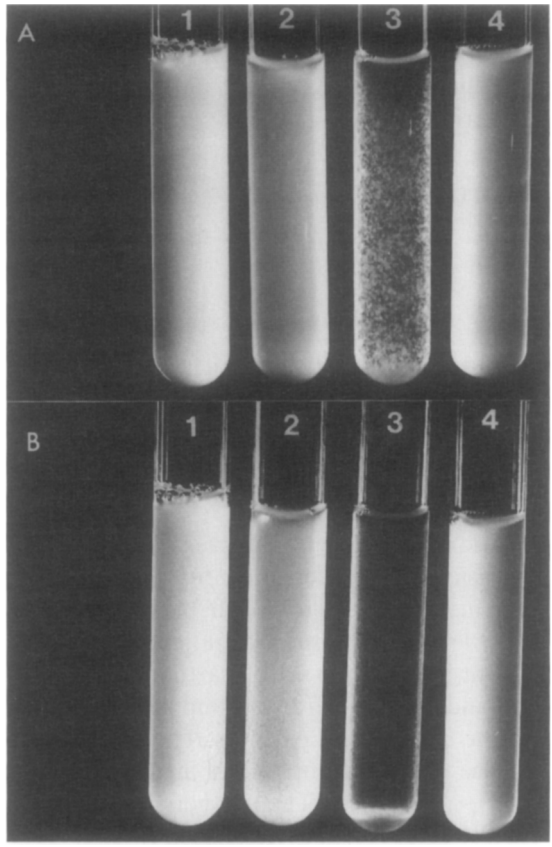

FIG. 1. The visual coaggregation assay is viewed immediately after vortex mixing (A) and after standing for 2 min (B): cell suspension of one partner cell type (tube 1); cell suspension of the other partner cell type (tube 2); mixture of equal volumes of the two cell types (tube 3); and reversal of coaggregation like that seen in tube 3 by adding a final concentration of 60 m*M* lactose (tube 4).

A coaggregation as shown in Fig. 1, tube 3, is the strongest kind and is given the highest visual score of plus 4 to indicate rapid and complete settling of large coaggregates leaving a water-clear supernatant. A coaggregation score of plus 3 is given when large coaggregates settle rapidly but the supernatant remains slightly cloudy; a score of plus 2 is given when coaggregates are formed immediately but remain suspended in a turbid background; a score of plus 1 indicates detectable but finely dispersed coaggregates; and a zero score is given to an evenly turbid suspension, that is, one that does not change appearance after the two suspensions are vortex

mixed. Coaggregates can be observed with 10-fold lower cell densities of the two cell types, but such observations approach the limits of visual detection of coaggregate formation, and measurements of coaggregation with such low densities are better made by using a radioactively labeled cell type (see later in this chapter).

The visual assay is particularly applicable for screening large numbers of strains for the ability to coaggregate. Over 1000 strains of human oral bacteria have been examined for potential partnership with other strains. In general, most strains do not coaggregate with members of their genus. However, some intrageneric coaggregation has been noted within the genus *Actinomyces,* and members of the genus *Streptococcus* exhibit extensive partnerships.[3]

The simplicity of the assay allows rapid evaluation of a set of coaggregation properties, which help to group strains with similar or identical properties. First, the range of partnerships is found. Then, the heat-inactivated cell type is determined by heating each cell type at 85° for 30 min and subsequently testing each for ability to coaggregate with either heated or unheated partner cells. Usually, heating or protease treatment yields identical results and identifies the partner bearing the protein adhesin. Proteinase K (P-0390; Sigma, St. Louis, MO) is used at 0.45 μg/ml for 1 hr at 50°. Identical results with other proteases such as Pronase, papain, subtilisin, chymotrypsin, and trypsin are obtained. Often, one cell type is sensitive to heating and protease treatments, whereas the other is insensitive. Occasionally, both cell types require heating (or protease treatment) to prevent coaggregation. If heating both partners at 85° for 30 min fails to abolish coaggregation, then heating is done at 99° for 45 min; in all cases tested so far, that treatment is sufficient to inactivate the cell type.

The third parameter used to characterize a coaggregation group is the effect of saccharide inhibitors on coaggregation. Coaggregations are divided into lactose-inhibitable and lactose-noninhibitable interactions. Some that are lactose-inhibitable are even more sensitive to other sugars like L-rhamnose.[4] Finally, the inability of a cell type to coaggregate with a coaggregation-defective (COG^-) mutant of its partner helps to place that cell type into a group comprising other strains exhibiting the same phenotype. Thus, such a group of strains exhibits a distinct coaggregation pattern with a set of partners. The simultaneous loss of coaggregation among the group to a COG^- mutant of one of the group's partners strongly suggests that the

[3] P. E. Kolenbrander, R. N. Andersen, and L. V. H. Moore, *Appl. Environ. Microbiol.* **56,** 3890 (1990).

[4] E. I. Weiss, J. London, P. E. Kolenbrander, A. S. Kagermeier, and R. N. Andersen, *Infect. Immun.* **55,** 1198 (1987).

adhesion-mediating component on the surface of the partner is recognized by all members of the group. It further suggests that the members of the group may possess a surface component that is functionally similar.[5,6]

By including these four properties, six coaggregation groups of human oral streptococci, six coaggregation groups of human oral actinomyces, and four coaggregation groups of human oral veillonellae have been delineated. These groups represent the coaggregation properties of greater than 90% of the human oral strains of *Streptococcus gordonii, S. oralis, S. sanguis, Actinomyces naeslundii, Veillonella atypica, V. dispar,* and *V. parvula.*

Visual Assay Procedure

The assay is routinely conducted in 10 × 75 mm glass test tubes to which equal volumes of the two cell suspensions are added. Usually the volume is 100 μl of each cell type. The appropriate cell density (about 1×10^9 cells/ml) is obtained turbidimetrically with a Klett–Summerson colorimeter by adjusting the turbidity to 260 Klett units at 660 nm (red filter) (Klett Manufacturing, New York, NY). This is equivalent to a value of 1 optical density unit with a spectrophotometer at 660 nm. Cells are suspended in coaggregation buffer, which consists of the following [dissolved in 1 m*M* tris(hydroxymethyl)aminomethane adjusted to pH 8.0]: $CaCl_2$ (10^{-4} *M*), $MgCl_2$ (10^{-4} *M*), NaN_3 (0.02%, w/v), and NaCl (0.15 *M*). Most cells can be stored for months or years in coaggregation buffer at 4° without loss of coaggregation phenotype.

After a brief vortex mixing of the two cell types for 5 to 10 sec, the tube is placed on a glass surface with an indirect light source below. The light source used in our laboratory is the illuminated magnifier No. 39-101 (Stocker and Yale, Beverly, MA). The tube is rocked gently to maximize the interaction between the two cell types. The change from an evenly turbid suspension before mixing to the formation of visible coaggregate, occurs immediately either during vortex mixing or during the gentle rocking procedure. The reversal of coaggregation by a sugar or saccharide is determined by adding the carbohydrate to a final concentration of 60 m*M* and rescoring the coaggregating pair. Infrequently, coaggregates are visible immediately after vortex mixing but become undetectable visually during the rocking procedure.

Coaggregation-Defective Mutants

An adaptation of the visual assay is useful in the selection of spontaneously occurring coaggregation-defective (COG^-) mutants.[7] The procedure

[5] P. E. Kolenbrander and J. London, *Adv. Microbial Ecol.* **12,** 183 (1992).
[6] R. N. Andersen, N. Ganeshkumar, and P. E. Kolenbrander, *Infect. Immun.* **61,** 981 (1993).
[7] P. E. Kolenbrander, *Infect. Immun.* **37,** 1200 (1982).

works most conveniently when the wild type of the potential COG^- mutant is made resistant to two antibiotics and is used as the parent strain. The parent is obtained by sequential selection of the wild type on the two antibiotic-containing agar media. The partner is cultured in a large volume (usually 1 liter), harvested, washed, and stored at 4° in coaggregation buffer containing azide.

On the day of the COG^- mutant selection, the partner is washed three times in sterile coaggregation buffer without azide and adjusted to 600 to 800 Klett units (about 1×10^{10} cells/ml) with the same buffer. The double antibiotic-resistant parent strain is cultured in 10 ml broth, harvested, and washed in coaggregation buffer without azide, and a final suspension of 0.5 ml is made in the same buffer. The dense suspension of partner cells is added dropwise (about 50 μl per drop) to the 0.5 ml suspension and mixed. Coaggregates are immediately observed (cf. Fig. 1A, tube 3) after the first drop. Usually two or three drops are added, and the suspension is mixed and centrifuged at low speed (400 *g* for 1 or 2 min). The resultant supernatant fluid should be enriched for COG^- mutants, and two or three more drops of partner suspension are added, followed by mixing and centrifugation. The procedure can be done at ambient temperature and is repeated until no coaggregates are seen after adding partner cells, which requires about 5 to 9 cycles of partner addition and centrifugation. The entire procedure is usually complete within 2 hr and is therefore applicable to selecting COG^- mutants of strains with moderate anaerobic requirements[4,8] but not requiring a sealed anaerobic chamber.

The final supernatant (usually less than 1 ml) is diluted in coaggregation buffer without azide and plated onto agar containing one of the two antibiotics. Colonies are transferred with a sterile toothpick to 100 μl of sterile coaggregation buffer without azide in a sterile microtiter well of a 96-well microtiter plate, called the master plate. After trituration with a sterile-tipped micropipettor, 25 μl of the colony suspension is transferred from the master plate to one or more replicate plates, 25 μl of partner cell suspension (about 5×10^8 cells/ml; 160 Klett units) is added, the plate is covered with a plastic adhesive film and shaken for 10 min on a microtiter plate shaker (DSG Titertek shaker; Flow Laboratories, McLean, VA), and finally the plate is viewed from below with a mirrored plate reader. A typical view is shown in Fig. 2. Most of the wells contain obvious coaggregates, but a few exhibit a smooth appearance and contain potential COG^- mutants (Fig. 2, wells A3, B6, D2, E10, F12, and H5). From the master plate, the potential COG^- mutant-containing wells are streaked for purity and then tested for antibiotic resistance to the second antibiotic by plating on the

[8] E. I. Weiss, P. E. Kolenbrander, J. London, A. R. Hand, and R. N. Andersen, *J. Bacteriol.* **169,** 4215 (1987).

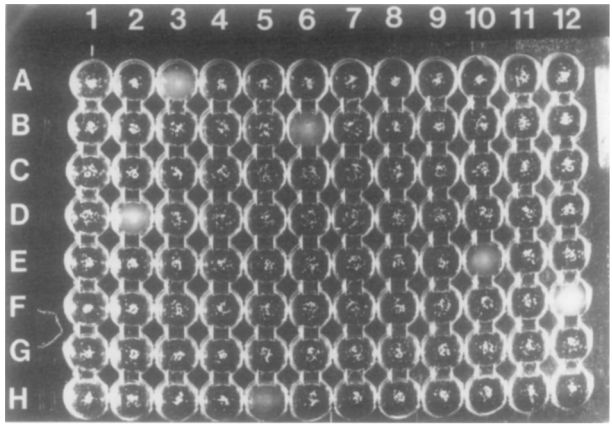

FIG. 2. Modification of the visual assay for coaggregation to screen for potential COG^- mutants of *Actinomyces naeslundii* ATCC 12104. A microtiter plate containing a mixture of *Streptococcus oralis* J22 and *A. naeslundii* ATCC 12104 is viewed from the bottom. The actinomyces cells came from colonies that resulted from plating a cell suspension which was enriched for COG^- mutants. A stock suspension of *S. oralis* J22 was added to the triturated actinomyces colony mass in each well. Coaggregates are clearly evident in all but six wells (A3, B6, D2, E10, F12, and H5). (Reprinted from Kolenbrander[7] with permission.)

antibiotic-containing medium. Resistance to both antibiotics is used as verification of genetic lineage of the COG^- mutant from the parent strain. By using this selection technique, we have isolated COG^- mutants of several species of the genera *Actinomyces, Capnocytophaga, Fusobacterium, Prevotella* (formerly *Bacteroides* in part), *Streptococcus,* and *Veillonella.*

Turbidimetric Assay

The turbidimetric assay was originally performed in glass tubes, and the turbidity was measured at 650 nm in a cuvette in a spectrophotometer. The assay was scaled down to accommodate testing of scarce compounds for inhibition of coaggregation[9] and has been further adapted for siliconized conical tubes that are also used for the radioactivity-based assay described in the next section. The turbidity can be determined spectrophotometrically at 650, 510, or 540 nm in a microtiter plate reader; turbidity read at the higher wavelength (650 nm) is a lower absolute value and the effects of cell size or chain length on turbidity are reduced, whereas the lower wavelength gives higher values and is more sensitive to small changes in turbidity. The assay is useful for measuring routine coaggregations of two cell types

[9] F. C. McIntire, L. K. Crosby, and A. E. Vatter, *Infect. Immun.* **36,** 371 (1982).

and for measuring the inhibitory effects of sugars on the coaggregations. Because it does not require radiolabeled bacteria, the assay is unable to identify the participation of a given cell type in a milieu. Therefore, multigeneric coaggregations and other coaggregations involving more than two cell types should be done with the radioactivity-based assay.

Turbidimetric Assay Procedure

Cells are suspended in 20 m*M* Tris-HCl, 0.1 m*M* $CaCl_2$, 0.02% (w/v) NaN_3, 0.15 *M* NaCl, pH 7.4 (TBS). Bacterial cell density is adjusted to about 1×10^{10} cells/ml. The assay is conducted in TBS plus 0.05% (v/v) Tween 20 (TBST), and the Tween 20 is added as a 10× concentrate in TBS to the siliconized tube. A 25-μl aliquot of cell suspension A or B is adjusted, respectively, to 100 μl in TBST and serves as the noncoaggregation control. Coaggregation is measured by adding 25-μl samples of cell suspensions A and B to an adjusted final volume of 100 μl, vortex mixing 10 sec, further mixing on a DSG Titertek shaker (Flow Laboratories) for 30 min, adding 200 μl TBST, centrifuging 200 rpm (about 10 *g*) for 1 min (Beckman Accuspin, Beckman Instruments, Palo Alto, CA), transferring 210 μl of supernatant to a flat-bottomed microtiter well, and measuring turbidity at 510 nm in a microtiter plate reader. The calculations for percent coaggregation and percent inhibition are as follows:

Percent coaggregation

$$= \frac{(A_{510}\text{ cell suspension A} + A_{510}\text{ cell suspension B}) - (A_{510}\text{ cell mixture A + B})}{(A_{510}\text{ cell suspension A} + A_{510}\text{ cell suspension B})} \times 100$$

When inhibitors or other molecules are tested for the effect on coaggregations, cell suspension A is vortex mixed with the chemical followed by mixing on the shaker for 30 min. Cell suspension B is added, vortex mixed, and finally mixed on the shaker for another 30 min. Calculations use the following equation:

$$\text{Percent inhibition} = \frac{\%\text{ coaggregation without inhibitor} - \%\text{ coaggregation with inhibitor}}{\%\text{ coaggregation without inhibitor}} \times 100$$

Radioactivity-Based Assay

The radioactivity-based assay is useful when it is important to know the extent of participation of a particular cell type in a multigeneric coaggregate (three or more genetically distinct coaggregation partners).[2] It also offers

quantitative measurement of coaggregation in the presence of varying concentrations of inhibitor molecules or coaggregation-blocking antibodies.[10,11] Cells are labeled with either [^{3}H]thymidine or [^{14}C]uracil at 10 or 2 μCi/ml growth medium, respectively. After 4 to 6 cell doublings, cells are harvested, washed by centrifugation, and suspended in coaggregation buffer. The radioactivity should be about 10^3 bacteria per count per minute (cpm).

Radioactivity-Based Assay Procedure

Coaggregates Formed in Suspension. To determine the ratio of unlabeled partner cells required to maximally coaggregate with the radioactively labeled cell type, increasing numbers of unlabeled partner cells are added to a constant number of labeled cells (Fig. 3). A 6- to 8-fold higher number of unlabeled partner cells is usually the ratio giving the maximum percentage of input radioactivity in coaggregates. The point of approaching the maximum number of radioactively labeled cells in coaggregates (i.e., saturating unlabeled partner) is used to conduct carbohydrate inhibition or antibody blocking of coaggregations. For comparison, an identical saturation curve using a radioactively labeled COG$^-$ mutant of the streptococcus that failed to coaggregate with the fusobacterium would yield values of 0 to 5% of input radioactivity in coaggregates even in the presence of a 10-fold excess of unlabeled fusobacteria.

The radioactive cell suspension (about 2×10^6 to 1×10^7 cells; ~2000 to 10,000 cpm) is mixed with a suspension of unlabeled potential partner strain in a siliconized conical tube (0.65 ml capacity, No. 505-195, PGC Scientifics, Gaithersburg, MD). The final volume is adjusted to 200 μl, and the suspension is vortex mixed for 5 sec followed by gentle rocking of the tubes (setting 5 for 5 min with a DSG Titertek shaker; Flow Laboratories). Coaggregates are pelleted by low-speed centrifugation [100 *g* for 1 min (1000 rpm)] in a Microfuge 12 (Beckman Instruments). One-half of the volume (100 μl) is removed and used for scintillation counting. The remaining 100 μl and pellet in the tube are centrifuged at 10,000 rpm (8000 *g*) for 5 min to pellet all cells, free and coaggregated. A 50-μl portion of the supernatant is removed and counted as above to determine the amount of non-cell-associated radioactivity, which is presumed to be due to leaching of radioactivity from the cells. The non-cell-associated value is subtracted from the total counts per minute in the supernatant, and after adjusting for volume differences (multiplying by 2) the percentage of input counts

[10] E. I. Weiss, J. London, P. E. Kolenbrander, R. N. Andersen, C. Fischler, and R. P. Siraganian, *Infect. Immun.* **56,** 219 (1988).

[11] P. E. Kolenbrander and R. N. Andersen, *Infect. Immun.* **58,** 3064 (1990).

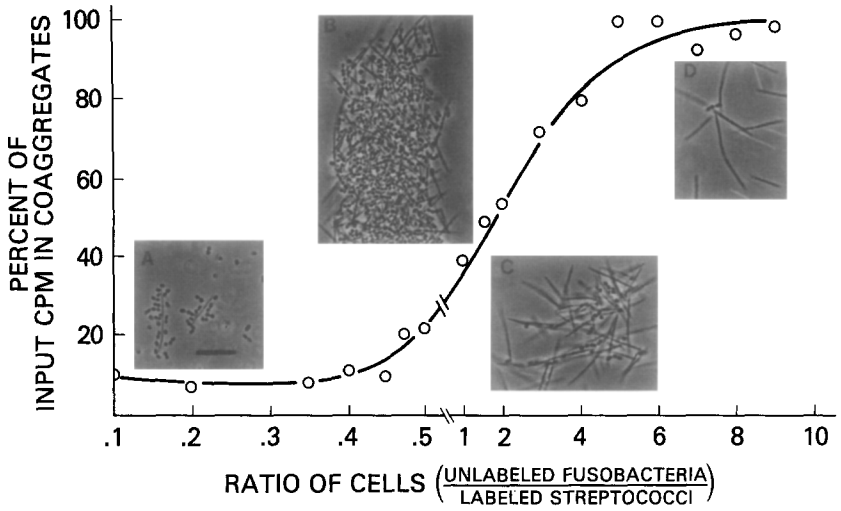

FIG. 3. Percentage of input radioactivity in coaggregates formed between radioactively labeled *Streptococcus oralis* C104 and unlabeled *Fusobacterium nucleatum* PK1909. The number of labeled cells is constant, and cells are mixed with increasing numbers of unlabeled cells. The logarithmic plot is used to expand the region of cell ratios between 0.5 and 4, in which the greatest changes in coaggregation occur. The phase-contrast micrographs show the typical cell-to-cell arrangements in the cell suspensions before centrifugation at 100 *g* for 1 min. (A) Corncob configuration at a cell ratio of 0.1 showing streptococci surrounding slender fusobacterial cells. (B) Tightly arranged networks of both cell types at a cell ratio of 0.5. (C) Loosely arranged networks of both cell types at a cell ratio of 4. (D) Rosette at a cell ratio of 10 showing streptococcal cells surrounded by fusobacteria. Note the numerous free cells in (A) and (D) and the lack of noncoaggregated cells in (B) and (C). Bar: 10 μm. (Reprinted from Kolenbrander[1] with permission.)

per minute remaining in the supernatant is calculated. Because this reflects the noncoaggregated cells, the value is subtracted from 100% to yield the percentage of input counts per minute in coaggregates.

This indirect method is the most convenient method to measure the amount of coaggregation. To test its validity the amount of radioactivity in the coaggregates can also be measured directly, and the same amount of coaggregation is observed.[2] The assay variability is ±5% of the average value of multiple samples, and the average percent recovery of input radioactivity is 100 ± 3%. A tube containing only buffer and the radioactive cell type is centrifuged with the experimental samples and serves as the control (no coaggregation). The buffer control value [sampled from the supernatant after centrifugation at 1000 rpm (100 *g*) for 1 min] of total noncoaggregated cells was about 90% of the input radioactivity (determined by sampling directly from the radioactively labeled cell suspension for scintillation

counting). For some coaggregations, where the size of the coaggregates is small, the conditions of centrifugation are 2000 rpm (300 *g*) for 2 min. In these experiments, the buffer control value of noncoaggregated cells is about 80% of the input radioactivity.

Coaggregates Formed by Accretion onto Partner Cell-Coated Microtiter Well Surface. An adaptation of coaggregate formation in suspension is possible by first coating a plastic surface with an unlabeled partner cell type, washing the surface with buffer, adding a radioactively labeled cell type, and measuring the amount of label accreted onto the surface.[12] Unlabeled bacteria are adjusted to a density of about 1×10^8 cells/ml in TNMC buffer (1 m*M* Tris-HCl, 0.15 *M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$; pH 8.0) and 50-μl samples (about 5×10^6 cells) are applied to the wells of flat-bottomed 96-well microtiter plates (MaxiSorp, A/S Nunc, Kamstrup, Denmark). The MaxiSorp surface has a high binding capacity and gives more consistent results than untreated microtiter plates. The plates are centrifuged at 800 *g* at 20° for 5 min to deposit the cells onto the bottom surface of the wells; after incubation at 4° for 16 hr, the liquid contents are discarded. Remaining binding sites are blocked by adding 200 μl of TNMC buffer containing 0.2% (v/v) Tween 20 to each well and incubating the plates at 4° for an additional 16 hr. A sample of 50 μl of labeled partner cell (2×10^7 cells; specific radioactivity should be about 10^3 cells per cpm) is added to the wells, and the plates are incubated at 37° for 2 hr on a rotary shaker at 200 rpm. The liquid contents of the wells are discarded, and the wells are washed four times with TNMC buffer containing 0.05% (v/v) Tween 20. Accreted cells are removed from the plastic surface by adding 100 μl of a solubilization solution containing 1% (w/v) sodium dodecyl sulfate (SDS) and 0.4 *N* NaOH, incubating the plates for 2 to 16 hr at room temperature, and transferring the liquid contents to scintillation vials for determination of radioactivity. The assays are performed in quadruplicate, and the controls are wells without the unlabeled partner. Under these conditions, the maximum percent labeled streptococci (input of 2×10^7 cells) accreted was reported to be 24 ± 3%, which is equivalent to about one labeled cell accreted per potential unlabeled actinomyces (input of about 5×10^6 cells) coated on the microtiter well surface.[12] Inhibition of accretion by carbohydrates can be tested by mixing the sugar solution (25 μl of 0.1 *M* stock solution or dilution in TNMC buffer, adjusted to pH 8.0 if necessary) with the labeled partner (25 μl containing 2×10^7 cells) in the wells containing the partner-coated surface.

It is important to determine the optimum number of unlabeled bacteria coating the surface of the microtiter well. Increasing the number of unla-

[12] H. F. Jenkinson, S. D. Terry, R. McNab, and G. W. Tannock, *Infect. Immun.* **61,** 3199 (1993).

beled bacteria severalfold above 1×10^8 cells/ml often reduces the number of bound radiolabeled partner cells. This reduction is probably due to piling up of unlabeled cells several layers thick and subsequent sloughing off of unlabled cell–labeled cell coaggregates during the final washing procedures.

Coaggregates Formed by Accretion onto Partner Cell Prebound to Saliva-Coated Spheroidal Hydroxylapatite Beads. Because the saliva-coated spheroidal hydroxylapatite (SHA) bead has been the model surface for human teeth, SHA has been used to determine potential interactions that may occur in the human oral cavity (see [43] in this volume). The initial phase of binding by oral bacteria to SHA has been described by Clark *et al.*[13] Subsequent accretion by a second cell type (cell type B), which cannot bind directly to SHA, to an SHA-bound partner (cell type A) is measured by first binding an unlabeled cell type (cell type A) to SHA and then adding a radioactively labeled partner cell (cell type B).[14] Demonstrating the accretion of cell type B directly to the already bound cell type A can be accomplished by using a galactoside-inhibitable coaggregation (between cell types A and B) and a galactoside-noninhibitable SHA binding by cell type A. After the adhesion of cell type B to the cell type A coating on the SHA, the release of accreted cell type B occurs by adding a galactoside to the cell types A/B-coated SHA. The specificity of adhesion is shown by release of only cell type B, even in experiments where cell type A is radiolabeled.

It is desirable to use cell types that exhibit large differences in the ability to bind SHA directly. We have used *Streptococcus gordonii* (formerly *S. sanguis*) DL1, which binds to SHA 400-fold better than does *Propionibacterium acnes* PK93.[14] The binding of streptococci is proportional over a 500-fold range of input cell numbers from 2×10^6 to 1×10^9, and the efficiency of binding of streptococci throughout this range is 50 to 60% of input cells. The SHA (50 mg) is precoated with 3×10^8 streptococcal cells (input of 5×10^8 cells), washed three times with coaggregation buffer, and mixed with increasing numbers of radiolabeled propionibacteria. The number of propionibacteria (75% of input) bound to streptococcal-coated SHA is proportional up to an input of 8×10^7 propionibacteria (Fig. 4A,B). With higher numbers of propionibacteria, saturation of binding to streptococcal-coated SHA is observed (Fig. 4B,C, open circles). Saturation is reached when only 1.5×10^8 propionibacterium cells (15% of 1×10^9 cells input, Fig. 4C, open circles) bound; this is about one-half of the number of streptococcal cells available on the SHA surface. Proportional increase in accretion of propionibacteria is regained by precoating SHA with 10-fold higher numbers of streptococci (Fig. 4C, filled circles), which allows 85% of the

[13] W. B. Clark, L. L. Bammann, and R. J. Gibbons, *Infect. Immun.* **19,** 846 (1978).
[14] J. E. Ciardi, G. F. A. McCray, P. E. Kolenbrander, and A. Lau, *Infect. Immun.* **55,** 1441 (1987).

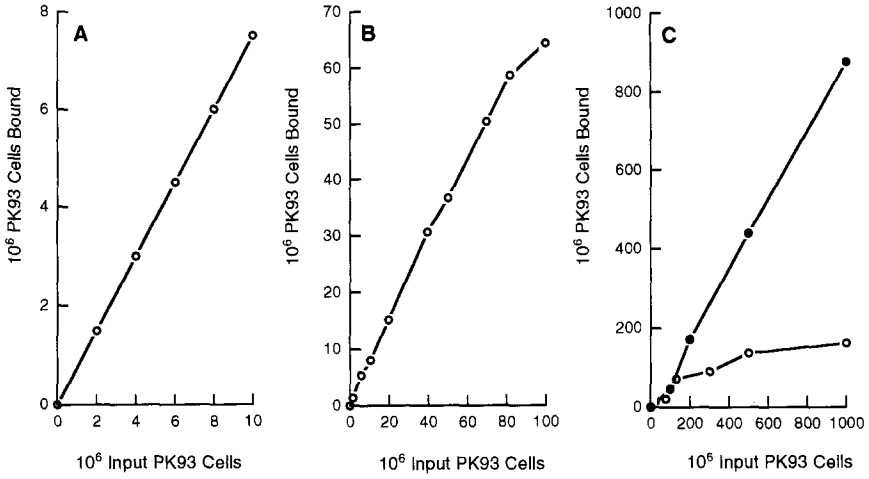

FIG. 4. Accretion of ^{3}H-labeled *Propionibacterium acnes* PK93 to unlabeled *Streptococcus gordonii* DL1-coated SHA. Input propionibacteria ranged between 2×10^6 and 1×10^9 cells, which were mixed with SHA precoated with either 3×10^8 (○) or 3×10^9 (●) streptococcal cells. Coating with 3×10^8 and 3×10^9 streptococcal cells was done by treating SHA with 5×10^8 and 4×10^{10} cells, respectively. (Data are replotted from Ciardi *et al.*[14] with permission.)

input propionibacteria to adhere. The inhibitory effects of carbohydrates on accretion of the propionibacteria is determined by a 30-min incubation of the carbohydrate solution with the streptococcal-coated SHA before adding the propionibacteria.

Summary

The oral bacterial community appears to use coaggregation as a major mechanism for interbacterial adhesion and colonization of the host. Methods for measuring and evaluating the specificity of adhesion vary from the visual observation of the phenomenon to quantitative analyses. Not only is aggregation specificity reflected in the choice of partners but also in the fact that many are inhibited by galactosides and sialic acid. Each coaggregation between any two partners within a multigeneric coaggregate is independent of the others and can be shown to be distinct by using the radioactivity-based assay. By using the visual assay, it has been shown that members of the 17 genera of most frequently isolated oral bacteria exhibit coaggregation. With the exception of oral streptococci and a few oral actinomyces, the 17 genera do not exhibit intrageneric coaggregation. As a dynamic

population, oral bacteria are in a constant flux of accretion and detachment, which are coupled to growth and death. This ecological community is amenable to intensive study, and the coaggregation assays described here are particularly suited to enhance progress in this study.

[32] Identifying and Isolating Fimbrial-Associated Adhesins of Oral Gram-Negative Bacteria

By JACK LONDON

Introduction

Studies have shown that most well-characterized fimbrial-associated bacterial adhesins are found in comparatively low numbers that vary from 150 to 1000 molecules per cell.[1–3] The dearth of these protein molecules can be fully appreciated by comparing the ratio of adhesin to fimbriae subunits, which has been estimated to range between 1:500 and 1:1000 for enteric bacteria.[3] Because of the relatively low numbers and close association with other surface components or structures, conventional protein purification techniques do not readily lend themselves to the isolation of substantial quantities of these surface molecules. The use of specific affinity matrices provides a simple approach to purifying adhesive proteins and maximizes their recovery from crude preparations of bacterial cell surface components. This chapter discusses the use of monoclonal and polyclonal antibody conjugates, agarose–sugar conjugates, and less conventional supports in the identification and isolation of adhesins from several gram-negative oral bacteria.

Background

The colonization of the oral cavity by indigenous bacteria can occur by attachment of bacteria to either host tissue or other bacterial species.[4,5]

[1] E. I. Weiss, J. London, P. E. Kolenbrander, A. R. Hand, and R. Siraganian, *J. Bacteriol.* **170,** 1123 (1988).

[2] P. Tempro, F. Cassells, R. Siraganian, A. R. Hand, and J. London, *Infect. Immun.* **57,** 3418 (1989).

[3] C. H. Jones, F. Jacob-Dubuisson, K. Dodson, M. Kuehn, L. Slonim, R. Striker, and S. J. Hultgren, *Infect. Immun.* **60,** 4445 (1992).

[4] P. E. Kolenbrander, *in* "Microbial Cell–Cell Interactions" (M. Dworkin, ed.), p. 303. American Society for Microbiology, Washington, D.C., 1991.

[5] P. E. Kolenbrander and J. London, *Adv. Microbial Ecol.* **12,** 183 (1992).

population, oral bacteria are in a constant flux of accretion and detachment, which are coupled to growth and death. This ecological community is amenable to intensive study, and the coaggregation assays described here are particularly suited to enhance progress in this study.

[32] Identifying and Isolating Fimbrial-Associated Adhesins of Oral Gram-Negative Bacteria

By JACK LONDON

Introduction

Studies have shown that most well-characterized fimbrial-associated bacterial adhesins are found in comparatively low numbers that vary from 150 to 1000 molecules per cell.[1–3] The dearth of these protein molecules can be fully appreciated by comparing the ratio of adhesin to fimbriae subunits, which has been estimated to range between 1:500 and 1:1000 for enteric bacteria.[3] Because of the relatively low numbers and close association with other surface components or structures, conventional protein purification techniques do not readily lend themselves to the isolation of substantial quantities of these surface molecules. The use of specific affinity matrices provides a simple approach to purifying adhesive proteins and maximizes their recovery from crude preparations of bacterial cell surface components. This chapter discusses the use of monoclonal and polyclonal antibody conjugates, agarose–sugar conjugates, and less conventional supports in the identification and isolation of adhesins from several gram-negative oral bacteria.

Background

The colonization of the oral cavity by indigenous bacteria can occur by attachment of bacteria to either host tissue or other bacterial species.[4,5]

[1] E. I. Weiss, J. London, P. E. Kolenbrander, A. R. Hand, and R. Siraganian, *J. Bacteriol.* **170,** 1123 (1988).

[2] P. Tempro, F. Cassells, R. Siraganian, A. R. Hand, and J. London, *Infect. Immun.* **57,** 3418 (1989).

[3] C. H. Jones, F. Jacob-Dubuisson, K. Dodson, M. Kuehn, L. Slonim, R. Striker, and S. J. Hultgren, *Infect. Immun.* **60,** 4445 (1992).

[4] P. E. Kolenbrander, *in* "Microbial Cell–Cell Interactions" (M. Dworkin, ed.), p. 303. American Society for Microbiology, Washington, D.C., 1991.

[5] P. E. Kolenbrander and J. London, *Adv. Microbial Ecol.* **12,** 183 (1992).

Many of the interactions are mediated by specific lectin-like protein adhesins that recognize appropriate carbohydrate receptors. Among these, galactoside-containing carbohydrates constitute the most commonly reported receptor molecules.[6] Protein to protein interactions, involving, for example, integrins and proteases, represent the second major group of colonizing factors, and other types of specific intermolecular associations have been suggested.

A simple test, the coaggregation assay,[7] is used to determine whether oral isolates exhibit an affinity for one another. If two cell types possess in one case a specific adhesin and in the other a complementary receptor, then mixing turbid suspensions of each results in the formation of settling aggregates. This versatile test can be used to identify and to characterize the interactions in either a qualitative or quantitative fashion. The test also provides a means of determining whether a simple compound (e.g., sugar) or antibody can inhibit the coaggregation of two bacterial partners. Inhibition of coaggregation by ascites fluid was the principal criterion used to select hybridomas for cloning into mice and led to the subsequent production of adhesin-specific monoclonal antibodies.[8]

Prior to attempting the production of adhesin-specific monoclonal antibodies, studies were performed to determine whether polyclonal antisera directed against intact wild-type cells bearing the adhesin of interest could be used to inhibit aggregation of two cell types. Sera containing high levels of precipitating antibody made inhibition screening difficult or impossible to perform. However, extensive adsorption of the sera with coaggregation-deficient cells yielded sera that could be used for the immunological detection of adhesin molecules or for inhibition studies.[9,10] The success of these experiments increased the probability of successfully preparing adhesin-specific monoclonal antibodies.

Preparation of Antigen

A suspension of an overnight culture of *Prevotella loescheii* PK1295 cells (2 g wet weight per 10 ml of coaggregation buffer[7]) is subjected to mild disruption by four 30-sec treatments with a Kontes (Vineland, NJ) Micro-Ultrasonic cell disruptor. The suspension is centrifuged at 35,000 *g*

[6] J. London, *Ann. Rep. Med. Chem.* **26,** 239 (1991).

[7] P. E. Kolenbrander, this volume [31], p. 385.

[8] E. I. Weiss, J. London, P. E. Kolenbrander, R. N. Andersen, C. Fischler, and R. Siraganian, *Infect. Immun.* **56,** 219 (1988).

[9] A. Kagermeier and J. London, *Infect. Immun.* **51,** 490 (1986).

[10] E. I. Weiss, P. E. Kolenbrander, J. London, A. R. Hand, and R. N. Andersen, *J. Bacteriol.* **169,** 4215 (1987).

for 15 min, and the resulting supernatant is passed through a 0.22-μm low-protein binding filter. The filtrate is then centrifuged at 230,000 *g* for 3 hr, the clear pellet is suspended in 20 m*M* Tris-HCl buffer, pH 7.5, and the preparation is examined by electron microscopy. Phosphotungstate staining reveals a few membrane vesicles, but the suspension is mainly composed of fimbriae which retain the ability to aggregate separately both partners of *P. loescheii,* namely, *Streptococcus oralis* 34 and *Actinomyces israelii* PK14. Following the addition of 0.25% CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) to the fimbriae-containing preparation, the suspension is passed over a Sephacryl 500 column (1 × 40 cm) previously equilibrated with coaggregation buffer without azide containing 0.25% (w/v) CHAPS. The fimbriae-enriched suspension, which elutes in the void volume of the column, is collected and concentrated to approximately 400 μg protein/ml.

Preparation of Monoclonal Antibodies

BALB/c mice receive an initial injection of the fimbriae-enriched preparation (40 μg protein/injection) in Freund's complete adjuvant in the foot pad and a total of four booster injections (30 μg of protein) every 21 days. One week after administration of the final injection, serum from each animal is tested for the ability to inhibit coaggregation with both partner cells. Following an adaptive procedure,[11] spleen cells are fused with the myeloma cell line X63-AG8-653, and the hybridomas are screened for production of coaggregation-inhibiting antibody as well as precipitating antibody. The latter is assayed qualitatively by a conventional capillary tube assay in which equal volumes of antigen are mixed with various dilutions of antibody. Hybridomas producing the highest levels of inhibiting or precipitating antibody are cloned in mice.

Ultimately, we isolated four lines of monoclonal antibody that are potent inhibitors of coaggregation between *P. loescheii* and *S. sanguis*, two lines that block coaggregation between *P. loescheii* and *A. israelii*, and four lines of precipitating antibody. In a standard coaggregation assay, the monoclonal antibodies completely inhibit interaction of the appropriate partner cells in the range of 0.1 to 10 μg protein; Fab fragments are inhibitory at even lower concentrations. However, neither the clones reacting with the streptococcal-specific adhesin (galactoside sensitive) nor those reacting with the actinomyces-specific adhesin (galactoside insensitive) agglutinate suspensions of fimbriae or intact *P. loescheii* cells. Three of the four monoclonal lines of streptococcal-specific antibody and one of two mononclonal lines of actino-

[11] J. F. Kearney, A. Rudbruch, B. Liesgang, and K. Rajewsky, *J. Immunol.* **123,** 1548 (1979).

myces-specific antibody react with adhesin on Western immunoblots. These are the clones chosen for affinity purification of the respective adhesins.

Purification of Galactoside-Specific Adhesin

1. Between 15 and 20 mg of monoclonal line 3AD6 or 5DB5 protein[8] is reacted with 1 g CNBr-Sepharose 4B to prepare a 3-ml volume of the affinity matrix. The matrix is washed several times with 0.2 *M* sodium borate buffer (BBS), pH 8.0, containing 0.8% NaCl and 0.02% NaN_3 and stored in the same buffer.

2. A sample of *P. loescheii* cells (8 to 10 g wet weight) is suspended in BBS containing EDTA (10 m*M*) and phenylmethylsulfonyl fluoride (PMSF, 5 m*M*) to a final volume of 30 ml. The suspension is subjected to either mild breakage (see above) or prolonged sonication in a Branson Model 350 sonifier operating at 75% of maximum power for 2–3 min; suspensions are cooled with a circulating ice–water bath.[12]

3. The suspension of disrupted cells is centrifuged (240,000 *g* for 60 min), and the clarified supernatant is concentrated to a volume of 4 to 6 ml by membrane filtration (10-kDa cutoff).

4. The concentrate is mixed with 3 ml of antibody–Sepharose 4B conjugate, and the mixture is rotated end-over-end at 4° for 12 hr. This method provides far more effective binding of the antigen than is obtainable by simply passing the adhesin preparations over a column containing the matrix. The Sepharose beads are collected and separated from the ultrasonic preparation by centrifugation (1200 *g* for 2 min at 4°).

5. The affinity matrix is washed once with 5 ml BBS containing protease inhibitors (EDTA and PMSF, as above), three times with BBS alone, once with BBS containing 1 *M* NaCl, once with BBS containing 0.25% CHAPS, and finally twice in 5 ml BBS.

6. Adhesin is quickly eluted from the matrix with three 5-ml washes of 5% acetic acid, maintaining matrix and eluates at 4° at all times. The matrix is brought to pH 8.0 with BBS for subsequent reuse, and the eluate is adjusted to pH 4.5–5.0 by the addition of 1 *M* Tris-HCl buffer, pH 6.8. The volume of the solution is reduced to 0.5–1 ml, and the buffer concentration is reduced to 20 m*M*, by ultrafiltration. By this procedure, between approximately 200 and 400 μg of pure adhesin (Fig. 1A) is obtained from extracts prepared by extensive sonic disruption, whereas mild sonic disruption yielded approximately 150 μg of adhesin protein. The product can be stored at 4° in 0.02% NaN_3 or frozen at −20°, remaining intact and active even after prolonged periods of storage.

[12] J. London and J. Allen, *J. Bacteriol.* **172,** 2527 (1990).

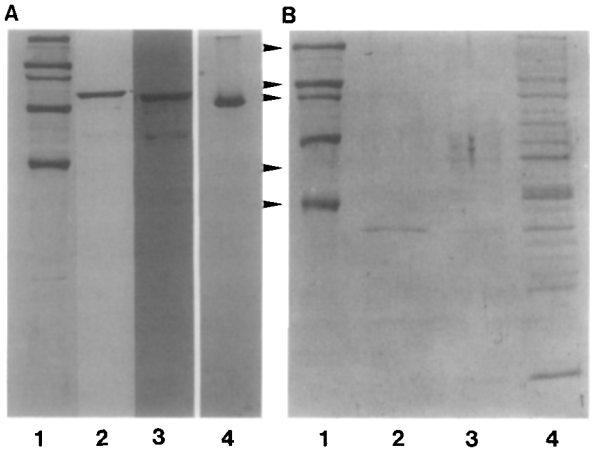

FIG. 1. (A) Gel electrophoresis of the galactoside-specific adhesin purified from monoclonal antibody–agarose conjugate. Lane 1, molecular weight standards: myosin, 200 kDa; β-galactosidase, 116 kDa; muscle phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; egg white ovalbumin, 45 kDa. Lane 2, denaturing gel of pure adhesin. Lane 3, immunoblot of purified adhesin developed with monoclonal antibody 3AD6. Lane 4, native gel electrophoresis of purified adhesin. (B) Denaturing gel electrophoresis of the actinomyces-specific adhesin. Lane 1, molecular weight standards as above; lane 2, intact-cell purified actinomyces-specific adhesin; lane 3, pooled and concentrated washes; lane 4, composition of starting material.

An essentially identical protocol is used to purify the nonlectin adhesin that is specific for *A. israelii.* For these purifications, monoclonal line 2AA1 is conjugated to CNBr-Sepharose 4B. However, significantly less adhesin is recovered (J. London and J. Allen, unpublished data, 1992), roughly 10 to 20% of the galactoside-specific adhesin.

Conventional Affinity Chromatography

Commercially available agarose or Sepharose supports to which lactose, galactose, or *N*-acetylgalactosamine has been conjugated are used in attempts to purify the *P. loescheii* galactoside-specific adhesin utilizing the batch procedures as described above. Employing column bed volumes of between 3 and 5 ml, extracts prepared by use of a Waring blendor are first transferred to the column. After extensive washing with 20 m*M* Tris-HCl buffer, pH 7.5, the adherent proteins are eluted with the appropriate concentrations of sugar solutions (25 to 50 m*M*). The sugars are removed from the eluates by dialysis, and the preparations are tested for the presence of the galactose-specific adhesin. This procedure yields nanogram quantities of adhesin from extract preparations that usually yield 200 to 300 μg of

adhesin protein using the monoclonal antibody conjugates. Furthermore, because of nonspecific adhesion, these preparations also contain trace contaminations of other proteins. The efficacy of other disaccharide galactosyl conjugates were not tested. These may be significantly more effective in purifying galactoside-specific adhesins.

Unconventional Affinity Chromatography

As mentioned earlier, the yield of the nonlectin actinomyces-specific adhesin is in the range of 15 to 30 μg protein per preparation of 8 g wet weight cells. An alternate procedure is designed to both improve the yield of adhesin and conserve monoclonal antibody. In general, gram-positive bacterial cells (e.g., streptococci and actinomyces) are significantly more resistant to lysis than their gram-negative counterparts. An attempt is made, therefore, to use intact cells of these species for affinity chromatography. A 60-ml suspension *A. israelii* cells (10^{10} frozen cells/ml) is washed in coaggregation buffer, made up to 6 ml in the same buffer, and placed in a 15-ml plastic test tube. Two milliliters of a Waring blendor preparation of *P. loescheii* cells (1.4 mg protein) is added to the cells, and the test tube is rocked gently for approximately 5 min. Adsorption of the adhesin to cells is signaled by the appearance of large aggregates.

The mixture is centrifuged for 10 min at 2300 *g*, and the supernatant fluid is removed and retained. The cell pellet is gently suspended in 10 ml of coaggregation buffer. Washing is accomplished by rocking of the mixture, and this cycle is repeated 3 to 4 times. In each case, the supernatants are collected and pooled. Finally, the pellet is washed two to three times with a solution of 5% acetic acid, neutralized, and suspended in coaggregation buffer. The eluate is quickly neutralized with 1 *M* Tris-HCl, pH 7.8, dialyzed against 10 m*M* Tris-HCl, pH 7.5, and freeze-dried. The residue is suspended in 250 ml of distilled water.

Essentially all of the protein in the suspension is recovered (96%) in the cumulative washes; the acetic acid eluate contains 25 μg protein. Denaturing polyacrylamide gel electrophoresis reveals that the eluate contains one major polypeptide (M_r approximately 43,000, Fig. 1B) and one minor protein band. The preparation also reacts with monoclonal antibody that recognizes the actinomyces-specific adhesin. Although the yield of the adhesin is not great, the preparation appears to be relatively pure. The same batch of cells can be used for several purification cycles without substantial decrease in yield.

Comments

The time invested in producing antiadhesin antibodies (monoclonal or polyclonal) is considerable. However, the procedures described here pro-

vide a reliable way to identify and isolate this elusive class of protein molecules. There are numerous benefits to possessing a preparation of adhesin-specific antibodies. For example, when appropriately labeled, they can be used to estimate the number of adhesins present on specific microorganisms. Alternatively, when coated on gold particles, they display the topographic arrangement of the proteins on the cell surface. Finally, they proved to be invaluable tools for assaying the cloning of adhesin genes, where they can be used to detect expression of the gene product.

[33] Analysis for Adhesins and Specific Cytoadhesion of *Trichomonas vaginalis*

By JOHN F. ALDERETE, ROSSANA ARROYO, and MICHAEL W. LEHKER

Introduction

Trichomonas vaginalis is a protozoan parasite that is one of the most common, clinically recognized sexually transmitted infectious agents. As with other mucosal pathogens, trichomonads must be capable of surmounting the mucus barrier and must cytoadhere to the squamous epithelial cells of the vaginal epithelium in order to overcome being expelled by the continuous fluid flow of the vagina. Indeed, in some patients, a significantly elevated vaginal discharge occurs during trichomoniasis. Is it possible, then, to determine whether cytoadhesion by the parasite, either to host cells in monolayer cultures as *in vitro* models or to freshly derived human vaginal epithelial cells, is specific, and, if so, can the molecules mediating host cell recognition and binding be identified?

What follows is a description of the assays developed and employed to establish the highly specific nature of *T. vaginalis* cytoadhesion and the identity of trichomonad surface proteins involved in this property. It is noteworthy that, prerequisite to the identification of important virulence factors, like adhesins, some degree of understanding is needed of the host factors of the vagina (or at the site of infection of a given microbial pathogen) that may provide environmental signals that regulate expression of the property being examined, in this case cytoadhesion and synthesis of adhesins.

Specificity and Nature of Cytoadhesion

Adhesion Assay with HeLa Cells on Glass Coverslips

HeLa epithelial cells (American Type Culture Collection, Rockville, MD) are maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin–streptomycin or gentamicin.[1] Cultures are kept in a 7% CO_2 atmosphere at 37°, and cells in monolayer cultures on round glass cov-

[1] J. F. Alderete and E. Pearlman, *Br. J. Vener. Dis.* **60,** 99 (1984).

erslips[1,2] are passaged and used for cytoadhesion assays. Cell suspensions are seeded at a density of 2.0×10^5 to 2.5×10^5 cells onto sterile, ethanol-washed glass coverslips (diameter 15 mm) placed in individual wells of 24-well Costar culture plates (Bellco Glass, Inc., Vineland, NJ). Cells are grown for 18 to 24 hr in DMEM containing 10% FBS, at which time the monolayers have approximately 4×10^5 cells and are over 90% confluent, determined by visualization via phase-contrast microscopy.

The parasites are cultivated in a complex medium consisting of Trypticase, yeast extract, and maltose (TYM) supplemented with 10% (v/v) heat-inactivated horse serum. Organisms are radiolabeled for 18 hr with 1–10 μCi/ml [^{3}H]thymidine.[1,2] Radiolabeled trichomonads grown in TYM–serum complex medium are washed three times in phosphate-buffered saline (PBS) and suspended to the desired densities in a medium mixture of 2 parts cell culture medium (DMEM) and 1 part trichomonal medium (TYM).[1,2] The DMEM–TYM medium mixture without serum is important because it is found to support both parasites and host cells in coincubation experiments at 37° in a 7% CO_2 atmosphere. Organisms and host cells are always equilibrated in the medium mixture at 37° prior to coincubation. Experiments involving different temperatures are done without a CO_2 atmosphere; however, the pH of the medium is monitored throughout the experimental period. The ratio of parasites to host cells (*P/C*) used for the experiments is optimized to be 5:1[1] such that, for the assay, 2×10^6 trichomonads in 1 ml of DMEM–TYM are added to the cell monolayer. After the time desired (maximum cytoadhesion for trichomonads occurs by 30 min), the glass coverslips are washed by immersion no less than 10 times in a glass beaker containing sterile PBS prewarmed to 37°. The coverslips are then air-dried and placed inside vials with scintillation cocktail for measurement of adherent radioactivity.

Cytoadhesion Assay in Microtiter Wells

In the modified adhesion assay using microtiter well plates, 4×10^4 HeLa cells are seeded in each well and incubated overnight (final cell numbers in each well averaged 8×10^4). To confluent monolayers is added 100 μl containing a 4×10^5 [^{3}H]thymidine-labeled parasites (*P/C* ratio of 5) suspended in a 2:1 mixture of DMEM–TYM medium, as has been described.[1,2] After incubation for 30 min (or the time desired) at 37° in a 7% CO_2 atmosphere, unbound parasites are removed, and wells with adherent trichomonads are washed three times with temperature-equilibrated DMEM. The microtiter well plates are air-dried, and radioactivity is measured by scintillation spectroscopy.

[2] J. F. Alderete and G. E. Garza, *Infect. Immun.* **50,** 701 (1985).

All experiments are performed in quadruplicate, and one sample is always visualized by phase-contrast microscopy to ensure that the results from radioactivity measurements represent *bona fide* radiolabeled organisms adherent to host cells. Parasite viability and motility are always monitored throughout the experiment.

Cytoadhesion Assay Using Vaginal Epithelial Cells

Using cells from the site of infection affirms the results derived from using HeLa cells in monolayer cultures as the *in vitro* model. The preparation of vaginal epithelial cells (VECs) is done through sequential filtration to remove debris and other host cells and to enrich for VECs.[3] Swabs from the vagina of control, uninfected women as well as of patients with trichomoniasis are immersed singly or combined in a 10-ml volume of PBS for processing. The VEC preparation is then passed through a 60-μm pore-size nylon filter, which removes the very large debris while allowing the VECs, bacteria, and other small diameter debris to pass. The flow-through is then filtered with an 8-μm pore-size nylon filter. The VECs are retained in this filter and are then gently suspended and washed twice in PBS. Once it became clear that glutaraldehyde fixation of VECs still allows for trichomonal parasitism under the same conditions, as established with HeLa cells in monolayer cultures,[3,4] VECs are routinely fixed at 4° with glutaraldehyde at a 2.5% (v/v) final concentration prepared in PBS and stored at 4°.[3] Cells from patients used for cytoadhesion experiments are purified at 4°, which allows for detachment of the adherent parasites from the host cells, and trichomonads are then removed from VECs during the filtrations.

The results from employment of the adhesion assays have been reported in a series of articles that, collectively, show the highly specific nature of *T. vaginalis* recognition and binding to host cells.[1–7] Furthermore, the cytoadhesion findings reinforce the idea that trichomonad surface proteins are mediators of specific host cell attachment.

Identification of Adhesins and Other Considerations

Ligand Assay

A method has been developed by which specific microbial surface proteins involved in cytoadhesion are identified. A detergent extract of radiola-

[3] J. F. Alderete, P. Demes, A. Gombosova, M. Valent, A. Janoska, J. Stefanovic, and R. Arroyo, *Infect. Immun.* **56,** 2258 (1988).
[4] J. F. Alderete and G. E. Garza, *Infect. Immun.* **56,** 28 (1988).
[5] R. Arroyo and J. F. Alderete, *Infect. Immun.* **57,** 2991 (1989).
[6] R. Arroyo, J. Engbring, and J. F. Alderete, *Mol. Microbiol.* **6,** 853 (1992).
[7] M. Lehker, R. Arroyo, and J. F. Alderete, *J. Exp. Med.* **174,** 311 (1991).

beled microorganisms is incubated with chemically stabilized host cells.[8,9] The assay has been employed successfully to identify the fibronectin-binding proteins of *Treponema pallidum*[8] and the adhesin of *Mycoplasma pneumoniae*.[9] The earlier work had showed that fixation of host cells still preserved the adhesin-binding receptors and, equally important, that detergents could be used which allowed for functionality of the receptor-binding epitopes of the adhesins. Clearly, the specificity by which *T. vaginalis* organisms recognize and bind to HeLa cells and VECs and especially the implication that surface proteins are mediating cytoadhesion prompted the use of the ligand assay for identifying the putative trichomonad adhesin proteins.

Parasites are radiolabeled intrinsically with [^{35}S]methionine or extrinsically through radioiodination using standard protocols.[4,6,7] After three washes in PBS, approximately 2×10^7 trichomonads are then incubated for 10 min at 4° in 0.5 ml of NET buffer [50 m*M* Tris-HCl, pH 7.4, 15 m*M* NaCl, and 5 m*M* ethylenediaminetetraacetic acid (EDTA)] containing 1 m*M* *p*-tosyl-L-lysine chloromethyl ketone (TLCK; a cysteine proteinase inhibitor).[5,6] Parasites are then solubilized by the addition of 1% (w/v) deoxycholate and trituration. To the extract is added 0.5 ml of TDSET buffer [10 m*M* Tris-HCl, pH 7.8, 0.2% (w/v) deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 10 m*M* EDTA, and 1% (v/v) Triton X-100] containing 1 m*M* TLCK, and the mixture is triturated gently.The detergent extract is then centrifuged at 100,000 *g* through a 10% (w/v) sucrose–phenol red cushion to remove any insoluble debris, and the supernatant (~1 ml) is added to a pellet of 10^6 HeLa cells or 10^6 VECs previously fixed with glutaraldehyde.[4,6] After an 18-hr incubation at 4°, the fixed host cells are then centrifuged at 600 *g* and washed three times with TDSET buffer containing 1 m*M* TLCK. To elute any specifically, avidly bound proteins, fixed host cells are finally boiled in electrophoresis dissolving buffer[10] for 3 min, then microcentrifuged to remove host cells, and released, radiolabeled trichomonad proteins are subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using 7.5 or 10% (w/v) separating acrylamide gels.[6,7,11,12]

For chemical stabilization of host cells used in the ligand assay, cells are washed in PBS and suspended to a density of 5×10^6 cells/ml in PBS. Then the cells are fixed with 2.5% (v/v) glutaraldehyde in PBS for 30 min to 1 hr and gently stirred at 4°. Cells are monitored continuously and triturated to avoid clumping. Fixed cells are then washed extensively at least five times with ice-cold PBS and incubated with 0.2 *M* glycine in PBS

[8] J. B. Baseman and E. C. Hayes, *J. Exp. Med.* **151,** 573 (1980).
[9] D. C. Krause and J. B. Baseman, *Infect. Immun.* **37,** 382 (1982).
[10] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).
[11] J. F. Alderete, *Infect. Immun.* **39,** 1041 (1983).
[12] J. F. Alderete, *Infect. Immun.* **40,** 284 (1983)

for at least 1 hr at 37°. The cells are then washed five times with TDSET and suspended to the desired density in individual microcentrifuge tubes for immediate use. Fixed cells are also kept at 4° for several days prior to use in the ligand assay.

Other Considerations

At least three considerations were important in the successful employment of the ligand assay for identification of putative adhesins. First, the ligand assay was employed using extracts of radiolabeled *T. vaginalis* organisms solubilized with different detergents. Zwitterionic detergents were initially favored because of efficient solubilization of trichomonad membrane protein immunogens, as evidenced by the successful utilization of radioimmunoprecipitation assays.[11-14] Extracts of *T. vaginalis* made with Zwittergent 3–12 detergent and several other detergents did not allow for detection of any trichomonad proteins specifically and avidly bound onto host cells. Only experiments performed with deoxycholate gave reproducible binding of four proteins which could be resolved by SDS–PAGE and autoradiography.[4] The detergent and conditions required for efficient solubilization of the adhesins with preservation of the receptor-recognition epitopes have since been modified, as described above, but still incorporate the essential feature of exposure of trichomonads to deoxycholate before addition of TDSET buffer.

Next, early attempts to identify the adhesins resulted in autoradiograms with very diffuse protein bands in four gel areas.[4] The diffuse nature of the proteins was subsequently determined to be due to trichomonal proteinases released during detergent solubilization. Although the early ligand assays incorporated inhibitors of proteinases, the inhibitors were without effect owing to the complexity and specificity of the proteinases of *T. vaginalis*.[15,16] Inclusion of the correct inhibitors during the detergent solubilization allowed for resolution of four proteins, as has been reported.[7] The analysis of the trichomonad cysteine proteinases[16] then prompted further experiments which showed the exquisitely sensitive nature of the adhesins to the proteinases.[6]

Finally, evaluation of fresh *T. vaginalis* isolates, for the cytoadhesion and ligand assays, as compared to laboratory strains that were being examined, immediately revealed dramatic differences in the levels of cytoadhesion

[13] J. F. Alderete, L. Suprun-Brown, L. Kasmala, J. Smith, and M. Spence, *Infect. Immun.* **49,** 463 (1985).
[14] J. F. Alderete, L. Suprun-Brown, and L. Kasmala, *Infect. Immun.* **52,** 70 (1986).
[15] K. A. Neale and J. F. Alderete, *Infect. Immun.* **58,** 157 (1990).
[16] G. H. Coombs, D. T. Hart, and J. Capaldo, *Parasitology* **86,** 1 (1983).

(Table I).[6,7] All fresh isolates yielded higher overall levels of cytoadhesion. The range of percent diferences in cytoadhesion between organisms grown in high- versus low-iron medium ranged from 14 to 67%. Some long-term grown isolates, like IR 78 and JH 31A, were relatively unresponsive to iron supplementing the medium. Clearly, isolates passaged *in vitro* for extended periods were no longer representative of wild-type, infecting *T. vaginalis* parasites in the overall level of cytoadhesion.

Elevated levels of cytoadhesion (Table I) and upregulation of adhesin synthesis (Fig. 1) occurred under iron-replete medium conditions.[7] All isolates seemed capable of responding to iron added to the medium. Four trichomonad proteins designated AP65, AP51, AP33, and AP23 were iden-

TABLE I
EXAMINATION OF *Trichomonas vaginalis* ISOLATES GROWN IN HIGH- AND LOW-IRON MEDIUM FOR LEVELS OF CYTOADHESION

Isolate[a]	Relative adhesion value[b]		
	High-iron	Low-iron	Reduction (%)
Fresh			
T038	4.96 ± 0.39	2.08 ± 0.23	58.0
T048	6.53 ± 0.84	2.16 ± 0.32	66.9
T023	2.31 ± 0.21	1.58 ± 0.17	31.6
T036	2.75 ± 0.31	1.82 ± 0.16	33.8
Long-term grown			
NYH 286	1.19 ± 0.14	0.58 ± 0.04	51.0
T005	1.46 ± 0.21	0.72 ± 0.12	50.6
T003	1.52 ± 0.14	0.96 ± 0.06	37.0
RU 375	1.50 ± 0.11	1.10 ± 0.09	26.0
IR 78	0.73 ± 0.08	0.60 ± 0.12	14.2
JH 31A	0.95 ± 0.12	0.78 ± 0.15	17.8

[a] Isolates grown for more than 30 days *in vitro* were considered long-term grown. Fresh clinical isolates were grown for less than 30 days *in vitro*.[6,7]

[b] *Trichomonas vaginalis* organisms were grown in high- or low-iron medium as described[7] in the presence of 2 μCi/ml [^{3}H]thymidine. At the late exponential growth phase, which occurred between 24 and 48 hr of growth, parasites were washed twice and added to confluent HeLa cell monolayers in microtiter wells, as described in the text.[7] Nonadherent trichomonads were removed by washing and associated radioactivity of the wells counted by scintillation spectroscopy. Adhesion of NYH 286 grown in normal complex growth medium was set as 1 for comparative purposes, as has been done before,[6,7] to show the greater levels of cytoadhesion by fresh isolates. Reproduced from the *Journal of Experimental Medicine*, 1991, *174*, 311–318, by copyright permission of the Rockefeller University Press.

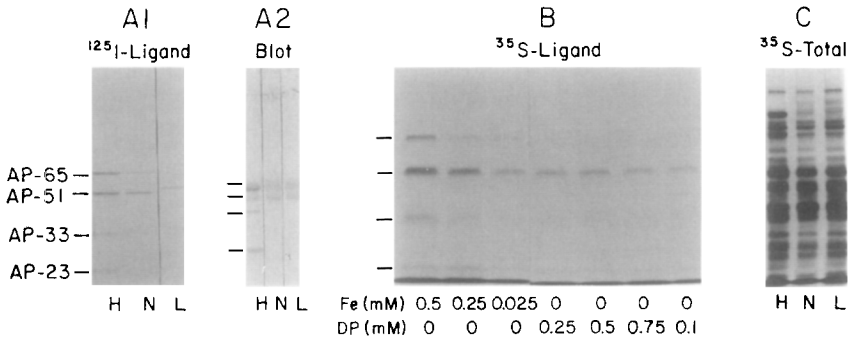

FIG. 1. Iron modulation of surface expression and synthesis of the four trichomonad adhesins.[7] *Trichomonas vaginalis* T048 cells were grown in high- (H) or low- (L) iron medium or in normal growth medium (N) as detailed.[7] Parasites were then used for a ligand assay to identify the adhesins as described in the text,[7] and acrylamide gels were analyzed by autoradiography using ^{125}I surface-labeled parasites (A1) and by fluorography using [^{35}S]methionine-labeled trichomonads (B) grown in TYM–serum medium at different iron concentrations. Duplicate gels of the experiment performed in (A1) were also blotted and probed using a pooled preparation of monospecific antiadhesin serum (A2). The fluorogram of total proteins of parasites grown in high-, normal-, and low-iron medium is shown in (C). Reproduced from the *Journal of Experimental Medicine,* 1991, **174,** 311–318, by copyright permission of the Rockefeller University Press.

tified as the trichomonad adhesins,[4,6,7] as evidenced by the autoradiographic profiles obtained from a ligand assay in which a detergent extract of surface-labeled parasites was incubated with fixed HeLa cells (Fig. 1, A1).[7] The four iodinated adhesins were present in larger amounts when the ligand assay was performed with organisms grown in a high-iron medium (lane H) when compared to parasites grown in either a normal growth medium (lane N) or a low-iron medium (lane L). Visualization of iodinated adhesins in the normal and low-iron grown trichomonads required a longer exposure of the gel to X-ray film for this ligand assay.

Immunoblot experiments were performed simultaneously on the adhesins from a ligand assay using antiadhesin antibodies known to inhibit cytoadhesion by *T. vaginalis*[6] (Fig. 1, A2). After their isolation from the fixed cells, electrophoresis, and blotting, adhesins on nitrocellulose were probed with a pooled preparation of each monospecific antiadhesin serum.[6] The four adhesins were readily detected in parasites grown in high-iron medium (Fig. 1, A2, lane H) but not in blots with adhesins from organisms grown in normal (lane N) or low-iron medium (lane L), confirming that the host cell-bound proteins that were upregulated with iron were the adhesins.

Iron influenced overall adhesin synthesis and not just surface expression of adhesins (Fig. 1B). Total synthesis of the adhesins was monitored in a ligand assay using detergent extracts of [^{35}S]methionine-labeled trichomonads. Synthesis of adhesins was dependent on the concentration of iron, and again organisms from a high-iron medium (lane H, Fig. 1B) had the most intense bands on fluorograms, indicative of the highest amounts of adhesins. Because fluorograms of total proteins were equally complex and comparable for both high-iron and low-iron medium grown organisms (Fig. 1C), the data show the regulation by iron of only the adhesins, and not all proteins. It was noteworthy that the routinely used complex TYM–serum medium employed for growing the parasites was deficient in amounts of iron needed for optimal adhesin synthesis.

Finally, when isolates and media were obtained from different laboratories, a large variation in levels of cytoadhesion and amounts of adhesins were obtained. In all cases, however, the same isolates grown in TYM–serum supplemented with iron exhibited enhanced synthesis of adhesins with correspondingly elevated levels of cytoadhesion. The significance of the latter results cannot be underscored, however, because the precise environmental cues responsible for expression of virulence factors must be understood in order for results among different laboratories to be reproducible.

Acknowledgments

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[34] Adhesion of Fungi

By KEVIN C. HAZEN and PATI M. GLEE

Introduction

Adhesion of a fungus to an appropriate surface precipitates a variety of biological events that could influence fungal survival and could ultimately jeopardize viability or integrity of the host surface. Following adhesion, fungal spores will elaborate vegetative growth structures or will produce structures that penetrate the host surface. Within the human host, conversion of mycelial elements or conidia to yeast cells and subsequent growth may result in destruction of host tissue and dissemination to vital organs.

Iron influenced overall adhesin synthesis and not just surface expression of adhesins (Fig. 1B). Total synthesis of the adhesins was monitored in a ligand assay using detergent extracts of [^{35}S]methionine-labeled trichomonads. Synthesis of adhesins was dependent on the concentration of iron, and again organisms from a high-iron medium (lane H, Fig. 1B) had the most intense bands on fluorograms, indicative of the highest amounts of adhesins. Because fluorograms of total proteins were equally complex and comparable for both high-iron and low-iron medium grown organisms (Fig. 1C), the data show the regulation by iron of only the adhesins, and not all proteins. It was noteworthy that the routinely used complex TYM–serum medium employed for growing the parasites was deficient in amounts of iron needed for optimal adhesin synthesis.

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Understanding the mechanisms of fungal adhesion to inanimate and animate structures is tantamount to the development of effective modalities for inhibiting (or enhancing) successful fungal colonization.

A variety of adhesion assays for fungi have been devised and are, as expected, dependent on the interest of the investigator. No one assay can be applied to all fungi because the substrata that are attacked by fungi differ; insect cuticle is distinctly different from human endothelium. However, the general designs of the adhesion assays closely mimic those used for studying bacterial adhesion. For this reason, it is worthwhile for the interested investigator to examine bacterial adhesion assays as they may translate to fungal systems relatively well. Caveats that must be considered when applying bacterial adhesion assay designs to fungi are addressed below. Information relative to the development of fungal adhesion assays are described along with details of two convenient assays.

Caveats for Fungal Adhesion Assays Compared to Bacterial Adhesion Assays

Fungi differ from bacteria in several important ways. For example, fungal cells are generally larger than bacteria and therefore are less subject to Brownian motion, are more easily sheared from a surface, and more easily settle out of suspension than bacteria. The composition of the cell wall is also different and is complex. Wall architecture and surface composition can change with cell cycle and with growth phase. Fungi form a variety of structures. The structures that are produced in an inanimate environment (e.g., hyphae in soil) may differ from the structures that are made when the fungus attacks an animate object (appressoria or haustoria on a leaf surface). A variety of surface appendages are also produced. Growth of molds involves multicellular strands, thus limiting the ability to assay single cells.

Once an investigator has considered how each fungal attribute will affect an adhesion assay, the difference between bacterial assay systems and fungal assay systems is negligible. It is the mechanical aspects of the assay that are then most affected. Such aspects include but are not limited to preparation of the fungal cells, quantitation of adhesion, and incubation conditions. Table I illustrates the effects of different mechanical aspects on the outcome of various adhesion assays.

Considerations for Constructing Fungal Adhesion Assay

Cell Preparation

An investigator usually knows which cellular form (conidia, asexual or sexual spore, hyphal elements) of a fungus is responsible for the initial

TABLE I

ASSAY PARAMETERS INFLUENCING FUNGAL ADHESION

Parameter	Organism	Result	Ref.
Assay medium	*Nectria haematococca*	Macroconidia produce adhesive glycoprotein when suspended in plant extract or potato–dextrose broth but are nonadherent in water	*a*
	Magnaportha grisea	Conidia release adhesive compounds on hydration	*b*
	Fusarium solani f. sp. *phaseoli*	Macroconidial attachment to root zones of a hydroponically grown host is influenced by nutrient solution pH	*c*
Inoculum density	*Beauveria bassiana*	At low inoculum density, conidia attach to specific areas of larval cuticle; at high densities there is coaggregation and less target specificity	*d*
	Candida albicans	Coadhesion to buccal epithelial cells occurs at high yeast densities	*e*
Length of incubation	*Candida albicans*	Incubation times of 30–60 min demonstrate increasing cell surface hydrophobicity for initially hydrophilic cells and results in penetration and disruption of endothelial cell surfaces	*f, g*
Host cell variability	*Candida albicans*	Hormonal or ABO blood group status of donor epithelial cells affect yeast attachment	*h, i*
	Nomurea rileyi	Preferential attachment of conidia to epicuticle and not endocuticle	*j*
Fungal growth medium	*Candida albicans*	Carbon source influences adhesion to plastic and epithelial cells	*k, l*

[a] Y. Kwon and L. Epstein, *Mol. Plant–Microbe Interact.* **6,** 481 (1993).
[b] J. E. Hamer, R. J. Howard, F. G. Chumley, and B. Valent, *Science* **239,** 288 (1988).
[c] A. C. Schuerger and D. J. Mitchell, *Phytopathology* **82,** 1311 (1992).
[d] S. Pekrul and E. A. Grula, *J. Invertebr. Pathol.* **34,** 238 (1979).
[e] R. King, J. Lea, and A. Morris, *Infect. Immun.* **72,** 667 (1980).
[f] K. C. Hazen, *Infect. Immun.* **57,** 1894 (1989).
[g] S. A. Klotz, D. J. Drutz, J. L. Harrison, and M. Huppert, *Infect. Immun.* **42,** 374 (1983).
[h] E. D. Theaker, D. B. Drucker, and A. C. C. Gibbs, *Arch. Oral Biol.* **38,** 353 (1993).
[i] M. A. Buford, J. C. Weber, and J. M. Willoughby, *J. Med. Vet. Mycol.* **26,** 49 (1988).
[j] D. G. Boucias, J. C. Pendland, and J. P. Latge, *Appl. Environ. Microbiol.* **54,** 1795 (1988).
[k] L. J. Douglas, J. G. Houston, and J. McCourtie, *FEMS Microbiol. Lett.* **12,** 241 (1981).
[l] J. McCourtie and L. J. Douglas, *Infect. Immun.* **32,** 1234 (1981).

contact with a substratum. Ideally then, it is desirable to use such structures for the adhesion assay. Asexual spores of many molds are most easily obtained by growing the mold on a solid medium, whereby the spores may be borne on aerial hyphae. The spores can be dislodged from the hyphae by washing the surface of the solid medium with sterile saline or other liquid. The spores can then be washed by centrifugation and suspended in the desired assay solution. Dry spores can sometimes be obtained by simply inverting a petri dish culture and tapping the dish while the lid is attached[1] or by scraping the surface of colonies with a spatula.[2] Highly hydrophobic spores, such as those of *Aspergillus fumigatus*, can also be pelleted by centrifugation, but the addition of a mild surfactant (such as Tween 20) may be necessary. Individual arthroconidia can be obtained by vigorous agitation of hyphae obtained from the surface of a colony[3] followed by filtration through glass wool to remove chains.

If the assay requires hyphal cells, then hyphal fragmentation must be performed. A variety of methods are available, such as glass bead breakage, Dounce homogenization, grinding with sand, and sonication. Unfortunately, the methods can also lead to cell lysis and, at best, result in a heterogeneous mix of cell units (one or more cells per fragment). Subsequent separation of the cell fragments from larger segments of hyphae may be required for a particular assay and can be accomplished by filtration through gauze, cheesecloth, or sintered glass filters.

Yeast cells can be obtained by standard culture methods. We recommend broth culture as colonies obtained on solid media are composed of highly variable cell populations. Synchronized yeast cell populations are desirable, but this is oftentimes impractical. Choosing cells from the mid-exponential phase of growth may help overcome this problem but can also lead to additional difficulties. For example, the opportunistic pathogen *Candida albicans* typically undergoes cell catenation at exponential phase such that a contiguous cell unit may comprise from two to eight or more blastoconidia (K. C. Hazen and B. W. Hazen, unpublished observations, 1988). In the case of molds, conidia harvested from a colony may also be heterogeneous.

The choice of cell type and stage of growth can have a significant impact on adhesion results. Yeast cells obtained at exponential phase may expose different surface macromolecules than cells freshly diluted into medium or stationary phase cells.[4] Similarly, the selection of growth medium will also

[1] R. P. Doss, S. W. Potter, G. A. Chastagner, and J. K. Christion, *Appl. Environ. Microbiol.* **59,** 1786 (1993).
[2] D. G. Boucias, J. C. Pendland, and J. P. Latge, *Appl. Environ. Microbiol.* **54,** 1795 (1988).
[3] M. H. Straver, J. W. Kijne, and G. Smit, *Trends Biotechnol.* **11,** 228 (1993).
[4] B. W. Hazen and K. C. Hazen, *Infect. Immun.* **56,** 2521 (1988).

affect which surface macromolecules will be exposed for adhesion interactions. For example, yeast cells grown in a medium containing a high glucose concentration may have a different surface composition than cells grown in low glucose.

One common practice in preparing cells for adhesion assays is to fix the cells with glutaraldehyde, formalin, or other similar agent, or to kill the cells with a chemical agent. Although this practice provides a means to prepare a common, convenient source of cells and prevents cell growth during the adhesion assay, it results in alterations in the cell surface[5] that may influence the outcome of the experiment. Whenever possible, such treatments should be avoided, and, if used, the particular surface feature under study should be assessed before and after the treatment to ensure that it has not been altered.

Assay Medium

Quantitation of attachment of airborne conidia onto host substrata is difficult owing to problems of assuring that a fixed number of spores have equal access to the host surface after release into the air. To overcome this, conidia may be aerosolized in microdroplets and sprayed, but this too is an unsatisfactory procedure. Alternatively, airborne spores may be suspended in a buffer and the suspension placed onto the target host substrata.[1,2] This method, however, does not mimic the actual natural process, and the choice of suspending medium can greatly misrepresent the natural fungus–host contact environment.

In the case of medically important fungi, the environment in which the fungal cell contacts the host is liquid. Therefore, the choice of suspending liquid for the assay system should be designed to mimic the natural medium. This ideal is not possible and necessitates that an investigator use an alternate medium. The choice of medium can affect the outcome of the adhesion assay.

Several factors can influence the ability of a fungal cell to adhere to the substratum. These factors include ionic composition (especially the presence of divalent cations), viscosity, pH, surface tension, osmolarity, and the presence of antagonists or agonist chemicals. One or more of these factors are frequently ignored in assays. Consideration of each factor in the adhesion interaction must be made when constructing an adhesion assay. For example, certain human tissues such as vascular endothelium are exposed to plasma which has a specific pH, surface tension, viscosity, osmolarity, etc. In tissue culture systems where an endothelial cell mono-

[5] S. A. Klotz and R. L. Penn, *Curr. Microbiol.* **16,** 119 (1987).

layer is produced, adhesion assay medium may contain only 5 to 10% serum (not plasma) or no serum components, resulting in different interfacial characteristics than are present at the blood–endothelium region. Furthermore, growth of the monolayer in a tissue culture medium may lead to alterations in the endothelial membrane surface that are unlike the surface *in vivo*. On the other hand, it is the intentional manipulation of these assay medium factors that can also provide insights into fungal adhesion to host substrata.

An additional consideration for assay media is that a medium should not induce growth or surface changes of the fungus or host substratum during the adhesion assay that could affect the final adhesion result. That is, the initial surface conditions of the fungus and host—not those that form during the assay incubation period—should be the surfaces under study. Changes in the surfaces during the assay could lead to spurious results and misinterpretations of the significance of the surface characteristics under study. However, if surface changes are likely to occur, the investigator should be aware of what those are.

Assay Incubation Conditions

Numerous incubation factors can influence the outcome of an adhesion assay. The choice of conditions should ideally reflect the natural environment in which the fungus and host substrata make contact. In addition, the length of incubation can have a significant impact on the adherents. For example, the human opportunistic pathogen *Candida albicans* has been shown to undergo surface changes within 30 min after cell suspension in defined or complex assay media at 37° or room temperature.[4,6] Thus, cells potentially nonadherent for a given substratum may undergo surface changes that then allow the cells to be adherent or vice versa. This point is frequently not considered in *C. albicans* adhesion assays. It behooves the investigator to assess the changes in the cell surface during the incubation period in order to better interpret the final adhesion data. In the case of *C. albicans*, it is recommended that the incubation period not exceed 15 min. Similar considerations should be made for spores, as resting spores may undergo surface changes during activation in a suitable assay medium if the incubation conditions are appropriate.

Many times an adhesion assay is performed under static conditions. Although this is appropriate in many situations, it may be inappropriate in others. For example, attachment of the blood-borne fungi to vascular endothelium *in vivo* occurs under high shear forces (~2 dynes or more).

[6] K. C. Hazen, *Infect. Immun.* **57,** 1894 (1989).

Thus, the adhesion mechanism must be able to overcome the shear force to accomplish adhesion. Several adhesion mechanisms may succeed under static conditions, but only one mechanism may function under shear stress.

Detection of Adhesion

Fungal cells are relatively easy to visualize by bright-field microscopy because of their large size (>1 μm) and high refractivity. However, many substrata may not provide sufficient contrast to allow cell visualization, necessitating alternative methods. One of the easiest methods is to stain the fungal cell. A variety of stains are possible, such as crystal violet, lactophenol cotton (China) blue, phloxine, and Congo red. Several potential problems must be considered. First, sufficient contrast between the substratum and the fungal cell is needed. Second, the staining procedure must not itself lead to loss of cells or alteration of the binding pattern when this pattern is an element of the adhesion assay. This consideration is also true when washing nonadherent cells from the substratum. Whenever possible, an investigator should prepare several test samples to determine the effect of the staining procedure on adhesion outcomes. For example, it has been noted that crystal violet staining of *C. albicans* cells attached to a microtiter plate can lead to a 10% loss of cells (K. C. Hazen and P. M. Glee, unpublished observations, 1993). Cells can also be stained with a fluorescent dye. Calcofluor white (λ_{ex} ~440 nm, λ_{em} ~510 nm) is one such stain. It binds to chitin and cellulose in the fungal cell wall but may also bind nonspecifically to connective tissue within animal host tissues. Immunoflorescence techniques are also useful. Once stained, the number of adherent cells per unit area of substratum can be assessed.

Manual counting of adherent cells can sometimes be facilitated by inducing microcolony to small colony formation.[6-8] For example, adherent yeast cells of *C. albicans* on an epithelial cell monolayer can be overlaid with molten corn meal agar. Viable cells will then produce microcolonies by 8 to 24 hr which can be easily visualized under low-power magnification. Manual counting can also be facilitated by computer techniques using image analysis software.[9] If adherent cells are first radiolabeled (preferably with an isotope that does not interfere with surface characteristics), then the entire substratum can be assayed for radioactivity. In some cases, this would entail simply transferring the substratum in the appropriate vessel for radiometric detection. In other cases, the substratum may have to be disrupted to release the adherent cells. Epithelial cell monolayers, which

[7] P. P. Antley and K. C. Hazen, *Infect. Immun.* **56,** 2884 (1988).
[8] J. E. Cutler and B. D. Thompson, *J. Immunol. Methods* **66,** 27 (1984).
[9] M. H. Riesselman, T. Kanbe, and J. E. Cutler, *J. Immunol. Methods* **145,** 153 (1991).

have been washed to remove nonadherent fungal cells, can be trypsinized to release the cells from the plastic support surface and then subjected to radiometric detection.

An alternative approach to visualizing the number of adherent cells is to determine the number of nonadherent cells. To accomplish this, the nonadherent cells can be determined by various methods including colony formation, radioactivity, protein/DNA/RNA concentration, etc. Although useful, this method may limit important insights about the adherent cell population. It is advisable to visualize the adherent cell population whenever possible.

Specific Assays

Two adhesion assays are described below. As mentioned above, many of the assays that are used to assess bacterial adhesion capabilities are also amenable to fungal cells. The assays presented below have been shown to be useful for individuals interested in understanding yeast adhesion mechanisms to human tissues and conidial attachment to plant or insect cuticle.

Ex Vivo Tissue Adhesion Assay

The *ex vivo* tissue adhesion assay has several advantages over cell monolayer methods for assessing fungal adhesion to host substrata.[9] In particular, it allows the use of essentially undisrupted host tissues and the determination of specific binding patterns. The latter is helpful as it can give an indication as to which host cells are recognized by the fungal cells.[10] Although the *ex vivo* assay involves equipment that is usually present in a microbiology research laboratory, it also requires a cryostat to cut tissue sections of 5–15 μm thickness at $-20°$. The general procedure for the *ex vivo* assay involves placing yeast cell suspensions onto sections of host tissue, incubating to allow adhesion, fixing cells, and washing the nonadherent cells from sections. The adherent cells can then be visualized with or without the aid of staining. The use of crystal violet helps visualize fungal cells but does not provide much differential staining of host tissues. To enhance tissue staining, a counterstain can be used. It is important to consider that once tissue sections have been cut and placed on glass slides, they must be used within 1 hr. This limitation may influence when and how fungal cells are prepared. We have found, for example, that yeast cells of *C. albicans* did not change in surface hydrophobicity characteristics for at least 2 hr when the cells are pelleted by centrifugation and kept on ice.

[10] T. Kanbe, M. A. Jutila, and J. M. Cutler, *Infect. Immun.* **60,** 1972 (1992).

Procedure

1. Remove desired tissues from host animal, cut into 0.2- to 1.5-cm pieces (if necessary), embed in OCT compound (Miles Diagnostic Division, Elkhart, IN), and snap freeze on dry ice. The frozen tissues should be stored at −70° until needed. If mouse spleens are to be utilized, up to two spleens can be placed in a block.

2. From each block, cut 10-μm-thick sections and pick the sections up on Gold Seal Rite-On Micro Slides (Becton Dickinson Labware, Lincoln Park, NJ). Two sections can be placed on each slide.

3. Draw a 20-mm-diameter circle with water-insoluble ink (Isolator TM marker pen, No. 754, Lipshaw, Pittsburgh, PA, or a black ink Sharpie pen) around each section. Place the tissue sections in a 4°–6° chamber (such as a cold room). Allow the sections to dry for 15 min but no more than 1 hr before use. Be sure that the slides are on a flat, level surface.

4. Fungal cells are washed to remove culture medium and suspended in a precooled (4°–6°) buffer, such as Dulbecco's phosphate-buffered saline (DPBS). The buffer must be osmotically compatible with the host cells but can be otherwise modified by the investigator. The appropriate cell or spore concentration will depend on the organism under study and will have to be determined by preliminary experiments. In the case of *C. albicans,* it has been found that hydrophobic and hydrophilic yeast cells did not aggregate on the tissues when tested at $1–5 \times 10^7$ and $1–2 \times 10^8$ cells/ml, respectively.

5. Place 100 μl of cell suspension evenly across each tissue section with a micropipettor.

6. If yeast cells are under investigation, incubate up to 15 min without agitation. The appropriate time for spores or mycelial fragments will depend on investigator interests, but fungal cell surface changes during incubation should be considered.

7. Nonadherent cells are removed by decanting the liquid off the slides and blot drying the excess liquid off the slide edges with absorbent paper.

8. Adherent cells are fixed to the tissue by immersing the slides for 30 min to 24 hr in 1.5% (w/w) glutaraldehyde in DPBS followed by rinsing in three or more changes of cold (10°–15°) tap water. If the quality of the tap water is unacceptable, Scott's tap water can be purchased commercially (Sigma, St. Louis, MO). Rinsing is accomplished by dipping the slide into water and agitating.

9. Attached cells can be viewed microscopically. Some water should be covering the sections. Alternatively, the slides can be air-dried following rinsing for crystal violet staining.

10. Air-dried slides are stained in Gram's crystal violet for 1 min, gently washed with tap water from a squeeze bottle or pipette until the runoff is

clear, dipped for 1 min in Gram's iodine, and rinsed. Gram's crystal violet and iodine are available commercially (Becton Dickinson, Cockeysville, MD). Sections can be counterstained with hematoxylin–eosin to help enhance histological features. Dehydrate the sections by dipping the slides in three changes of 95% (v/v) ethanol (5 min each) and air-drying. Coverslip the sections after placing a drop of Permount (Fisher Scientific, Pittsburgh, PA)–xylene mixture (2 parts Permount, 1 part xylene). Sections can also be stained with periodic acid–Schiff reagent.[11]

11. Examine slides. Adhesion can be evaluated quantitatively by manual counting or computerized image analysis.[9]

Comments. The *ex vivo* assay as described above can be altered to suit the interests of the investigator. Fixed yeast cells can sometimes be used, allowing preparation of the cells possibly several days or weeks before the assay is to be performed. For example, formalin-fixed hydrophilic yeast cells have been shown to bind to spleens in a manner similar to unfixed yeast cells.[11] The choice of assay incubation temperature of 4°–6° was selected to minimize surface changes of the host and fungal cells.[12] If the incubation time is minimized (15 min for *C. albicans*), then the incubation temperature can be elevated to 37°. However, numerous researchers have found that more cells will bind to the tissues, although in the same pattern, when the incubation temperature is increased. Individuals interested in using the assay to assess specific adhesins are referred to Kanbe *et al.*[10]

Insect and Plant Cuticle Adhesion Assays

Entomopathogenic fungi attack insects by attachment of conidia to the cuticle, followed by production of germ tubes which penetrate the cuticle and invade the host hemocoel. Plant pathogenic fungi also attach to host cuticle and produce invasive structures such as haustoria or appressoria. Adhesion mechanisms involved in attachment to the cuticle can be studied by overlaying washed cuticle with spore suspensions or tapping dry conidia (collected in a petri dish) onto the cuticle segment.[1,2] Cuticle segments should be attached to glass microscope slides with waterproof adhesive tape or double-sided adhesive tape. Insect larval cuticle segments can also be suspended in buffer along with conidia. In this case, care should be taken to make sure that the epicuticle surface is discernible. Nonadherent conidia can be removed by gentle washing or, in the case of dry conidia, with a gentle stream of nitrogen gas. Attached conidia can be stained with lactophenol blue.

[11] J. E. Cutler, personal communication, 1993.

[12] J. E. Cutler, D. L. Brawner, K. C. Hazen, and M. A. Jutila, *Infect. Immun.* **58,** 1902 (1990).

The assays are similar to the *ex vivo* assay described above. Cuticle tissue can be treated either before or after attachment to glass slides with various reagents (enzymes, glutaraldehyde, etc.) to test their effects on adhesion.

[35] Galactose-Specific Adhesion Mechanisms of *Entamoeba histolytica:* Model for Study of Enteric Pathogens

By KEVIN C. KAIN and JONATHAN I. RAVDIN

Introduction

Adhesion to cell surfaces is a central event in the pathogenesis of many microbial infections. Microorganisms frequently subvert host cellular receptors, including cell surface carbohydrates, in order to adhere and initiate infection. Because glycoproteins and glycolipids are prevalent on the surface of eukaryotic cells, microbial lectins provide an efficient means to recognize and adhere to target cells. Examples of lectin–carbohydrate interactions involved in the pathogenesis of infectious diseases include the interaction of the influenza hemagglutinin with sialic acid-containing carbohydrates on the surface of target cells[1,2] and a 35-kDa Gal-α1,4-β-galactose-specific lectin of the P fimbriae found in *Escherichia coli* strains that cause pyelonephritis.[3]

Approaches to Lectin–Carbohydrate Interactions

Various experimental approaches can be used to determine if microbial lectins are involved in the processes of recognition and adhesion. Indirect methods are based on the premise that if cellular adhesion is demonstrated to be carbohydrate-dependent, a lectin is involved. Carbohydrate-dependent adhesion can be studied by several methods, such as measuring the ability of oligosaccharides to inhibit microbial adhesion or enzymatically modifying cell surface carbohydrates. In addition, recognition and adhesion assays can be performed using glycosylation-defective mutant cells that have altered surface carbohydrates. However, whereas these methods sug-

[1] N. Sharon and H. Lis, *Science* **246,** 227 (1989).
[2] J. C. Paulson, in "The Receptors" (P. M. Conn, ed.), p. 131. Academic Press, New York, 1985.
[3] H. Hoschützky, E. Lottspeich, and K. Jann, *Infect. Immun.* **57,** 76 (1989).

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Adhesion to cell surfaces is a central event in the pathogenesis of many microbial infections. Microorganisms frequently subvert host cellular receptors, including cell surface carbohydrates, in order to adhere and initiate infection. Because glycoproteins and glycolipids are prevalent on the surface of eukaryotic cells, microbial lectins provide an efficient means to recognize and adhere to target cells. Examples of lectin–carbohydrate interactions involved in the pathogenesis of infectious diseases include the interaction of the influenza hemagglutinin with sialic acid-containing carbohydrates on the surface of target cells[1,2] and a 35-kDa Gal-α1,4-β-galactose-specific lectin of the P fimbriae found in *Escherichia coli* strains that cause pyelonephritis.[3]

Approaches to Lectin–Carbohydrate Interactions

Various experimental approaches can be used to determine if microbial lectins are involved in the processes of recognition and adhesion. Indirect methods are based on the premise that if cellular adhesion is demonstrated to be carbohydrate-dependent, a lectin is involved. Carbohydrate-dependent adhesion can be studied by several methods, such as measuring the ability of oligosaccharides to inhibit microbial adhesion or enzymatically modifying cell surface carbohydrates. In addition, recognition and adhesion assays can be performed using glycosylation-defective mutant cells that have altered surface carbohydrates. However, whereas these methods sug-

[1] N. Sharon and H. Lis, *Science* **246,** 227 (1989).
[2] J. C. Paulson, in "The Receptors" (P. M. Conn, ed.), p. 131. Academic Press, New York, 1985.
[3] H. Hoschützky, E. Lottspeich, and K. Jann, *Infect. Immun.* **57,** 76 (1989).

gest that a lectin–carbohydrate interaction is occurring, they are not definitive. Ultimately, to prove that a microbial lectin participates in cellular adhesion requires direct evidence of the interaction between the isolated putative lectin and its associated ligand. In addition, the lectin must be surface accessible, and purified lectin and its corresponding ligand, and antibodies generated to either the lectin or the ligand, must specifically block the interaction between the microbe and physiologically relevant target cells.

Adhesion of *Entamoeba histolytica* trophozoites to colonic mucins and epithelium is an essential first step in colonization and invasion. Adhesion is also required for amebic contact-dependent cytolysis of epithelial and immune effector cells, a central event in amebic pathogenesis.[4,5] Attempts have been made to understand the molecular basis of amebic adhesion in order to provide insight into the mechanisms of invasive disease and to identify new therapeutic interventions. In this chapter, we discuss the evidence indicating that adhesion of *Entamoeba histolytica* is mediated by a galactose-inhibitable lectin and present a method used to analyze the subunit structure of the adhesion protein. These investigations serve as a model to approach lectin–carbohydrate interactions of other pathogenic microorganisms.

Cell–Cell Interactions

Adhesion to intestinal mucins, epithelium, and neutrophils is essential to the pathogenicity of *E. histolytica.* Initial efforts to characterize the nature of amebic adhesion focused on cell–cell interactions between amebic trophozoites and various target cells. Ravdin and Guerrant,[5] working with Chinese hamster ovary (CHO) cells, were the first to demonstrate that amebic adhesion was carbohydrate-dependent and might be mediated by a lectin–carbohydrate interaction. They developed a rosetting assay in which CHO cells are bound by *E. histolytica* trophozoites and rosettes quantified in a hemacytometer chamber (Fig. 1). Amebic lysis of bound CHO cells is prevented by incubation at 4°. Using the assay adhesion and contact-dependent cytolysis of CHO cells by trophozoites is shown to be completely blocked by millimolar concentrations of galactose and *N*-acetylgalactosamine (GalNAc), but not by other oligosaccharides, including *N*-acetylglucosamine, glucose, or mannose.[5] Pretreatment of ameba, but not target cells, with galactose or GalNAc results in complete inhibition of adhesion, indicating that the galactose-binding adhesin is on the ameba rather than the CHO cell. In addition, glycoproteins containing terminal

[4] J. I. Ravdin, B. Y. Croft, and R. L. Guerrant, *J. Exp. Med.* **152,** 377 (1980).
[5] J. I. Ravdin and R. L. Guerrant, *J. Clin. Invest.* **68,** 1305 (1981).

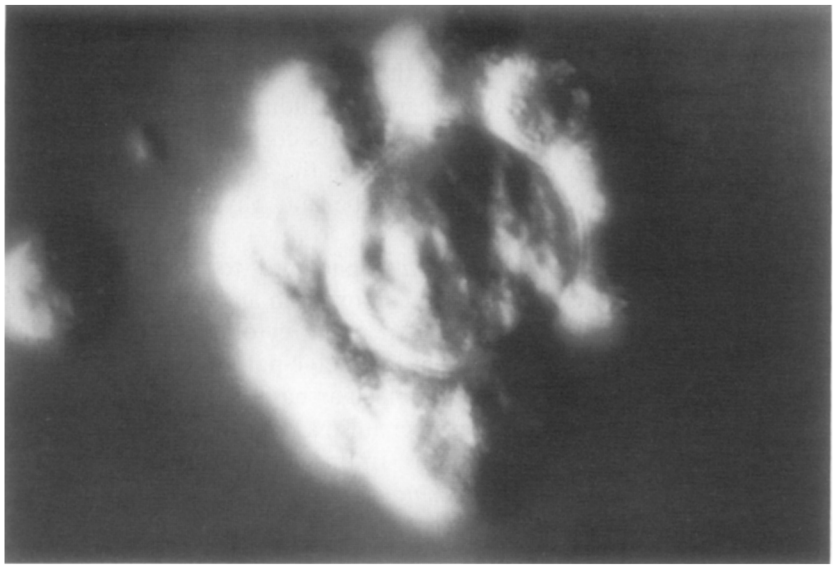

FIG. 1. Axenic *E. hystolytica* trophozoite (center) with multiple adherent Chinese hamster ovary (CHO) cells at 4°, forming a rosette. Photomicrograph was taken with a Zeiss Axiomax using interference contrast optics. Magnification: ×1000. (Reprinted with permission from the *Journal of Clinical Investigation*.[5])

galactose residues, such as asialofetuin (ASF) and asialoorosomucoid (ASOR), are shown to be 1000-fold more effective than galactose in inhibiting amebic adhesion to CHO cells (Fig. 2). The studies have been extended to show galactose-inhibitable adhesion of trophozoites to biologically relevant targets, such as erythrocytes, neutrophils, mononuclear cells, bacteria, and Chang hepatocytes.[4–8]

In addition to adhesion, cell-contact dependent cytolysis has also been shown to be dependent on the galactose-inhibitable lectin using two different strategies. In the first, galactose or GalNAc is shown to inhibit amebic lysis of CHO cells completely as indicated by trypan blue dye exclusion, release of indium-111 oxine or fluorescent dyes from labeled target cells, and cell sorting experiments (Fig. 3). In the second approach, amebic binding and cytolysis of CHO cell glycosylation mutants are compared using cell sorter methodology. Each mutant expresses a unique array of N- and/or O-linked cell surface carbohydrates. Amebic adhesion has been shown

[6] J. I. Ravdin, C. F. Murphy, R. A. Salata, R. L. Guerrant, and E. L. Hewlett, *J. Infect. Dis.* **151,** 804 (1985).
[7] R. Bracha and D. Mirelman, *J. Exp. Med.* **160,** 353 (1984).
[8] R. Bracha and D. Mirelman, *Infect. Immun.* **40,** 882 (1983).

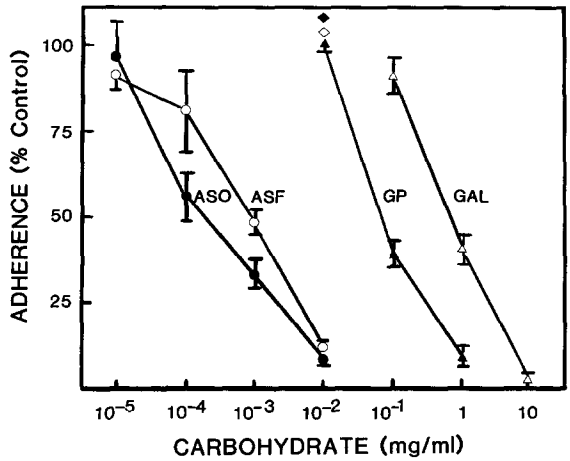

FIG. 2. Carbohydrate inhibition of amebic adhesion to target cells. Adhesion was expressed as the percentage of the adhesion observed in paired studies performed in control medium (where 60% of amebas had at least three adherent CHO cells on vortex suspension of the pellet). Data represent means ± SE of 6–11 determinations. Carbohydrate concentrations were determined by phenol–sulfuric acid method. GAL, (△), Galactose; GP (▲), asialofetuin glycopeptide; (◇), fetuin; (◆), orosomucoid; ASF (○), asialofetuin; and ASOR (●), asialoorosomucoid. (Reprinted with permission from the *Journal of Clinical Investigation.*[12])

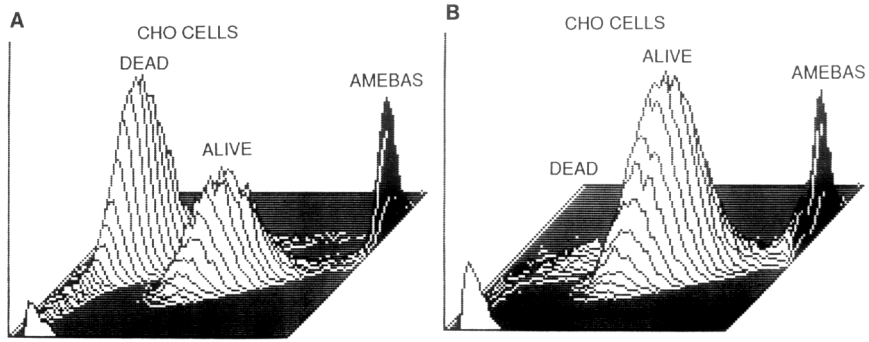

FIG. 3. Galactose-inhibitable extracellular cytolysis of target CHO cells by *E. histolytica* trophozoites as determined by cell sorting. Target CHO cells (2×10^4) were centrifuged and then incubated for 2 hr at 37° with (B) and without (A) galactose (20 mg/ml). After incubation, cells were placed on ice, galactose (10 mg/ml) was added to all tubes, and cells were vortexed to elute adherent CHO cells from amebas. Data are presented as populations of viable CHO cells and dead but still intact CHO cells as separated by size and density in a cell sorter (ordinate, cell number). With galactose present (B), amebic extracellular cytolysis of target CHO cells is completely inhibited. (Reproduced with permission from the *Journal of Infectious Diseases.*[8b])

[8b] J. I. Ravdin, *J. Infect. Dis.* **159,** 420 (1989).

to be reduced to mutants deficient in N-linked carbohydrates and almost undetectable to mutants with absent N- and O-linked carbohydrates (1d1D. Lec1 mutant). In contrast, adhesion is enhanced for mutants with exposed terminal galactose residues (Lec2 and Lec3 mutants) or for wild-type CHO cells treated with sialidase to expose galactose residues.[9] These and further studies have begun to define the ligand for the amebic adhesion protein. Using additional CHO N-linked glycosylation mutants and oligosaccharides, Ravdin and Murphy[10] and Saffer and Petri[11] have demonstrated that the preferred ligand for the amebic adhesin is galactose in a β-1,4 linkage with *N*-acetylglucosamine (*N*-acetyllactosamine), although galactose in a β-1,6 linkage and α-linkage also weakly inhibits adhesion.

Direct Protein–Carbohydrate Interactions

To obtain direct evidence that the adhesin protein of *E. histolytica* is a lectin, it is essential to isolate the putative molecule, determine its carbohydrate specificity, and establish that the molecule is central to the adhesion process. Two strategies to isolate the lectin have been undertaken. In the first approach, galactose-affinity chromatography is used to take advantage of the carbohydrate-binding activity of the adhesin. Metabolically labeled amebic proteins are applied to an ASOR affinity column, and, following extensive washing, a 260-kDa protein is eluted with galactose under nonreducing conditions. After reduction with 2-mercaptoethanol, the protein is shown to consist of a 170-kDa heavy subunit and a 35-kDa light subunit joined by disulfide bonds.[12] In the second approach, monoclonal antibodies generated to amebic trophozoites are screened for the ability to block adhesion to CHO cells, and hence the ability to recognize the adhesin protein. The ASOR-affinity-purified protein is demonstrated to be the galactose-inhibitable adhesin lectin in a number of ways: (1) the same metabolically labeled protein is purified by immunoaffinity chromatography with adhesion-inhibitory monoclonal antibodies; (2) by Western blotting, adhesion-inhibitory monoclonal antibodies are shown to recognize the 170-kDa heavy subunit purified by galactose-affinity chromatography; (3) the protein purified by both ASOR- and immunoaffinity chromatography exhibits galactose-inhibitable binding to CHO cells, competitively inhibiting adhesion by amebic trophozoites; (4) polyclonal antisera generated to the purified protein completely inhibits CHO cell adhesion by amebic trophozo-

[9] J. I. Ravdin, P. Stanley, C. F. Murphy, and W. A. Petri, *Infect. Immun.* **57,** 2179 (1989).
[10] J. I. Ravdin and C. F. Murphy, *J. Protozool.* **39,** 319 (1992).
[11] L. D. Saffer and W. I. Petri, *Exp. Parasitol.* **72,** 106 (1991).
[12] W. A. Petri, R. D. Smith, P. H. Schlesinger, C. F. Murphy, and J. I. Ravdin, *J. Clin. Invest.* **80,** 1238 (1987).

ites; and (5) the surface location of the adhesion protein is confirmed by indirect immunofluorescence.[12] These studies have established that the purified adhesion protein is a lectin.

Further efforts to characterize the biochemistry of the lectin–ligand interaction and to evaluate the role of the lectin as a vaccine candidate antigen were limited by the insufficient quantities of purified lectin available from parasite sources. For this reason, recombinant methods have undertaken to synthesize the adhesin protein. The 170-kDa subunit of the galactose lectin is cloned and sequenced from two pathogenic strains of *E. histolytica* by screening expression libraries with rabbit antilectin antisera.[13,14] The amino acid sequences of the two reported 170-kDa subunit genes are only 87.6% homologous, suggesting the presence of two or more gene variants encoding the 170-kDa subunit. The deduced amino acid sequence suggests that the galactose lectin is an integral membrane protein with a large extracellular domain which can be divided into three regions on the basis of amino acid composition. At the amino terminus is a 187-amino acid domain rich in cysteine (3.2 mol %) and tryptophan (2.1 mol %). The second domain (residues 188 to 378) contains alternating hydrophobic and hydrophilic stretches of amino acids and is cysteine-free. The third domain is a cysteine-rich domain which contains 10.8 mol % cysteine. It has been shown that the tertiary structure conferred by the high cysteine content plays an important role in protease resistance of the lectin and may be important for parasite survival in the gut.[14] The carboxyl-terminal domain is a putative cytoplasmic tail that is highly conserved between the two described variants. Tyrosine residue 1261, present in both variants, is surrounded by a region that shares homology with the autophosphorylation site of the epidermal growth factor receptor. It has been suggested that phosphorylation of the carboxyl-terminal domain may be the mechanism of activation of the lectin and that the lectin may interact with other cytoplasmic proteins during signal transduction.[14] The 170-kDa subunit has been shown to be glycosylated. However, there is a difference in the number of potential glycosylation sites between the two described members of the lectin gene family, suggesting that they may be functionally distinct.[13–15]

Monoclonal antibodies specific for functionally distinct epitopes of the 170-kDa subunit have been identified.[16] Several antibodies inhibit adhesion of trophozoites to CHO cells and human colonic mucins, contact-dependent

[13] E. Tannich, F. Ebert, and R. D. Horstmann, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 1849 (1991).

[14] B. J. Mann, B. E. Torian, T. S. Verdic, and W. A. Petri, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 3248 (1991).

[15] B. J. Mann and W. A. Petri, *Parasitol. Today* **7,** 173 (1991).

[16] W. A. Petri, T. L. Snodgrass, T. F. H. G. Jackson, V. Gathiram, A. E. Simjee, K. Chadee, and M. D. Chapman, *J. Immunol.* **144,** 4803 (1990).

cytolysis, and amebic resistance to complement lysis. However, monoclonal antibodies to two epitopes enhance adhesion 3- to 5-fold by causing a marked increase in galactose-binding activity of the lectin. The enhancement of adhesion by monoclonal antibodies suggests the possibility that amebas regulate their adhesion to galactose-containing compounds via changes in lectin activity.

The carbohydrate-binding site of the molecule has not yet been determined. The locations of adhesion-inhibiting and -enhancing epitopes have been mapped to the cysteine-rich extracellular region of the 170-kDa subunit.[17] The results suggest, but do not prove, that the galactose-binding site is contained within the cysteine-rich region of the heavy subunit. The sequence of the 170-kDa subunit does not share any significant sequence similarities to other known carbohydrate-binding domains or lectins identified thus far, suggesting that the galactose adhesin of *E. histolytica* represents a novel type of carbohydrate-binding protein.[13–15] However, there is some homology between the heavy subunit and the human complement receptor CR1, CD59, C8, and C9 complement components, and β1 integrins. The heavy subunit has been shown to share an epitope with β2 integrins,[18] a family of human glycoproteins involved in many adhesive interactions.

Purification of the lectin permits the direct determination of its carbohydrate specificity. Binding studies using purified lectin and CHO N-linked glycosylation mutants and wild-type CHO cells in the presence of different oligosaccharides confirm whole-cell experiments indicating that the adhesion lectin recognizes terminal α and β N-linked but not O-linked galactose residues.[10,11] Most of the adhesion work to this point had dealt with the *in vitro* CHO cell system, and as *E. histolytica* colonizes the large bowel, it remains to be demonstrated that the adhesin lectin mediated adhesion to physiologically relevant ligands. This evidence has been provided by the work of Chadee *et al.*[19] who studied the interactions of amoebic trophozoites and purified adhesin lectin with explants of colonic mucosa and human and rat colonic mucins. Using purified carbohydrate-rich colonic mucins, Chadee and co-workers[16] have been able to abrogate completely amebic adhesion to CHO cells and rat colonic epithelial cells at concentrations as low as 1 μg/ml, indicating that mucins are 10,000-fold more effective by weight than galactose or GalNAc in blocking amebic adhesion. The studies suggest that colonic mucins rich in galactose and GalNAc residues are bound by the adhesin lectin. When galactose and GalNAc

[17] B. J. Mann, C. Y. Chung, J. M. Dodson, L. S. Ashley, L. L. Braga, and T. L. Snodgrass, *Infect. Immun.* **61,** 1772 (1993).

[18] S. A. Adams, S. C. Robinson, V. Gathiram, T. F. H. G. Jackson, T. S. Pillay, R. E. Kirsch, and M. W. Makgoba, *Lancet* **341,** 17 (1993).

[19] K. Chadee, W. A. Petri, D. J. Innes, and J. I. Ravdin, *J. Clin. Invest.* **80,** 1245 (1987).

residues are removed by enzymatic treatment, mucins are no longer able to block adhesion. ^{125}I-Labeled mucins are shown to bind to amebic trophozoites, and the binding is inhibitable by galactose or monoclonal antibodies to the adhesin lectin. Finally, as further evidence of the interaction between the isolated lectin and mucins, mucins fixed to a solid phase are able to affinity-purify the adhesin lectin from soluble amebic protein. These studies indicate that colonic mucins are the high-affinity receptor for the adhesin lectin, and although mucins may serve as a nonimmune defense against *E. histolytica* invasion, they may also facilitate colonization of the large bowel.

The compilation of these studies provides the indirect and direct evidence required to establish that amebic adhesion is mediated by a lectin. The approach used to identify and characterize the adhesin lectin of *E. histolytica* provides a logical strategy for the study of other lectin–carbohydrate interactions. This work began with the examination of cell–cell interactions and moved to competitive inhibition studies with oligosaccharides, glycosylation mutants, and enzymatic alteration of cell surface carbohydrates. The studies were succeeded by those providing direct evidence of involvement of a lectin in amebic adhesion. This was made possible by the purification of the putative lectin and the determination of its carbohydrate specificity. At present, research efforts focus on understanding the molecular basis of the interaction of the lectin and its ligand. One novel scheme to characterize the subunit structure of the adhesin lectin is presented in the last section. In this approach, full-length and overlapping regions of the 170-kDa heavy subunit are synthesized *in vitro* and refolded by microsome-free protein disulfide-isomerase in order to identify the carbohydrate-binding domain of the adhesin lectin of *E. histolytica.*

Synthesis and Characterization of Adhesin Lectin Using Expression Polymerase Chain Reaction

Introduction

In vitro transcription and translation are powerful analytical tools to examine genes and the structure–function relationships of the proteins they encode. Standard methods for *in vitro* transcription and translation are limited by their requirements for cloning and subcloning into specialized vectors, bacterial amplification, plasmid purification, and restriction enzyme digestion. A PCR-based expression system, called the expression-PCR (E-PCR), to synthesize functional protein from DNA of interest without these constraints has been developed.

The polymerase chain reaction (PCR) has greatly facilitated the ability to generate, manipulate, and mutate DNA.[20,21] Numerous protocols describe the alteration of PCR primers in order to incorporate sequence changes, additions, or mutations in the final amplified PCR product. PCR primers can be designed with restriction enzyme sites,[22] translation initiation and termination signals,[23] or altered coding sequences for site-directed mutagenesis.[24] The E-PCR extends this approach by incorporating transcription and translation initiation signals into an "*in vitro* expression cassette" (EC) that can be linked to any amplified gene or gene segment.[25,26] This method eliminates the laborious handling required prior to *in vitro* transcription and translation and permits the generation of functional protein from DNA or RNA in as little as 8 hr. This approach has now been used to map the galactose-specific binding domain of the *E. histolytica* adhesin lectin.

Principles of Method

Description of Expression-Polymerase Chain Reaction. Expression-PCR (Fig. 4) is a procedure for installing transcription and translation signals to genes of interest, allowing their efficient expression *in vitro.* The signals are encoded on the *in vitro* expression cassette (EC), which is a synthetic oligonucleotide containing a T7 RNA polymerase promoter site, the 5′-untranslated leader sequence of the coat protein mRNA from alfalfa mosaic virus (AMV–UTL), and an initiation codon (ATG). The AMV–UTL has been shown to stimulate translation efficiency by up to 35-fold.[27] The EC is spliced to any gene of interest during thermal cycling, resulting in PCR products that can be used directly for *in vitro* transcription followed by *in vitro* translation.

To express the desired gene fragment, it must first be joined to the EC by a process called "splicing by overlap extension."[28] The gene of interest

[20] R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. N. Arnhein, *Science* **239,** 1350 (1985).
[21] H. A. Erlich, D. Gelfand, and J. J. Sninsky, *Science* **252,** 1643 (1991).
[22] S. J. Scharf, G. T. Horn, and H. A. Erlich, *Science* **233,** 1076 (1986).
[23] K. D. MacFerrin, M. P. Terranova, S. L. Schreiber, and G. L. Verdine, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1937 (1990).
[24] R. Higuchi, B. Drummel, and R. K. Saiki, *Nucleic Acids Res.* **16,** 7351 (1988).
[25] K. C. Kain, P. A. Orlandi, and D. E. Lanar, *BioTechniques* **10,** 366 (1991).
[26] K. C. Kain, P. A. Orlandi, J. D. Haynes, B. K. L. Sim, and D. E. Lanar, *J. Exp. Med.* **178,** 1497 (1993).
[27] S. A. Jobling and L. Gehrke, *Nature* (*London*) **325,** 622 (1987).
[28] R. M. Horton, H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease, *Gene* **77,** 61 (1989).

is first amplified using gene-specific sense and antisense primers. The sense primer contains a sequence of 9–15 bases at the 5′ end (overlap region; Fig. 4) that is homologous to the 3′ end of the *in vitro* EC. The *in vitro* EC and the amplified gene segment are then mixed and reamplified, generating a recombinant DNA template that can be used directly for *in vitro* transcription. Transcripts, produced by the addition of a T7 RNA polymerase, may be used for *in situ*, Northern, and Southern hybridizations, tran-

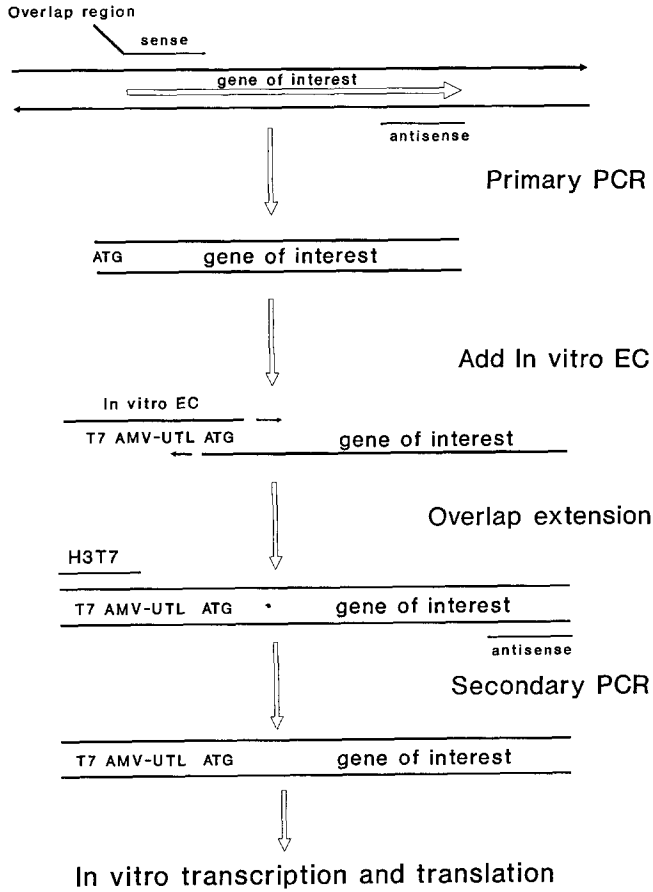

FIG. 4. Schematic of expression-PCR. T7, T7 RNA polymerase promoter site; AMV-UTL, 5′-untranslated leader sequence from alfalfa mosaic virus; EC, *in vitro* expression cassette. The H3T7 primer is the sense primer complementary to the 5′ end of the expression cassette.

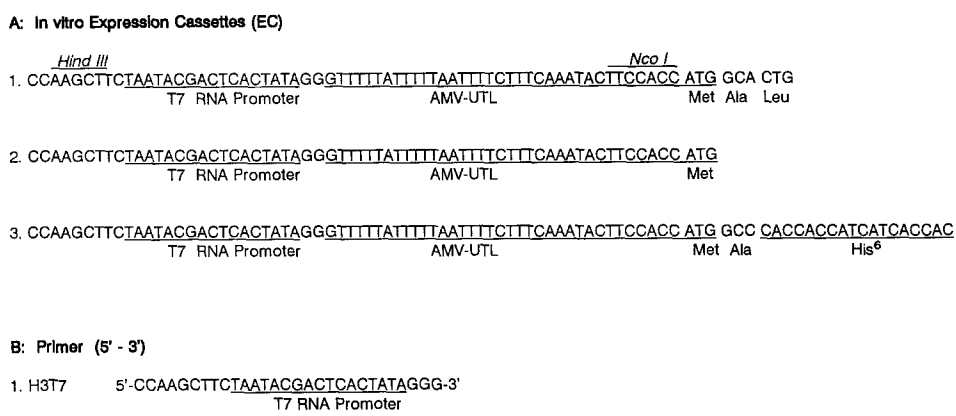

FIG. 5. (A) *In vitro* expression cassettes; (B) primer (5′–3′). See text for discussion.

scription mapping, and RNA processing studies.[29] In addition, they may be added directly to cell-free translation systems to produce pure radiolabeled protein in sufficient quantity for analysis.

In Vitro Expression Cassette. The EC can be synthesized either as a single-stranded DNA fragment or by designing overlapping oligonucleotides with complementary 3′ ends encompassing the length of the EC as described originally.[24] The original *in vitro* expression cassette (Fig. 5A, cassette 1) consisted of the T7 promoter site, the AMV–UTL, and the 9-base overlap region at the 3′ end coding for the amino acids methionine, alanine, and leucine. This 9-base overlap region was chosen so that polypeptides generated by E-PCR could be labeled with ^{35}[S]methionine and/or ^{3}[H]leucine. The EC has been modified to end at the initiation codon so that protein may be generated without extraneous amino acid incorporation (Fig. 5A, cassette 2). In addition, the *in vitro* expression cassette has been designed (Fig. 5A, cassette 3) so that expression-PCR products can be rapidly purified using polyhistidine–metal chelation chromatography.[30]

Posttranslational Refolding. Although translation may be accomplished by several cell-free systems, obtaining the correct tertiary structure, particularly for proteins with extensive disulfide bonds such as the adhesin lectin, may be more problematic. Disulfide bonds may be processed cotranslationally with protein disulfide-isomerase (PDI) associated with microsomes,[31,32]

[29] D. A. Melton, P. A. Krieg, M. R. Regagliati, T. Maniatis, K. Zinn, and M. R. Green, *Nucleic Acids Res.* **12,** 7035 (1984).
[30] E. Hochuli, H. Dobeli, and A. Schacher, *J. Chromatogr.* **411,** 177 (1987).
[31] G. Scheele and R. Jacoby, *J. Biol. Chem.* **257,** 12277 (1982).
[32] N. J. Bulleid and R. B. Freedman, *Nature* (*London*) **335,** 649 (1988).

but the epitopes or biological activity of the final product may require lysis of the microsomes, a process which may itself affect the conformation of the synthesized protein. Microsome-free PDI has been shown to refold *E. coli* recombinant proteins *in vitro* to establish enzymatic activity.[33]

Protocol

Primer Design. To be expressed, the gene of interest is first amplified with sense and antisense gene-specific primers. The sense primer is designed to include the 12-base sequence ACT TCC ACC ATG at the 5′ end. The remainder of the oligonucleotide should be gene-specific and in reading frame with the ATG codon. The 12-bases at the 5′ end permit overlap extension with the *in vitro* expression cassette. The antisense primer may be entirely gene-specific or can be modified to include a restriction site to facilitate cloning if desired. The primary amplification reaction contains 1× PCR buffer, 50 μM deoxynucleoside triphosphates (dNTPs) (nucleotide concentration lowered to enhance fidelity[34]), 50 pmol of each primer (sense and antisense), 1 mM $MgCl_2$ (to improve priming specificity), and 10 ng of target DNA in a 100-μl volume. The reaction is layered with mineral oil, denatured at 95° for 5 min, then lowered to 80° and "hot-started" by adding 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI).[35] The cycling profile is generally 25 cycles of 94° for 30 sec, 50° for 30 sec, and 72° for 1–6 min, depending on the T_m of the primers used and the gene segment to be amplified. The primary PCR product is then separated on a 2% NuSieve (FMC Bioproducts, Rockland, ME) agarose gel, and the DNA band is excised and either purified (Gene Clean; Bio101, La Jolla, CA) or melted at 65° and used directly in subsequent reactions.

Installation of in Vitro Expression Cassette. The expression cassette is now spliced to the gene of interest by a two-step PCR which includes an initial overlap extension program followed by a secondary PCR reaction (Fig. 4). In the overlap extension program, 30 fmol of the *in vitro* EC and approximately 1–10 ng of the primary PCR product are added (higher concentrations of both the template and the EC can be used without adverse effect) to a 100-μl reaction containing 1× PCR buffer, 50 μM dNTPs, 1 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase, but no primers, and denatured at 94° for 5 min followed by 5–10 cycles at 94° for 30 sec, 25° for 30 sec, and 72° for 1–6 min. This is then linked to a soak file at 80°. When the

[33] K. Okumura, Y. Miyake, H. Wakayama, T. Miyake, K. Murayama, K. Seto, H. Taguchi, and Y. Shgimabayashi, *Agric. Biol. Chem.* **52,** 1735 (1988).

[34] M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.), "PCR Protocols: A Guide to Methods and Applications." Academic Press, San Diego, 1990.

[35] D. Thompson, *Promega Notes* **35,** 3 (1992).

temperature has equilibrated at 80°, 50 pmol of the H3T7 primer (Fig. 5B), complementary to the 5′ end of the *in vitro* EC, and 50 pmol of the antisense gene-specific primer are added. The reaction is then denatured at 94° for 5 min, followed by 20–30 cycles at 94° for 30 sec, around 50° for 30 sec, and 72° for 1–6 min depending on the T_m of the gene-specific primer and the DNA template length. The final PCR product is the recombinant DNA template containing the *in vitro* EC spliced in frame to the gene or gene segment of interest. The final E-PCR template is extracted with chloroform, precipitated with ethanol, and suspended in approximately 25 μl of RNase-free water; it can be used for *in vitro* transcription.

In Vitro Transcription and Translation. Approximately 800 ng DNA template produced by E-PCR is added to a 20-μl transcription reaction (Promega) as per the manufacturer's instructions [except without dithiothreitol (DTT)] and incubated at 37° for 60 min. The mRNA product is confirmed by agarose gel electrophoresis. The DNA template is then digested with 1 unit of RQ1 DNase (Promega) at 37° for 10 min followed by chloroform extraction, ethanol precipitation and suspension in 10 μl of RNase-free water. Alternatively, approximately 5 μl of the transcription reaction may be added directly to the translation reaction. Approximately 750 ng of mRNA is added directly to an *in vitro* wheat germ extract or rabbit reticulocyte lysate (RRL) (Promega) according to the manufacturer's instructions (except without DTT in the RRL) and incubated at 25° or 30°, respectively, for 1 hr. Protein product can be labeled with ^{35}[S]methionine or ^{3}[H]leucine during the translation reaction. The efficiency of protein synthesis can be measured by analyzing 2-μl samples of the reaction for trichloroacetic acid-precipitable radioactivity, as measured by liquid scintillation counting or by analysis with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Synthesized proteins can frequently be used without additional purification.

Posttranslational Refolding. In vitro translated proteins can be refolded by treatment with microsome-free PDI. Aliquots of synthesized protein are treated with 0.2 m*M* glutathione, 2 m*M* reduced glutathione, and 20 μg/ml of PDI (Takara Biochemical, Berkeley, CA) in 100 m*M* sodium phosphate buffer (all are final concentrations) adjusted to pH 7.5 in a total volume of 30 μl and incubated at 25° for 30–60 min.

Analysis of Translated Products: Cell Binding Studies

To identify the carbohydrate-binding domain of the adhesin lectin, overlapping fragments of the heavy subunit are synthesized using E-PCR and *in vitro* translation combined with posttranslational refolding with microsome-free PDI. Synthesized fragments of the 170-kDa subunit are then analyzed

for biological activity including CHO cell binding assays. Specificity of binding to CHO cells is determined by competitive inhibition with galactose and native lectin. For the studies, CHO cells, harvested by trypsin and suspended in minimum essential medium (MEM), are washed twice with phosphate-buffered saline (PBS) without saccharides, PBS with 2% (55 m*M*) glucose, 2% (55 m*M*) mannose, or 2% (55 m*M*) D-galactose, or PBS with increasing concentrations of purified unlabeled native lectin. The cells are pelleted between washes at 1000 *g* for 3 min at 4° and suspended in the appropriate PBS, saccharide, or lectin solution. The final suspension of cells is counted on a hemacytometer, and densities are adjusted to 10^6 cells/ml. Around 500,000 cells are placed into 12 × 75 mm polystyrene culture tubes. The PDI-treated *in vitro* translated protein [100,000 counts/min (cpm)/reaction] is added and incubated with the cells at 4° for 60 min. After incubation, the cells are washed twice in the appropriate PBS, saccharide, or lectin solution at 4°, each time being pelleted at 1000 *g* for 3 min. The washed cells are then layered onto a 4:1 mixture of silicone oil (Accumetric, Elizabeth, KY) and mineral oil (Sigma, St. Louis, MO) in a 1.5-ml microcentrifuge tube. The cells are pelleted at 9000 *g* for 1 min. The tips of the microcentrifuge tubes are cut off and dropped into a scintillation vial with 10 ml of scintillation fluid and counted. In addition, refolded lectin is assessed for reactivity with neutralizing monoclonal antibodies as determined by immunoprecipitation in order to identify protective epitopes.

Discussion

The E-PCR approach is a rapid, simple method for the *in vitro* synthesis of proteins from genes without cloning. It eliminates the need for laborious procedures and permits the researcher to obtain experimental results in a matter of days. In its simplest format, E-PCR allows the rapid production of radiolabeled, biotinylated, or enzyme-linked transcripts from genomic, plasmid, or reverse-transcribed DNA for use as probes in Northern, Southern, and *in situ* hybridizations and in RNA processing and RNase mapping studies. Because transcripts made by E-PCR contain an UTL sequence that enhances translation efficiency, they also function as efficient templates for *in vitro* translation. Entire genes may be expressed; alternatively, because the initiation codon is contained with the expression cassette, specific domains within genes can be expressed. The initiation and termination of translation can be determined by operator design, rather than by the availability of restriction enzyme sites. The resulting radiochemically pure proteins are extremely useful for a variety of purposes that include studies on the subunit structure of proteins, epitope mapping, and mutagenesis studies. Because an entire population of amplified molecules are expressed

using this approach rather than a single clone, PCR incorporation errors that might occur in a minor subpopulation are generally not a problem. The E-PCR allows the efficient expression of many genes or gene fragments, especially those encoding proteins less than 100 kDa. However, some gene-specific sequences may be problematic to splice and express efficiently. The use of linked transcription/translation systems may increase the yield of synthesized protein.[34]

Using this approach to study the structure and function of the *E. histolytica* adhesin protein, it was determined that the epitope of the neutralizing monoclonal antibody 1G7 maps to the cysteine-rich domain of the 170-kDa subunit and that *in vitro* synthesized and refolded full-length 170-kDa subunit and a fragment containing only amino acid residues from the cysteine-rich region bind to CHO cells. Binding to CHO cells is competitively inhibited by galactose and purified native lectin but not by mannose or glucose. Galactose-dependent binding of the entire 170-kDa subunit is lost when portions of the cysteine-rich extracellular region are deleted, providing direct evidence that the carbohydrate-binding domain is located in the cysteine-rich region of the heavy subunit.[36]

An advantage of *in vitro* transcription and translation is the ability to produce mutant protein by altering the DNA template. Standard approaches to site-directed mutagenesis require a significant input of time to create a series of mutants, clone them, and then select for the desired mutants prior to characterization. That strategy often takes days to weeks before individual mutants are defined. By incorporating expression-PCR with the site-directed mutagenesis procedures,[24,37] it is possible to generate mutants in one day that can be screened the next.

Proteins synthesized in the E-PCR can often be used directly in biological assays. However, the addition of a tag, such as polyhistidines, to the expression cassette allows synthesized proteins to be purified by commercially available nickel columns[30] if desired.

Conformation and posttranslational modification of proteins are frequently important for biological activity and recognition by monoclonal antibodies. *In vitro* translation is a eukaryotic system which, when combined with pancreatic microsomes, performs many posttranslational modifications of proteins, including expression of signal peptidase activity, core glycosylation, phosphorylation, and disulfide bond formation. Disulfide bond formation may be further enhanced *in vitro* by the addition of oxidized glutathione cotranslationally or the use of microsome-free PDI (Takata).

[36] P. Wan, J. I. Ravdin, and K. C. Kain, *Annual Meeting of the American Society of Tropical Medicine and Hygiene* **49,** 127 (1993) (abstract).

[37] S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease, *Gene* **77,** 51 (1989).

In summary, the approach described offers advantages for researchers performing domain mapping, epitope mapping, and site-directed mutagenesis because the system offers the potential for rapid identification of biologically important domains and constructs for further analysis. The E-PCR combined with *in vitro* translation and refolding with PDI demonstrated that the 170-kDa subunit of the adhesin lectin is sufficient for galactose-inhibitable binding. Furthermore, the galactose-inhibitable binding can be localized to the cysteine-rich region of the 170-kDa subunit both by the lack of galactose-inhibitable binding in constructs deficient of portions of the cysteine-rich region and by galactose-inhibitable binding of a peptide containing only the cysteine-rich region. These observations confirm earlier indirect evidence obtained by monoclonal antibody mapping suggesting that the carbohydrate-binding domain is located in the cysteine-rich region of the heavy subunit.[36]

Acknowledgments

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[36] Adhesion and Interaction of *Candida albicans* with Mammalian Tissues *in Vitro* and *in Vivo*

By ESTHER SEGAL and HANA SANDOVSKY-LOSICA

Introduction

Attachment of microorganisms to animal or human tissues is believed to be a significant step in the interaction between pathogen and host, initiating the evolution of infection.[1] Host–pathogen interaction can be demonstrated in a number of *in vitro* systems and corroborated *in vivo* in some experimental animal models.

Among the fungal pathogens, yeast species have been studied the

[1] I. Ofek and R. J. Doyle, "Bacterial Adhesion to Cells and Tissues," p. 1. Chapman & Hall, New York, 1994.

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Among the fungal pathogens, yeast species have been studied the

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most.[2,3] The yeasts, being unicellular organisms, can be handled and standardized with relative ease, similarly to bacteria, and are best fitted for such experimental studies. With few exceptions, such as *Cryptococcus neoformans,* which adheres to glial and lung cells,[4] the majority of both the *in vitro* and *in vivo* adhesion experimentations in animal models have employed *Candida* species,[2,3] particularly *Candida albicans.*

Adhesion can be visualized *in vitro* microscopically by mixing exfoliated human or animal epithelial cells, various cell lines, or tissue segments with yeast suspensions. Furthermore, it can be quantitated by determining the number of adhering yeasts or the number of mammalian cells binding the microorganisms.[5] Adhesion *in vitro* can also be demonstrated and quantitated by use of radiolabeled yeasts or specific antibodies.

Comparison of the various methods is difficult. Experimental conditions in reference to fungal growth, host receptor cells, and the adhesion reaction may greatly influence the outcome of the yeast–host cell interaction. In addition, microscopical evaluation, which is a commonly used method, may be tainted by subjective judgment. Thus, in view of the lack of standardized experimental conditions and evaluation methods, critical analysis in regard to advantages and limitations would seem unjustified.

Only a few *in vivo* studies have been described. Most employ laboratory animals, such as rodent species, including mice, rats, and guinea pigs.[6] *In vivo* adhesion has been studied in vaginal, oral, cutaneous, gastrointestinal, and some other systems, attempting to imitate experimentally the relevant clinical entities of the particular anatomical sites. It should, however, be emphasized that such models are frequently difficult to interpret and not always easily comparable with the clinical entity.

This chapter concentrates on the most commonly used methods in the studies of *in vitro* adhesion of *Candida* to epithelial cells, cell lines, and tissue segments as well as the available *in vivo* models. Emphasis is given to those techniques with which the authors are familiar. More detailed description will include the *in vitro* methods of adhesion to human buccal and vaginal cells, corneocytes, to HeLa cell lines, and murine gastrointestinal (GI) tissue segments. The section on *in vivo* interactions includes murine models of vaginal infection and GI colonization which eventually leads to systemic disease, in naive and compromised animals.

[2] D. Rotrosen, R. A. Calderone, and J. E. Edwards, *Rev. Infect. Dis.* **8,** 73 (1986).
[3] R. A. Calderone and P. Braun, *Microbiol. Rev.* **55,** 1 (1991).
[4] G. J. Merkel and K. Cunningham, *J. Med. Vet. Mycol.* **30,** 115 (1992).
[5] M. J. Kennedy, *Mycopathologia* **109,** 123 (1990).
[6] N. J. Kennedy, *Curr. Top. Med. Mycol.* **3,** 315 (1989).

In Vitro Adhesion

Host Cells

Epithelial Cells. The *in vitro* adhesion test can be performed with various exfoliated human epithelial cells, such as buccal (BEC), vaginal (VEC), uroepithelial, and corneocytes (skin epidermal cells).[2] The more common types used are BEC or VEC.

Samples of VEC or BEC are generally collected from healthy donors by gently rubbing the oral or vaginal mucosa, using a sterile cotton swab. Cells are pooled from several donors and suspended in phosphate-buffered saline, pH 7.2 (PBS; containing 10–50 m*M* phosphate), rinsed two to three times by centrifugation, and suspended to the desired density (usually 10^6 cells/ml).

Human corneocytes are obtained by gently scraping the flexor area of the forearm with a Teflon policeman. The policeman is dipped in PBS supplemented with 0.1% (v/v) Triton X-100 to obtain a uniform suspension of epithelial cells. The scraped area is then gently rubbed with a cotton swab dipped in 0.1% (v/v) Triton X-100 in PBS to obtain additional corneocytes.

Epithelial cells can be collected from healthy individuals representing a normal population or from different populations whose medical state can influence the interaction with the fungus. The hormonal state of women can be included among the factors affecting that interaction.[7] The hormonal state can be recognized by vaginal cell cytology, as three types of VEC are known, (superficial, intermediate, and parabasal), each representing a different hormonal state.[8] Other factors affecting VEC adhesion include use of oral contraceptives, diabetes, pregnancy, and menopause.[7] Various conditions, such as diabetes, obesity, or endocrinopathies, may influence the adhesion of BEC or corneocytes, as well.[9]

Cell Lines. A variety of cell lines can be used, such as CCL-6,[10] HeLa, endothelial cells,[2] and fibroblasts.[3] HeLa, a cell line which originated from a human carcinoma of the cervix, has been used in a number of adhesion assays, including those from the authors laboratory.[11]

HeLa cells are maintained as monolayers in tissue culture flasks in M199 medium supplemented with 10% (v/v) fetal calf serum (FCS). The adhesion

[7] E. Segal, A. Soroka, and N. Lehrer, *Zentralbl. Bakteriol. Hyg. Ser. A* **A257,** 257 (1984).
[8] D. D. Gold, "Textbook of Gynecologic Endocrinology," p. 133. Harper & Row, New York, 1968.
[9] A. Srebnik and E. Segal, *Acta Derm.-Vernereol.* **70,** 459 (1990).
[10] S. A. Klotz, *J. Infect. Dis.* **158,** 636 (1988).
[11] A. Kalo and E. Segal, *Can. J. Microbiol.* **34,** 224 (1988).

reaction is performed in a multiwell tissue culture plate containing a sterile coverslip (24 × 24 mm) in each well. HeLa cell suspension is introduced into each well (30 mm diameter) and incubated at 37° in an atmosphere of 95% air and 5% CO_2 (v/v) until a confluent monolayer is obtained.

Tissue Segments. In vitro adhesion tests can also be performed with various animal tissue segments or explants of endothelial, oral, or GI origin.[5] Gastrointestinal tissue[12] segments or disks can be cut from the various parts of the GI system using a sterile metal tissue punch (0.9 cm diameter). Tissue disks can be obtained from naive animals or those subjected to immunosuppressive or compromising treatments (such as irradiation or anticancer chemotherapy). Tissues from such animals enable the study of the effects of these treatments on adhesion.

Candida

Most of the tests are performed with *Candida albicans,* as this is the major pathogen among *Candida* species. However, assays with other *Candida* strains have also been described.[2]

Growth conditions can vary, involving different media, temperatures, and other growth parameters. Growth conditions should be considered when comparing adhesion values, because these can affect the cell wall of the organism, the main site involved in the adhesion process.[5] Most studies used Sabouraud's dextrose agar (SDA) to maintain the *Candida.* However, the growth media for adhesion may vary among different investigators.[5] Our laboratory uses yeast extract (YE) broth to grow the culture for the adhesion reaction. *Candida* is grown in YE broth at 28° with constant shaking for 18 hr. These conditions yield cultures composed primarily of blastospores at the late exponential growth phase.

Candida blastospores are harvested by centrifugation and washed, usually three times, to remove media remnants. The washing buffer can vary and can affect adhesion although PBS is commonly used. After the last washing, blastospores are suspended to the desired density (generally 10^8 cells/ml).

In nonmicroscopic adhesion assays radiolabeled yeasts are used. Yeasts grown as described above are washed with PBS supplemented with $CaCl_2$ (1 m*M*) prior to the labeling process. Twenty microliters of [^{3}H]leucine (specific activity 30–50 Ci/mmol) should be added to the yeast suspension (10 ml). The suspension is then incubated at 37° for 3 hr in a CO_2 incubator. After incubation the yeast suspension is washed at least five times and suspended to the initial cell density.

[12] H. Sandovsky-Losica and E. Segal, *J. Med. Vet. Mycol.* **27,** 345 (1989).

Performance of Assay

Adhesion to Epithelial Cells. Equal volumes (0.2–0.5 ml) of epithelial cells (vaginal, buccal, or corneocytes) and fungi are mixed together in test tubes (plastic or siliconized glass) and incubated on a rotator at 37° for the desired time, usually 1–2 hr. The preferred ratio between yeast cells and epithelial cells is 100:1, although other ratios have been used as well. At a higher yeast cell density, there may be formation of clumps which make microscopic evaluation difficult. Lower yeast densities, on the other hand, lead to the possibility of decreased fungal availability, and therefore decreased adhesion.

Adhesion can be evaluated by microscopy or by use of radioactivity. Microscopic evaluation may include the determination of the number of host cells to which the fungi attach and/or the total number of adhering microorganisms per a given number of host cells (generally 100). Epithelial cells are counted and the number of adhering cells is expressed as percentage of adhesion. An adherent BEC or VEC is considered as such if it is covered with at least 20 yeasts, whereas for other epithelial cells different criteria may be employed. Nonmicroscopic evaluation, which is based on the use of radiolabeled *Candida*, quantitates the number of adhering yeasts in a sample, from which quantitation per single host cell can be calculated.

It is possible to separate nonadherent yeasts from adherent ones by passing the adhesion mixture through a Millipore (Bedford, MA) filter (12 μm pore size). For microscopic evaluation the filter is air-dried, stained (by Gram or other stain), and read microscopically. For nonmicroscopic evaluation the filters are placed in scintillation fluid and radioactivity is assessed.

It is also possible to evaluate adhesion microscopically directly from the adhesion mixture. Wet mounts are prepared on microscopic slides, covered with coverslips, and read microscopically. This method is applicable particularly for quantitation of the number of epithelial cells with adherent *Candida*. In addition, it requires the use of very stringent evaluation criteria. An adherent cell is considered as such only when a relatively high yeast number of siblings are adherent, thereby avoiding the possibility of chance contact.

Adhesion to Cell Lines: HeLa Cells. After a confluent monolayer of HeLa cells is obtained (as described above) and washed with PBS, 2 ml of yeast suspension is added. The plate is incubated for 30 min at 37°, washed to remove nonadherent yeasts, and subjected to gram staining.[11] Adhesion level is determined by microscopic counts of the adhering yeasts (at $\times$1000 magnification). A graticule of equal squares, mounted in the focus of the ocular, allows standardized counts of yeasts. The area of the graticule at

$\times 1000$ magnification is 0.0025 mm^2. Four hundred of these areas (representing 1 mm^2) are counted for each sample. Thus, the number of adhering yeasts is expressed per 1 mm^2 area.

Adhesion to Gastrointestinal Tissues. Adhesion to GI tissues of laboratory animals, particularly mice, has been extensively studied. Tissues are removed from sacrificed animals, rinsed, and cut (by sterile metal punch, diameter of 9 mm) into disks, which are placed in the yeast suspension (1 ml, 10^7–10^8 yeasts). If the adhesion reaction is performed with labeled yeasts, PBS supplemented with $CaCl_2$ is used as the adhesion medium, whereas in the case of nonlabeled yeasts PBS is used.

The adhesion mixture is incubated on a rotator for 2 hr, usually at 37°; but these conditions can change to 1 hr and/or 28°, respectively. Following the completion of the adhesion reaction, tissue disks are rinsed to remove nonadherent yeasts and aggregates. Then the tissue disks are homogenized (tissue grinder), and the homogenate is plated (following dilution) on Sabouraud's agar plates. The plates are incubated until *Candida* colonies appear (generally 48 hr). The number of organisms per tissue disk is estimated by enumeration of *Candida* colony-forming units (cfu). If labeled yeasts are used, after the reaction is terminated, the tissues should be rinsed and then digested. Chemical digestion is recommended, using 70% (w/v) perchloric acid and 30% (w/v) H_2O_2 and incubating in a water bath at 80°–90°, until full digestion is achieved. After cooling of the digested tissues, scintillation cocktail is added and the radioactivity is determined. Percent adhesion is estimated by dividing the counts per minute (cpm) remaining within the tissue disk by the cpm of the yeast inoculum (1 ml) introduced to the adhesion mixture.

Adhesion to Epithelial Cells Assessed by Enzyme-Linked Immunosorbent Assay. The enzyme-linked immunosorbent assay (ELISA) technique is based on detection with specific anti-*Candida* antibodies of yeasts adhering to epithelial cells fixed to a microtiter plate. The described technique is a modification of the method reported by Ofek *et al.*[13] for evaluation of bacterial adhesion. Microtiter plates should be pretreated with lysine and glutaraldehyde before plating the epithelial cells, in order to increase the adsorption of the cells to the inert surface. Then 0.1 ml of washed epithelial cell suspension (BEC, preferred density of 10^6 cells/ml) is placed in each well. The plates are dried overnight at 37°. For fixation of the cells to the plastic, glutaraldehyde is added. Following a washing step, 0.1 ml yeast suspension is added (10^8 cells/ml) to each well, and the plates are incubated at 37° for 1–2 hr. After incubation and washing of the plates in order to

[13] I. Ofek, H. S. Courtney, D. M. Schifferli, and E. H. Beachey, *J. Clin. Microbiol.* **24,** 512 (1986).

remove nonadherent yeasts and aggregates, the plates are covered with absolute methanol for fixation.

For detection of the adherent yeasts specific anti-*Candida* serum is added for 1 hr of incubation at 37°. Detection of adherent yeasts is problematic, as highly specific anti-*Candida* antibodies are needed. It is thus of great importance to select the most specific anti-*Candida* antibodies and to test whether they react with epithelial cells (not covered with yeasts). Following a washing step to remove excess antibodies, a peroxidase conjugated anti-rabbit immunoglobulin (if anti-*Candida* is produced in rabbits) is added (1 hr of incubation, 37°). After an additional washing the substrate (*o*-phenylenediamine dihydrochloride, 0.4 mg/ml) is added. The color is allowed to develop for 15–30 min and then measured at 405 nm with a microtiter plate reader. The intensity is proportional to the number of adherent yeasts. Controls consist of wells without yeasts to ensure that the primary antibody does not cross-react with the epithelial cells.

In Vivo Interactions

Vaginal Candidiasis

Although *Candida* is not normally found in the vagina of rodents, candidal vaginitis can be produced in rats and mice by inoculation of fungi into the vagina. The hormonal status of the animal is important for the development of the infection, intensity, and duration, as detailed below. It should be added that the rodent model is not entirely comparable with human vaginitis, as no typical signs of the human infection, such as vaginal discharge, are noted in the animal model. Thus, it would be more correct to define the animal infection as colonization by the fungus, which can be demonstrated either microscopically as an interaction with the vaginal mucosa of the animals and/or by isolation of the microorganism from the colonized tissues. The following section describes adhesion in murine candidiasis.

Vaginal Infection in Naive Mice. Because, as indicated above, the hormonal state is of importance for induction of infection, mice should be evaluated for hormonal status prior to inoculation of the fungus. This can be done by microscopy of vaginal smears. Vaginal smears or lavages are taken, and the cytology is examined. The murine hormonal cycle is 4–5 days long and consists of four stages characterized by different types of exfoliated vaginal cells. The estrus stage, which is the optimal stage for induction of infection, is characterized by the dominance of cornified epithe-

lial cells in the vaginal smear (Fig. 1). Mice at the estrus stage are inoculated with *Candida* using a wooden spatula. The *Candida* inoculum (grown on SDA plates at 28° for 48 hr) can vary from 10^7 to 10^{10} per mouse; in naive mice the larger inoculum is required.

Followup of the infection is based on microscopy and culture of vaginal smears/lavages. Yeasts adhering to epithelial cells are noted as early as 2 hr after *Candida* inoculation, as demonstrated both by direct microscopy of vaginal smears and by histopathological sections of vaginal tissue from sacrificed animals. Six hours after infection, development of hyphae which penetrate into the epithelial cells can be seen. Twenty-four hours after fungal inoculation a vaginal smear will demonstrate hyphal elements and yeasts interacting with the vaginal mucosa (Fig. 2), accompanied by the presence of an inflammatory host response, as detected in histopathological sections. The fungal colonization can be quantitated by culture, via enumeration of *Candida* colony-forming units (cfu) isolated from vaginal lavages on SDA plates. In the naive mouse model, 24 hr after fungal inoculation is the point at which infection is assessed, based on microscopic demonstration of fungal elements, particularly hyphae, and the isolation of *Candida* cfu in culture. About 50% of naive mice inoculated under the described

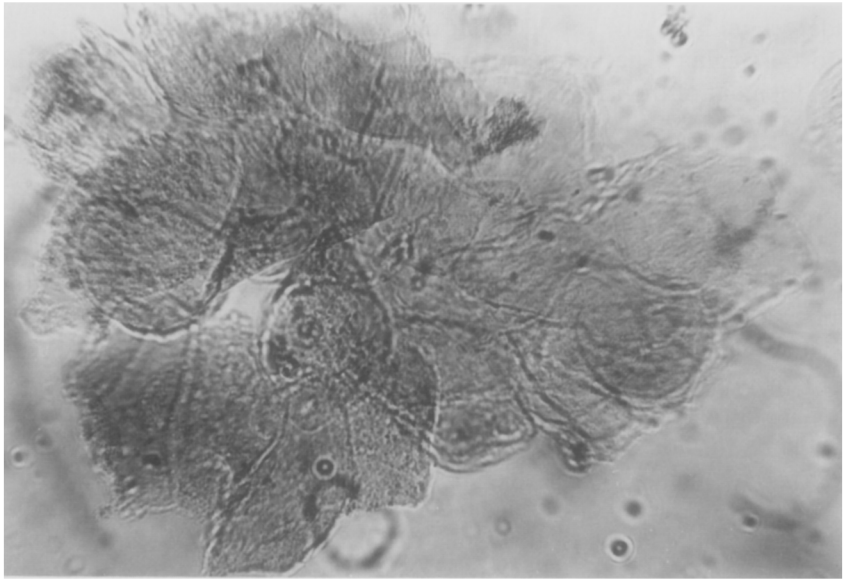


FIG. 1. Vaginal smear from a mouse at the estrus stage. Wet mount stained with methylene blue (×400). Note the predominance of cornified epithelial cells.

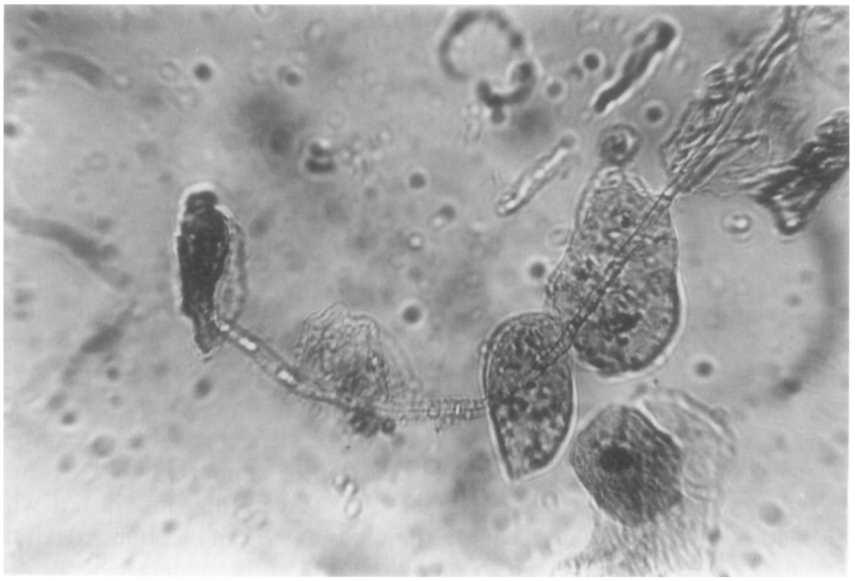


FIG. 2. Vaginal smear from a mouse inoculated with *C. albicans*. Wet mount stained with methylene blue (×400). Note the presence of fungal elements interacting with vaginal epithelial cells.

conditions will present with infection, which is self-resolving within 4–5 days.

Vaginal Infection in Hormone-Treated Mice. A constant estrus state is a condition predisposing to the development of a severe, persistent vaginal infection, which is prolonged, not self-resolving, and appears in an increased rate in mice infected with *Candida*. A constant estrus phase can be produced by estrogen injections.[14] Estradiol benzoate dissolved in sesame oil is injected subcutaneously (0.25 mg in 0.1 ml per mouse). Estrus phase, as determined microscopically by the exclusive presence of nonnucleated cornified epithelial cells in vaginal smears, appears about 1 week after estradiol injection. A constant estrus stage can be obtained by repeated weekly estradiol injections (in this specific model, six such courses are repeated, resulting in an estrus stage maintained for 6 weeks). Mice at the estrus phase are inoculated vaginally with 10^7–10^{10} *Candida* blastospores using a wooden spatula, as described above. Hormone-treated animals develop infection following inoculation with the lower *Candida* dose. The course of infection is evaluated microscopically and by plating vaginal lavage samples on SDA plates. The infection in these animals is of greater severity,

[14] E. Segal, L. Gottfried, and N. Lehrer, *Mycopathologia* **102,** 157 (1988).

as expressed by the numerous long, branched hyphal elements interacting with host mucosa, observed in vaginal smears, and by the high number of *Candida* cfu isolated in culture. The infection can affect almost all of the inoculated animals (as assessed 24 hr after fungal inoculation) and can last up to 5–6 weeks.

Vaginal Infection in Diabetic Mice. Diabetes is an additional condition which predisposes to vaginal *Candida* infection. Mice can be rendered diabetic by intraperitoneal injection of 160–200 mg/kg of streptozotocin, a diabetogenic substance acting on pancreatic β cells. Streptozotocin is freshly prepared in citrate buffer and PBS. Diabetes is monitored by blood and urine sugar levels. A sugar blood level of 200 mg/dl with or without urine sugar (over 0.1 mg/dl, with or without acetone of 0.1 mmol/liter) is an indication of a diabetic state. High blood sugar levels (over 400 mg/dl) with or without ketoacidosis (demonstrated by the presence of acetone in the urine) can lead to mortality among the animals. It is therefore recommended for experimental models to be carefully monitored with respect to sugar levels. In this model a diabetic state can be obtained within 2–7 days after streptozotocin injection, and it can be maintained for 4–5 weeks (surveillance period of this model). On determination of diabetes, the mice are inoculated intravaginally with 10^7–10^{10} organisms per mouse. The infection is evaluated microscopically and by enumeration of cfu in vaginal lavage following culture of the lavage on SDA. Diabetic mice develop infection with the low *Candida* inoculum. Almost all of the inoculated animals present with infection. The infection is very severe (using the same criteria for assessment as described above), is persistent, and can last up to 6–7 weeks.

Gastrointestinal Candidiasis

Gastrointestinal candidiasis has been described in a number of animal models including mice, rats, rabbits, and hamsters,[15] although *C. albicans* is not a natural inhabitant of the GI channel in rodents. In these models the pathogenesis of GI candidiasis was studied with respect to the interaction of the fungus with GI host tissues. The majority of investigations were carried out in mice, including naive adult animals maintained under either conventional, specific pathogen-free (SPF) or germ-free (GF) conditions. Other studies involved infant animals or animals with a defect in the immune system such as nude mice.

The animals are inoculated with the fungi by the oral route using various methods. These include direct inoculation into the mouth or stomach and

[15] M. N. Guentzel, G. T. Cole, and L. M. Pope, *Curr. Top. Med. Mycol.* **1,** 57 (1985).

supplementation of microorganisms in the food or water. Another approach involves inoculating infant mice by a natural way through their mothers, which harbor *Candida* in the gastrointestinal channel or milk. A variety of *Candida* species administered in different doses have been investigated, but most studies employ *C. albicans.* Below a detailed discussion of the procedure for naive and compromised mice is presented.

Gastrointestinal Candidiasis in Naive Mice. Oral inoculation of *Candida* to naive mice usually results in a short-lived GI colonization, characterized by a low number of fungal isolations from various parts of the GI channel with no dissemination into viscera. The following model[16] uses ICR, 6-week-old mice, kept under conventional conditions. The mice are inoculated by supplementing the drinking water (containing 5% w/v sucrose) with 5×10^7 *C. albicans* organisms/ml administered for 24 hr. The mean calculated fungal inoculum per mouse is, under these conditions, 2.5×10^8 organisms, estimated from the average water consumption of 5 ml/mouse. The *C. albicans* suspension is prepared from a culture grown in yeast extract broth for 18 hr at 28° under constant shaking, which results in a culture at the late exponential growth phase.

Followup of development of GI colonization is performed on various days after yeast inoculation. Animals are sacrificed, and the GI tract is removed, rinsed, and segmented into the esophagus, stomach, small intestine, and cecum. Tissue disks are cut with a sterile metal punch (as described above for *in vitro* assays). The stomach tissue segments should always include parts of secreting, nonsecreting, and cardial atrium line tissue. The esophagus should be taken as a whole organ because of its small size.

The GI tissue disks are homogenized separately, each in 1 ml of sterile distilled water, using a tissue homogenizer. Diluted samples of the homogenates are plated on SDA containing chloramphenicol (20 μg/ml) and incubated at 28° for 24–48 hr. Colonies growing on the SDA plates should be identified as *C. albicans* microscopically and by other conventional techniques.

Growth of more than 10 colonies per 0.1 ml of tissue homogenate from at least one part of the GI tract indicates candidal GI colonization. As growth is ascertained from tissue homogenates without lumen contaminations, it is believed to represent those fungi which associated with the tissues of the host. Candidal GI colonization in the naive mice is short-lived (14 days), with relatively low fungal cfu. Of the GI parts, most fungal isolations were obtained from stomach tissue homogenates.

Presence of fungi can be demonstrated microscopically in histopathological sections or in tissue homogenates. Histopathology of GI tissues can be

[16] H. Sandovsky-Losica and E. Segal, *J. Med. Vet. Mycol.* **30,** 219 (1992).

made by fixation of the organs, embedding in paraffin, cutting of 6-μm-thick sections, and staining with periodic acid–Schiff base (PAS). Direct examination of tissue homogenates for detection of fungal elements can be performed by the use of Calcofluor (a fluorochrome with affinity for chitin) staining and examining tissue mounts in a fluorescence microscope equipped with a UV light source (λ_{ex} 340 nm, λ_{em} 460 nm). Microscopic observation may indicate interaction of the fungus with GI mucosa, particularly in the stomach.

Gastrointestinal Candidiasis and Systemic Infection in Compromised Mice. To get a persistent or a massive GI infection to develop in a proportion of the animals into systemic candidiasis, naive animals have to be subjected to various treatments. One of these is administration of antibiotics, which changes the endogenous GI microbiota, thus enabling the fungus to proliferate in the GI channel. Others are immunosuppressive or anticancer treatments, which change the immunological status of the animal and cause damage to the GI mucosa, which again, facilitates fungal proliferation. Some models involve a combination of antibacterial and immunosuppressive treatments.

The *in vivo* model was developed[16] through the use of the same immunosuppressive treatments, as described above for *in vitro* adhesion of *C. albicans* to GI tissues. Specifically, conventional ICR mice are subjected to one of the following treatments: (a) cobalt irradiation, single dose (whole body), 4 or 6 Grey (Grey = 100 rad); (b) methotrexate, intraperitoneal injection, 3 mg/mouse; or (c) 5-fluorouracil, intravenous injection into the tail vein, 200 mg/kg body weight. Inoculation of the animals with the fungi is done on the fourth day after suppressive treatment, as done with naive mice, by supplementing the drinking water with *C. albicans.* This model of GI *Candida* infection develops into fatal systemic candidiasis, including involvement of visceral organs such as the liver, spleen, and kidneys.[16]

Surveillance for morbidity and mortality is carried out on different days after candidal inoculation. Morbidity (GI colonization and/or systemic disease) can be assessed by microscopic demonstration of fungi in GI or visceral organs and by enumeration of *C. albicans* cfu in the organs. For enumeration of *Candida* in GI tissues, the process is the same as for naive mice. For enumeration of *Candida* in visceral organs, liver (200 mg) and kidneys are removed and processed as described previously for GI tissues. Systemic *Candida* infection is defined as growth of more than 10 cfu per 0.1 ml of tissue homogenate. Detection of circulating candidal antigen in blood samples through the use of a latex agglutination test is another measure of systemic infection. Mortality is monitored, generally, for 30 days after candidial inoculation. Postmortem studies, including examination

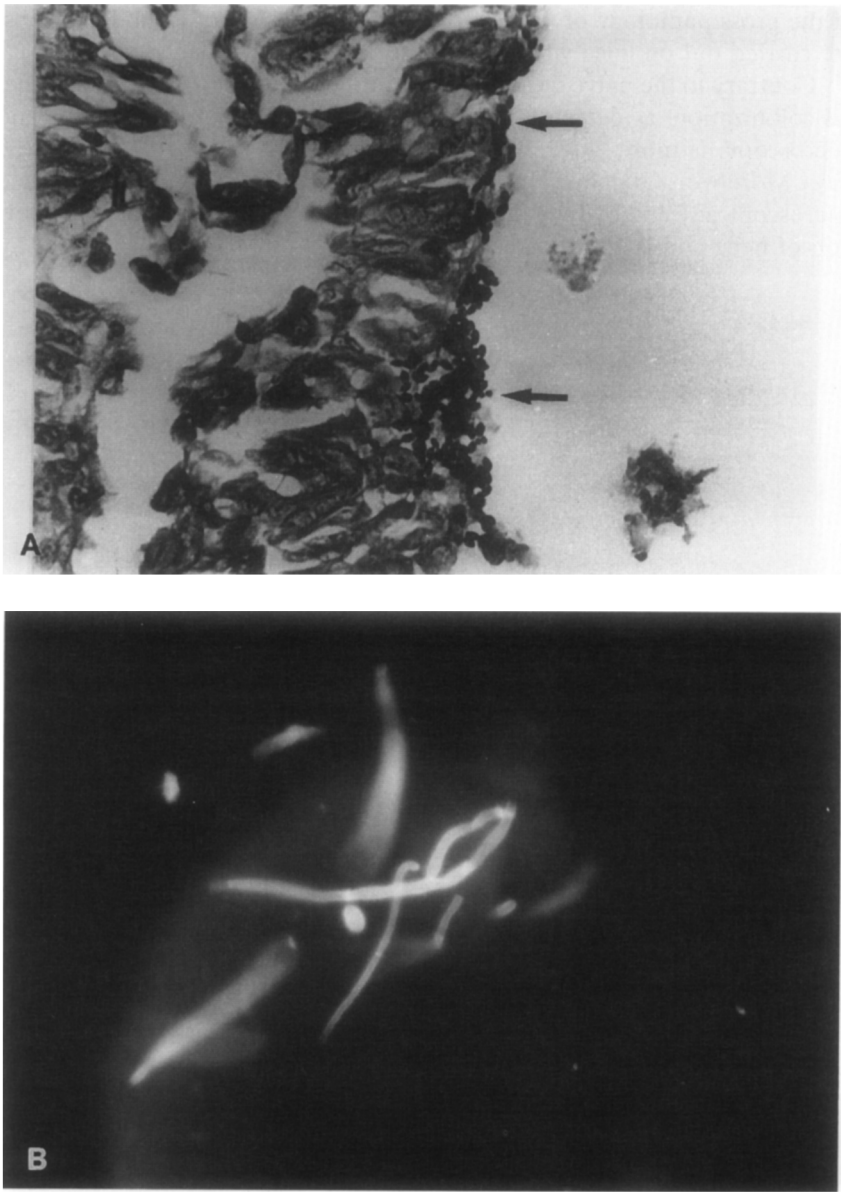


FIG. 3. Interaction of *C. albicans* with stomach tissue from a compromised mouse. (A) Histopathological section stained by PAS (×400); note yeasts interacting with tissue (arrows). (B) Tissue homogenate stained with Calcofluor (×400); note fungal elements.

of the gross pathology of various organs for detection of candidal lesions, are carried out on mice which expire during the surveillance period.

Contrary to the naive murine model, in the compromised murine model GI colonization, as detected by enumeration of colony-forming units and microscopy, is more extensive and persistent (Fig. 3), lasting at least 30 days. Moreover, GI colonization develops into systemic infection (detected microscopically and by fungal isolation from the viscera) in a large proportion of mice and is accompanied by mortality.

[37] Use of Flow Chamber Devices and Image Analysis Methods to Study Microbial Adhesion

By H. J. BUSSCHER and H. C. VAN DER MEI

Introduction

One of the important steps in the formation of a microbial biofilm on an implanted biomaterial, a tooth, a rock in a river or stream, or on a ship hull is the transport of microorganisms toward the substratum surface.[1,2] In a river or stream, transport of microorganisms to rocks will be mainly through the flow of water ("convection"). Alternatively, transport of marine organisms to a ship hull will be controlled by the speed of the ship through the water. However, in an environment such as the oral cavity, organisms suspended in saliva have almost no convective transport mechanism available to reach a tooth surface, and transport is diffusion limited.

In general, increased fluid flow toward or parallel to a substratum surface will yield an increase in mass transport and therewith a more rapid establishment of stationary numbers of adhering organisms. However, when fluid flow exceeds a critical limit, resulting wall shear rates will be so high as to prevent adhesion[3,4] (see also Table I).

Often experimental methods to study microbial adhesion neglect the influences of fluid flow,[5] including transport and wall shear rate aspects. For instance, in the MATH (microbial adhesion to hydrocarbons) assay[6] a suspension of microorganisms is vortexed together with a small amount of hydrocarbon to bring the organisms in contact with the resulting hydrocarbon microdroplets in suspension. Obviously the kinetics, that is, the mass transport, is not well controlled in this method. Another frequently employed method to study adhesion of oral microorganisms to saliva-coated hydroxylapatite is based on mixing hydroxylapatite beads with a microbial suspension in a tumbling device.[7] Neither the shear rates nor the mass

[1] K. C. Marshall, *in* "Bacterial Adhesion"(D. C. Savage and M. Fletcher, eds.), p. 133. Plenum Press, New York and London, 1985.
[2] M. C. M. Van Loosdrecht, J. Lyklema, W. Norde, and A. J. B. Zehnder, *Microbiol. Rev.* **54,** 75 (1990).
[3] P. R. Rutter and B. Vincent, *in* "Physiological Models in Microbiology" (M. J. Bazin and J. I. Prosser, eds.), p. 27. CRC Press, Boca Raton, Florida, 1988.
[4] H. Morisaki, *J. Gen. Microbiol.* **137,** 2649 (1991).
[5] J. Sjollema, H. J. Busscher, and A. H. Weerkamp, *J. Microbiol. Methods* **9,** 79 (1989).
[6] M. Rosenberg, *FEMS Microbiol. Lett.* **22,** 289 (1984).
[7] R. J. Gibbons, I. Etherden, and E. C. Moreno, *J. Dent. Res.* **64,** 96 (1985).

TABLE I
EFFECT OF WALL SHEAR ON ACCUMULATION OF MICROORGANISMS ON SURFACES[a]

Microorganism	Substratum	To remove attached organisms		To prevent attachment	
		τ_x (N/m^2)	F_{drag} (N)	τ_x (N/m^2)	F_{drag} (N)
Bacillus cereus	Glass	—	—	28	—
Bacillus cereus	Siliconized glass	—	—	52	—
Streptococcus sanguis	Glass	—	—	28	—
Pseudomonas fluorescens	Steel	12	—	6–8	—
Bacillus sp.	Quartz	—	—	—	10^{-14}–10^{-12}

[a] Adapted from Rutter and Vincent[3] and Morisaki.[4]

transport in this system is amenable to an easy understanding. In stagnant devices, mass transport can sometimes be so low as to lead to the erroneous conclusion that microbial adhesion is absent (see Fig. 1).

Although the use of controlled flow devices, such as the parallel plate

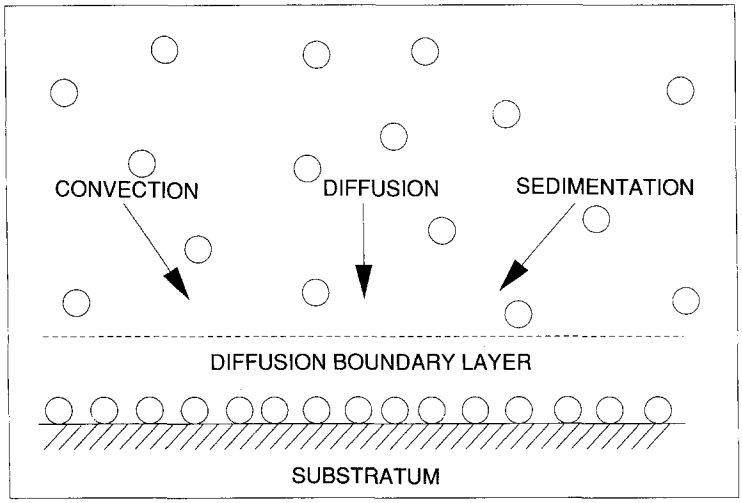


FIG. 1. Approach of bacteria to a substratum. Owing to adhesion of microorganisms to a substratum surface, a zone with a relatively low number of suspended organisms is created, often extending beyond the range of the interaction forces. Adhesion can continue only if additional organisms are transported toward the substratum by, for example, convection, diffusion, or sedimentation.

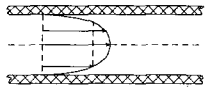
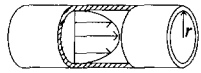
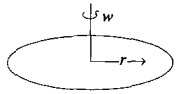
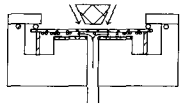
type of collector	schematic drawing	real-time observation
parallel plate chamber		yes
cylindrical channel		no
rotating disk		no
stagnation point flow collector		yes

FIG. 2. Schematic presentation of various types of controlled flow devices encountered in the current literature on particle (microbial) adhesion to solid surfaces.

flow chamber or the stagnation point flow chamber (see Fig. 2 for a limited overview of various devices), was introduced several decades ago,[8,9] the advantage of controlling the operating shear forces is not seldom lost when substrata with adhering microorganisms are removed from the system for enumeration. Major artifacts are created by "dipping in suspension fluid," or "rinsing under flow" to remove loosely bound or unattached organisms, expressions that can be found in the great majority of papers dealing with microbial adhesion to surfaces.[10–12] Simple calculations illustrated in Fig.

[8] P. Rutter and B. Vincent, *J. Gen. Microbiol.* **120,** 301 (1980).
[9] Z. Adamczyk, *Colloids Surf.* **35,** 283 (1989).
[10] H. J. Busscher, J. Sjollema, and H. C. Van der Mei, *in* "Microbial Cell Surface Hydrophobicity" (R. J. Doyle and M. Rosenberg, eds.), p. 335. American Society for Microbiology, Washington, D.C., 1990.
[11] W. G. Pitt, M. O. McBride, A. J. Barton, and R. D. Sagers, *Biomaterials* **14,** 605 (1993).
[12] H. J. Busscher, G. I. Doornbusch, and H. C. Van der Mei, *J. Dent. Res.* **71,** 491 (1992).

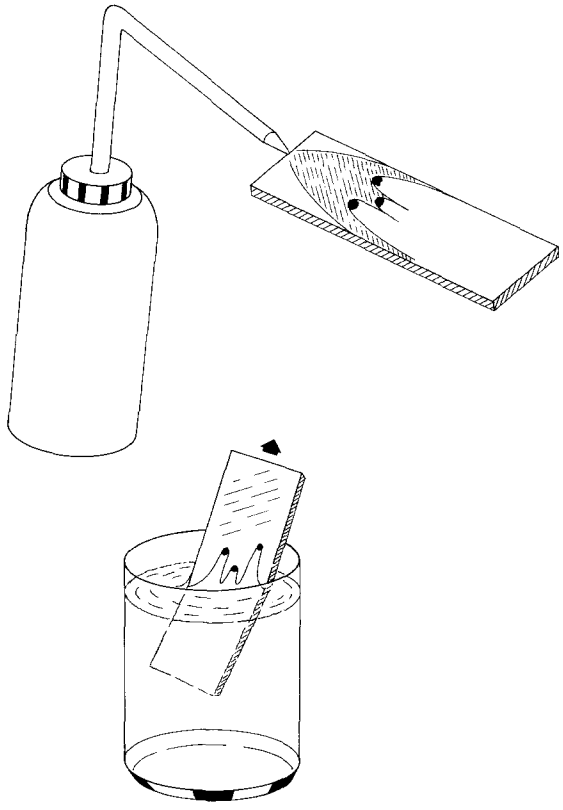


FIG. 3. Schematic presentation of the two major sources of error in the enumeration of microbial adhesion to solid surfaces. (*Top*) "Slight rinsing": An average flow of water exerts an approximate force of 10^{-9}–10^{-10} N per cell. (*Bottom*) "Dipping": The force exerted by dipping is approximately 10^{-7} N per cell.

3 demonstrate that the removal forces involved in passing an adherent organism through a liquid–air interface ("dipping") are approximately 10^{-7} *N*, whereas for an average flow of aqueous fluid in "slight rinsing" a removal force of about 10^{-9}–10^{-10} *N* is estimated. Comparison of these forces with those in Table I, clearly show that dipping or slight rinsing will remove a sizable fraction of adherent microorganisms, whether unattached, loosely bound, or tightly adhering. Unfortunately, this fraction is unknown and dependent on the magnitude of the cell–substratum interaction forces, the chemical heterogeneity, and the roughness of the substratum surface.[13]

[13] M. A. Hubbe, *Colloids Surf.* **16,** 249 (1985).

Enumeration artifacts as described above can be avoided effectively when controlled flow devices are used in combination with *in situ* observation methods. Ultralong working distance objectives and computerized image analysis methods enable "life" measurement of microbial adhesion without any interference.[14]

In this chapter, we summarize experiences obtained over a 10-year period in working with a parallel plate flow chamber and image analysis techniques to study microbial and particle adhesion to solid surfaces. The original choice for the parallel plate flow chamber over other devices described (see Fig. 2) was made on the following grounds: apart from sedimentation effects, deposition is homogeneous over the substratum surfaces; the design is fairly simple; the hydrodynamics of the parallel plate flow chamber can easily be described; the substratum does not move during the experiment, enabling real-time, *in situ* observation; proteinaceous or synthetic coatings on the substrata can be applied under the same flow conditions as during the adhesion experiment; rinsing solutions can be applied under the same flow conditions as during adhesion to study desorption; when desired, small substrata (e.g., dental enamel chips) can be embedded in the bottom (or top) plates of the chamber.

This chapter describes (1) the design of a parallel plate flow chamber, (2) the image analysis system and image handling employed, (3) the hydrodynamics and kinetics of particle deposition in a parallel plate flow chamber, (4) selected examples of the use of the parallel plate flow chamber for studying microbial adhesion, and (5) advantages and disadvantages of the system.

Design of Parallel Plate Flow Chamber

Figure 4 shows the construction of the parallel plate flow chamber (external dimensions 16 × 8 × 2 cm, length by width by height).[14,15] The chamber consists of a nickel-coated brass bottom part and a poly(methyl methacrylate) top part (a metal top plate is used when a sterilizable chamber is needed) which encloses two plates having the dimensions 7.6 × 5.0 × 0.2 cm, separated from one another by two Teflon spacers. The effective chamber dimensions are 7.6 × 3.8 × 0.06 cm, although the height of the chamber can be varied by the thickness of the spacers. The width-to-height ratio should be larger than 5 in order to exclude side-wall effects.

[14] J. Sjollema, H. J. Busscher, and A. H. Weerkamp, *J. Microbiol. Methods* **9,** 73 (1989).

[15] T. G. Van Kooten, J. M. Schakenraad, H. C. Van der Mei, and H. J. Busscher, *J. Biomed. Mater. Res.* **26,** 725 (1992).

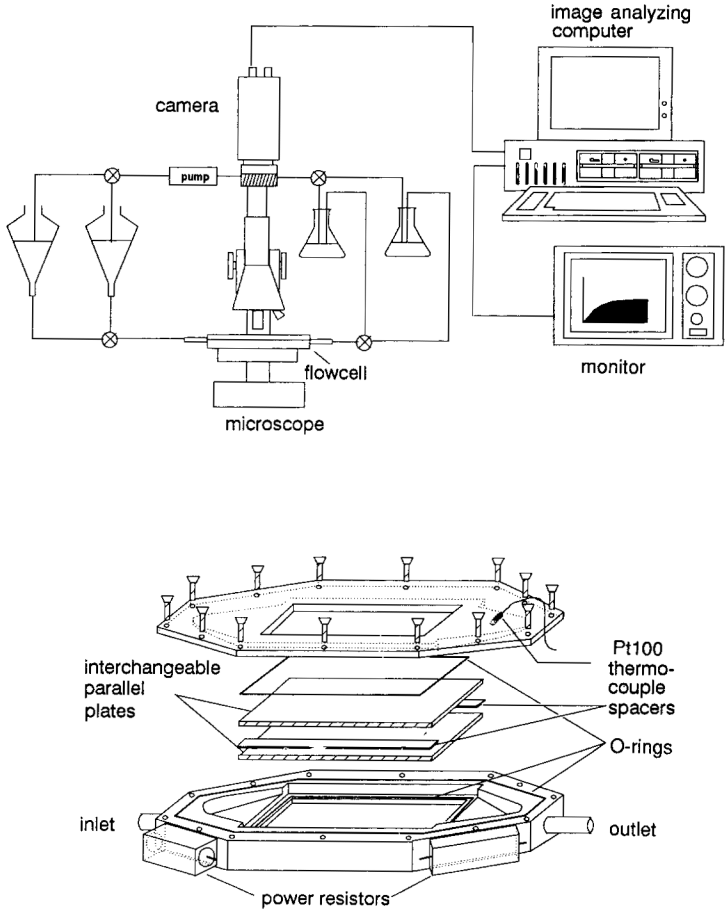


FIG. 4. Schematic overview of the parallel plate flow chamber system and detailed view of the chamber itself.

The top and bottom plates must be reasonably stiff to maintain a constant channel depth and plan parallelity during the flow experiment, whereas the application of phase-contrast microscopy requires transparent bottom and top plates. To allow the detection of adhering microorganisms on opaque materials, such as enamel, certain polymers, and steel, incandescent dark-field illumination is directed under a very low angle onto the bottom plate of the channel by means of a lens supported on a slide.[14] Furthermore, it is possible to use fluorescence microscopy, provided it is ascertained that the required fluorescent dyes do not affect the adhesive properties of the microorganisms. The establishment of a laminar flow between two plates

is enhanced by the design of the chamber, that is, the width and height of the inlet and outlet regions gradually change to the chamber dimensions.[16] The chamber itself can be heated, if necessary, by four 33-Ω power resistors, mounted on the sides of the bottom part and connected in parallel to a power supply (10 V). Feedback is provided by a Pt100 thermocouple, assembled in the top part in the downstream compartment of the flow chamber.

The entire flow chamber is placed on the stage of a phase-contrast microscope (Olympus BH-2) equipped with a 40× objective with an ultralong working distance (Olympus ULWD-CD Plan 40 PL) (see Fig. 4). A CCD camera (CCD-MX High Technology, Eindhoven, The Netherlands) is mounted on the phase-contrast microscope and is coupled to an image analyzer (TEA, Image-Manager, Difa Breda, The Netherlands), installed in a 66 MHz IBM AT personal computer. With this setup, direct observation of the deposition process *in situ* is possible without any additional shear forces acting on the deposited bacteria. Thus the spatial arrangement of deposited bacteria with respect to one another is fully preserved. A pulse-free flow can be created by hydrostatic pressure and the suspension recirculated by a roller pump. The pump can also be driven externally by an analog voltage signal, which gives the possibility of a controlled pulsatile flow in the chamber.[17] By means of a valve system it is possible to connect flasks which contain, for example, buffer, reconstituted human whole saliva, or bacterial suspension, with the flow chamber without passing an air–liquid interface over the adsorbed conditioning film and/or adhering organisms.[18]

Image Analysis System and Image Handling

Enumeration of the total number of adhering bacteria as well as the determination of the deposition and desorption rate is usually done on the bottom plate of the flow chamber.[19] Sometimes experiments are done on the top plate to exclude sedimentation contributions to the mass transport.[20] During a deposition experiment, images are grabbed as rapidly as possible. An image is built up of 512 × 512 pixels, with each pixel representing one byte, that is, 256 gray values ranging from 0 (black) to 255 (white). The magnification of the system is such that the total field of view is 0.017 mm^2 and the area of one bacterium equals approximately 6 pixels (depending,

[16] A. McQueen and J. E. Bailey, *Biotechnol. Lett.* **11,** 531 (1989).
[17] T. G. Van Kooten, J. M. Schakenraad, H. C. Van der Mei, and H. J. Busscher, *J. Biomater. Sci. Polym. Ed.* **4,** 601 (1993).
[18] I. H. Pratt-Terpstra, A. H. Weerkamp, and H. J. Busscher, *J. Gen. Microbiol.* **133,** 3199 (1987).
[19] J. M. Meinders, J. Noordmans, and H. J. Busscher, *J. Colloid Interface Sci.* **152,** 265 (1992).
[20] J. Sjollema, H. J. Busscher, and A. H. Weerkamp, *Biofouling* **1,** 101 (1988).

of course, on the microorganisms under study). To distinguish between adherent bacteria and in focus moving bacteria, two successively (~1 sec time interval) grabbed images are added or multiplied.[21] Because the gray value of the background is substantially higher than the gray value of the bacteria, this procedure yields higher gray values for moving bacteria than for adhering bacteria.

To eliminate artifacts caused by dust or dirt on the lenses and camera, an added or multiplied out-of-focus image is subtracted from the images, yielding a uniformly gray background. Furthermore, to reduce noise and to amplify the gray value difference between bacteria and background, a Laplace filter procedure is carried out. Finally the image is "thresholded," in which all pixels with gray values below a certain, adjustable threshold are made white (i.e., gray value 255) and the background is made black. At this point the black and white images and the times at which they were taken are written to a hard disk for later analysis.

In its simplest form, this analysis yields the number of adherent microorganisms per unit area at time t, $n(t)$. However, in our so-called history mode, the times of arrival and detachment of each adherent microorganism are also determined. This is done by creating "adsorption" and "desorption" images, in which the gray values are used to indicate the times of arrival and desorption. In this method it is assumed that no adhesion will occur on exactly the same position from which another organism has just detached.[19] From this information a residence time-dependent desorption rate coefficient can be calculated according to

$$\beta(t-\tau) = \sum_{j=1}^{N-1} \frac{1}{N-j-1} \sum_{i=j+1}^{N} \frac{\Delta n_{\text{des}}(t_i)}{\Delta n_{\text{ads}}(\tau_{i-j})(t_i - t_{i-1})} \tag{1}$$

Furthermore, when the spatial arrangement of the adhering microorganisms is to be studied, an additional distance transform separating adherent multiplets into individual adhering organisms can be done, yielding (x, y) coordinates of each adhering microorganism. (See Appendix at the end of this chapter for nomenclature.)

Hydrodynamics and Kinetics of Particle Deposition in Parallel Plate Flow Chamber

A fully developed, laminar flow between two parallel plates obeys the Poiseuille law and adapts a parabolic flow profile:

$$v(z) = \frac{3}{2}\left(\frac{Q}{2bw}\right)\frac{z}{b}\left(2 - \frac{z}{b}\right) \tag{2}$$

[21] J. M. Meinders, H. C. Van der Mei, and H. J. Busscher, *J. Microbiol. Methods* **16,** 119 (1992).

However, there are several criteria to be met before such a well-defined flow actually develops. A first criterion for laminar flow concerns the Reynolds number[22,23]:

$$Re = \rho \frac{Q}{(w + 2b)\mu} < 2000 \tag{3}$$

However, this is not the only criterion because the length of the chamber must be sufficiently long for laminar flow to develop and all effects from the inlet to have disappeared. The establishment length for laminar flow through a rectangular cross section can be calculated from

$$Le = (\text{constant})\ 2b(Re) \tag{4}$$

Proportionality constants in the literature range from 0.013[22] to 0.044.[23] From the Reynolds criterion, the maximal volumetric flow rate that can be allowed in our flow chamber is calculated to be approximately 100 cm^3 sec^{-1} requiring an establishment length between 2 and 6 cm.

The wall shear rate owing to the parabolic flow profile is

$$\sigma = \left.\frac{dv}{dz}\right|_{z=0} = \frac{3}{2}\frac{Q}{b^2 w} \tag{5}$$

When σ is multiplied by the absolute viscosity μ of the suspending fluid, which is usually taken as 10^{-3} kg m^{-1} sec^{-1}, one obtains the wall shear stress τ_x. Multiplying σ by the area of the adhering particles exposed to the flow yields an estimate of the hydrodynamic force acting on an adhering particle (see also Table I).

The kinetics of microbial deposition can be surprisingly different from that of inert particles, among other reasons because of the presence of structural surface features as fibrils and fimbriae, cell surface heterogeneities, or biosurfactant production.[20,24] Owing to the use of real-time automated image analysis, the data density in time will always be sufficiently high to allow calculation of (1) an initial deposition rate:

$$j_0 = \left.\frac{dn(t)}{dt}\right|_{t=0} \tag{6}$$

describing the initial rate of arrival of microorganisms at the surface; and (2) the number of adherent microorganisms in a stationary state n_∞. (Note:

[22] R. A. Van Wagenen and J. D. Andrade, *J. Colloid Interface Sci.* **76,** 305 (1980).
[23] B. D. Bowen, *J. Colloid Interface Sci.* **106,** 367 (1985).
[24] J. Sjollema, H. C. Van der Mei, H. M. Uyen, and H. J. Busscher, *J. Adhes. Sci. Technol.* **4,** 765 (1990).

We are referring explicitly to situations where microbial growth is excluded.)

Because adherent microorganisms prevent adhesion of other depositing organisms in a certain area (the so-called blocked area),[25,26] the adsorption rate decreases:

$$j_{ads}(t) = j_0[1 - A_1 n(t)] \tag{7}$$

For inert particles, the blocked areas are mainly determined by the repulsive interactions between adherent particles. For adherent microorganisms, larger blocked areas may arise, for instance, from biosurfactant production by an adherent organism, rendering unfavorable adhesion conditions. Oppositely, when cooperative effects prevail, smaller blocked areas may be found.

To describe the kinetics of microbial deposition fully, a desorption rate has to be introduced as well:

$$j_{des}(t) = \beta n(t) \tag{8}$$

In Eq. (8) desorption is assumed to be independent of the time an organism is adhering to the substratum surface, and the adhesion is assumed to acquire its final strength on initial contact between the organism and the substratum surface.[27] From Eqs. (7) and (8) the deposition rate $j(t)$ can be calculated as

$$j(t) = j_{ads}(t) - j_{des}(t) \tag{9}$$

and accordingly

$$n(t) = \int_0^t j(t)dt = n_\infty(1 - e^{-(j_0 A_1 + \beta)t}) \tag{10}$$

Usually j_0, n_∞, and $(j_0 A_1 + \beta)$ can be directly obtained from the measured time dependence of the number of adhering microorganisms $n(t)$, and accurate estimates for β can be derived from the measured $j_{des}(t)$ and for A_1 from the measured $j_{ads}(t)$. Optimization of the calculated parameters can then be done by iterative procedures.[19]

The approach just outlined and more or less summarized in Eq. (10) assumes that desorption does not depend on the residence time of an adhering organism, that is, "bond aging" does not occur. This is seldom true, as we observed bond aging even for the adhesion of inert polystyrene particles to glass,[28] possibly because of progressive removal of interfacial

[25] T. Dabros and T. G. M. Van de Ven, *J. Colloid Interface Sci.* **89,** 232 (1982).
[26] P. Schaaf and J. Talbot, *J. Chem. Phys.* **91,** 4401 (1989).
[27] T. Dabros and T. G. M. Van de Ven, *J. Colloid Interface Sci.* **93,** 576 (1983).
[28] J. M. Meinders and H. J. Busscher, *Colloids Surf. A* **80,** 279 (1993).

water, deformation, or rotation of an adhering particle to make the most favorable heterogeneity on its surface contact the glass. This type of process can easily be envisaged to control the desorption behavior of adhering microorganisms as well, possibly influenced in addition by biosurfactant production and the metabolic activity of the organisms.

Using the analysis method as described allows direct determination of the residence time-dependent desorption rate coefficient $\beta(t - \tau)$. It has been proposed[25] that $\beta(t - \tau)$ should be an exponential function of residence time:

$$\beta(t - \tau) = \beta_\infty - (\beta_\infty - \beta_0)\, e^{-\delta(t-\tau)} \tag{11}$$

Now, the expression for $j_{\text{des}}(t)$ is

$$j_{\text{des}}(t) = \int_0^t \beta(t - \tau) j(\tau) d\tau \tag{12}$$

and consequently[25]

$$n(t) = \int_0^t j(t)dt = \frac{j_0}{\Delta}\left\{\left(1 + \frac{\delta}{p_1}\right) e^{p_1 t} - \left(1 + \frac{\delta}{p_2}\right) e^{p_2 t} - \left(\frac{\delta}{p_1} - \frac{\delta}{p_2}\right)\right\} \tag{13}$$

with

$$p_{1,2} = \frac{1}{2}\left\{-(\beta_0 + \delta) \pm \Delta\right\} \tag{14}$$

$$\Delta = \{(\beta_0 + \delta)^2 - 4\delta\beta_\infty\}^{1/2} \tag{15}$$

As $\beta(t - \tau)$ can be directly determined, β_0, β_∞, and δ can be easily calculated from Eq. (11), and j_0 can be readily obtained from the measured time dependence $n(t)$ employing iterative procedures.

When $\beta_0 < \beta_\infty$ the bond (adhesion) strength weakens during aging, whereas when $\beta_0 > \beta_\infty$ the bond strengthens during aging. Direct measurement of blocked areas is possible from analysis of the spatial arrangement of adhering microorganisms by means of pair distribution functions.[29] The local pair distribution function $g(x, y)$ can be obtained from an image as illustrated in Fig. 5 and is given as

$$g(x, y) = \frac{\rho(x, \Delta x, y, \Delta y)}{\rho_0} \tag{16}$$

The area where $g(x, y) < 1$ represents the blocked area A_1.

Mass transport of particles in controlled flow devices is amenable to theoretical predictions. The mass transport in the parallel plate chamber can

[29] H. J. Busscher, J. Noordmans, J. Meinders, and H. C. Van der Mei, *Biofouling* **4**, 71 (1991).

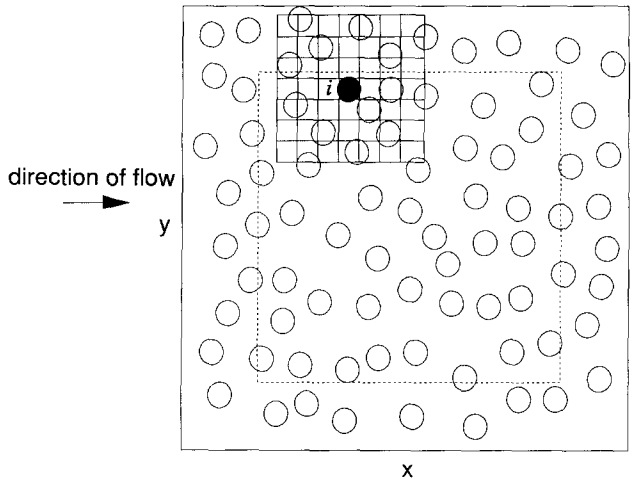


FIG. 5. Analyses of the spatial arrangements of adhering microorganisms by pair distribution functions. By moving the grid while taking each adhering organism i once as a center organism, the average local density $\rho(x, \Delta x, y, \Delta y)$ can be obtained.

be calculated by solving the convective-diffusion equation, which describes mass transport in terms of convection, diffusion, and the interaction forces operating. Often, however, solving the convective-diffusion equation is too difficult, and approximate solutions are chosen. In the Smoluchowski–Levich approximation, the attractive Lifshitz–van der Waals forces between a particle and a substratum surface are thought to be counterbalanced by the hydrodynamic drag which a particle experiences when approaching a substratum surface,[30] while electrostatic interactions are neglected. Accordingly, a theoretical deposition rate can be calculated, which for the parallel plate flow configuration, is[31,32]

$$j_0^* = \frac{D_\infty c}{a_h \Gamma(4/3)} \left(\frac{2}{9}\frac{bPe}{x}\right)^{1/3} \tag{17}$$

Although the assumptions outlined in the Smoluchowski–Levich approach are seldom completely met, j_0^* is a convenient starting value for comparison of experimental j_0 values. The ratio j_0/j_0^* is sometimes referred to as the deposition efficiency α_d and denotes how many of the microorganisms that arrive at a surface actually manage to adhere successfully.[25,32,33]

[30] H. Brenner, *Chem. Eng. Sci.* **16,** 242 (1961).
[31] B. D. Bowen, S. Levine, and N. Epstein, *J. Colloid Interface Sci.* **54,** 375 (1976).
[32] Z. Adamczyk and T. G. M. Van de Ven, *J. Colloid Interface Sci.* **80,** 340 (1981).
[33] Z. Xia, L. Woo, and T. G. M. Van de Ven, *Biorheology* **26,** 359 (1989).

Examples of Use of Parallel Plate Flow Chamber for Studying Microbial Adhesion

Example 1: Detachment of Adherent Microorganisms by Air–Water Interface

The major advantage of using the parallel plate flow chamber with *in situ* observation is that enumeration of adhering microorganisms can be carried out without the passage of an air–liquid interface.[10] After each experiment, however, we deliberately pass an air–liquid interface over the adhering organisms to determine the numbers of cells adhering so strongly that they can withstand the interfacial shear of 10^{-7} N (see also Fig. 3). In this way, valuable information can be obtained about the strength of microbial adhesion and detachment mechanisms.

Figure 6 shows various examples of a microscopic field of view prior to and after the deliberate passage of an air–liquid interface. As can be seen, the percentage of adhering organisms detached by the interface can be quite variable. In a study involving the detachment of *Streptococcus mutans* from glass and pellicle-coated glass,[12] it was found that different strains had different detachment levels from bare glass (Fig. 7). Obviously, different strains have different physicochemical surface properties and therewith different detachment levels from bare glass. From the observation that differences in detachment levels disappeared on pellicle-coated glass (Fig. 7), it was concluded that here detachment was through failure in the pellicle mass, which is mechanistically very important.

Example 2: Influence of Electrostatic Interactions on Deposition Efficiency

Microorganisms are transported in the parallel plate flow chamber by convection and diffusion. Depending on the interaction forces beween the substratum and the arriving microorganism, adhesion will be the result. The ratio between the experimentally measured j_0 and a theoretically calculated j_0^* is generally referred to as the deposition efficiency α_d.[32,33]

Figure 8 shows the deposition efficiencies of six coagulase-negative staphylococcal strains[34] to different substratum surfaces as a function of the electrostatic interactions for the regime where electrostatic repulsion prevails (i.e., the product of the microbial and substratum zeta potentials

[34] H. C. Van der Mei, P. Brokke, J. Dankert, J. Feijen, and H. J. Busscher, *J. Dispersion Sci. Technol.* **13,** 447 (1992).

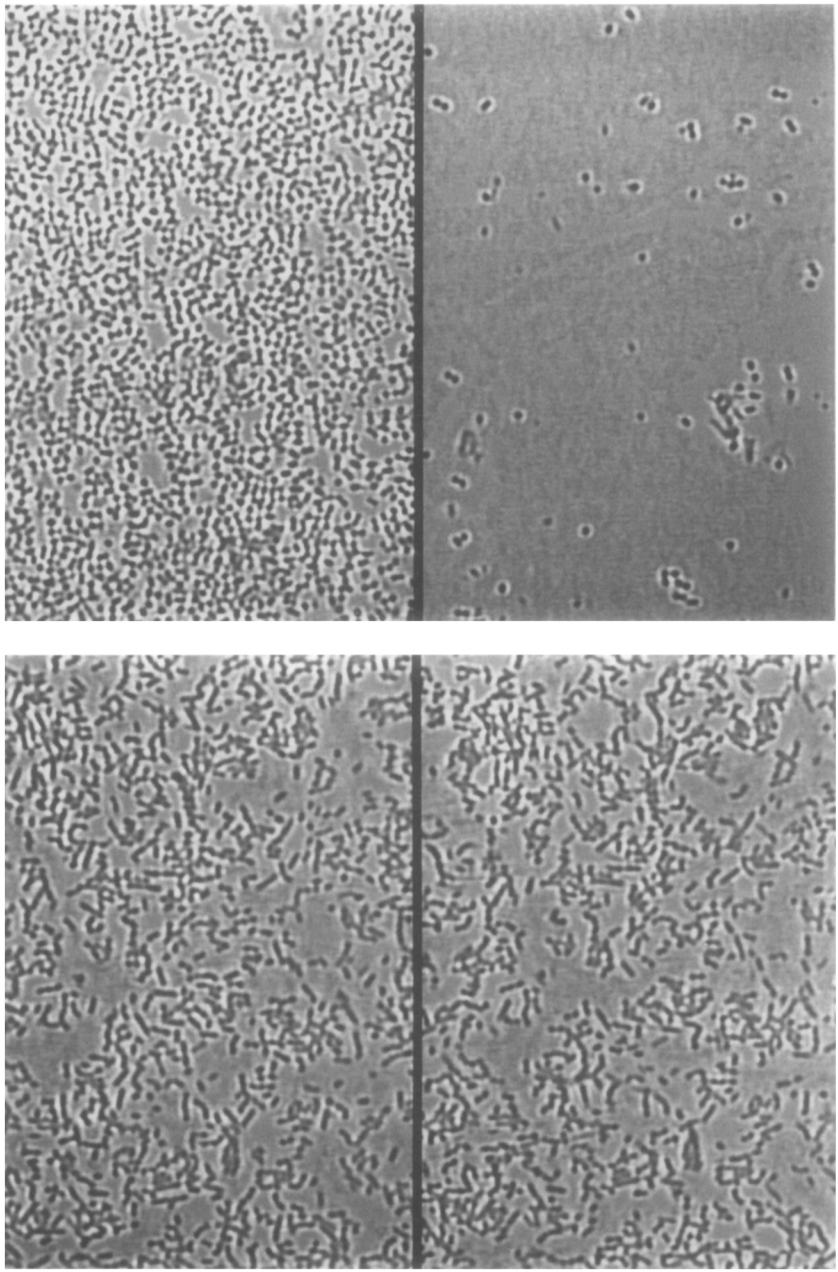


FIG. 6. Examples of predrain, *in situ* images of adhering microorganisms (left) and after drain images after passing an air–liquid interface over the adhering organisms (right). Note that the images are taken at exactly the same substratum location. (*Top*) *Streptococcus mutans* adhering and detaching on glass from a low ionic strength buffer. (*Bottom*) *Enterococcus faecalis* 1131 adhering and detaching on glass from a low ionic strength buffer.

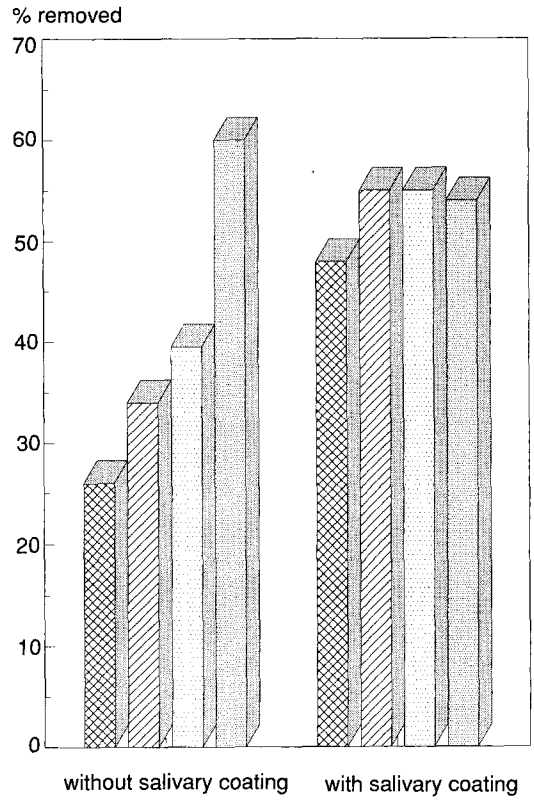


FIG. 7. Percentages of microorganisms detached by an air–liquid interface on bare and pellicle-coated glass. Different bars refer to different strains of *S. mutans*. For details, see Busscher *et al.*[12]

is greater than 0 mV^2). Figure 8 demonstrates two important features. First, owing to the high and reliable data density, accurate estimates of j_0 can be determined, revealing that deposition rates are linearly dependent in the repulsive interaction regime with electrostatic repulsion. Second, because mass transport is amenable to theoretical calculation in the parallel plate flow chamber, $j_0/j_0^* = 1$ arises as a reference value for high and low deposition. In the absence of such a reference value, data are more or less meaningless; furthermore, expression of deposition data in terms of a deposition efficiency allows better comparison of results obtained in various systems. (Note: Different expressions for j_0^* in the Smoluchowski–Levich approach exist for other flow chamber devices.)

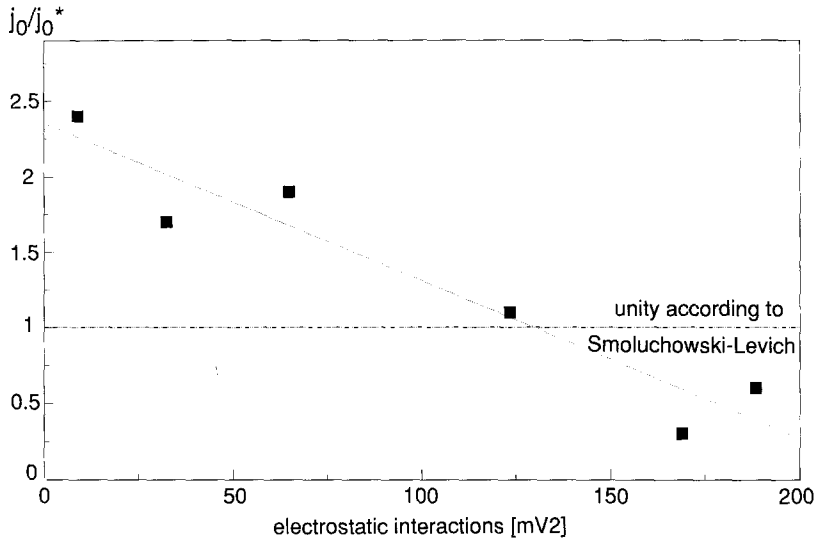


FIG. 8. Deposition efficiencies j_0/j_0^* of coagulase-negative staphylococci on various collector surfaces as a function of electrostatic repulsion, expressed as the product of the microbial and substratum zeta potentials (mV^2). Under the experimental conditions employed, $j_0^* = 1513\ cm^{-2}\ sec^{-1}$. For details, see Van der Mei *et al.*[34]

Example 3: Microbial Cooperativity from Local Pair Distribution Functions

Microbial cooperativity is a mechanism microorganisms can utilize to colonize effectively a substratum surface by which the presence of one adhering organism stimulates the adhesion of another.[35] Usually the existence of cooperative effects is concluded from Scatchard analysis of adhesion data through plotting the ratio bound/unbound organisms versus the number of bound organisms.[36] There is no general agreement in the literature whether it is valid to apply the Scatchard analysis to microbial adhesion data.[37,38] Because nearest-neighbor collection is one of the corollaries of

[35] R. J. Doyle, W. E. Nesbitt, and K. G. Taylor, *FEMS Microbiol. Lett.* **15,** 1 (1982).
[36] R. J. Doyle, J. D. Oakley, K. R. Murphy, D. McAlister, and K. G. Taylor, *in* "Molecular Basis of Oral Microbial Adhesion" (S. E. Mergenhagen and B. Rosan, eds.), p. 109. American Society for Microbiology, Washington, D.C., 1985.
[37] I. M. Klotz, *Science* **217,** 1247 (1982).
[38] A. Levitzki and D. E. Koshland, Jr., *Curr. Top. Cell. Regul.* **1,** 1 (1976).

cooperativity,[39] local pair distribution functions should be different in the absence or presence of cooperative effects. Figure 9 shows examples of the local pair distribution functions for a strain (*Streptococcus salivarius* HB) adhering by cooperative mechanisms and for a strain (*S. salivarius* HB-C12) lacking this ability. Clearly, the collection of nearest neighbors is far less for the strain which lacks the ability to employ cooperative mechanisms. By comparing results from Scatchard analyses with those of nearest-neighbor analyses, Van der Mei *et al.*[40] concluded that both yielded consistent conclusions, despite the indirect character of the Scatchard analysis. At this point it is emphasized that the nearest-neighbor analysis may only be carried out when it is absolutely ascertained that the spatial arrangement of the adhering organisms is preserved as existing during adhesion, that is, the passage of an air–liquid interface prior to observation must be avoided. Clearly, to this end, a device with *in situ* observation possibilities is required.

Example 4: Influence of Flow on Spatial Arrangement of Adhering Microorganisms

Long-range interaction forces between flowing and adhering microorganisms affect the spatial arrangement of the adhering organisms. When a flowing organism is approaching an adhering one, repulsive electrostatic interactions decelerate the flowing organism as long as it is upstream of the adhering one, therewith giving it an increased chance to adhere.[41] While moving over and downstream of an adhering microorganism, the flowing organism is being pushed into higher flow lines and accelerated,[42] therewith decreasing its chance to adhere. The above events become evident in the local pair distribution functions. In Fig. 9, a local pair distribution function of adhering *S. salivarius* HB-C12 on glass at low (Fig. 9B) and high (Fig. 9C) ionic strength is shown. At low ionic strength there is considerable electrostatic repulsion between flowing and adhering organisms, resulting in an asymmetrical distribution function with a depleted zone downstream of the central particle. At high ionic strength, however, the electrostatic interaction between organisms is virtually negligible, resulting in a symmetrical local pair distribution function without a depletion zone.

[39] J. Sjollema, H. C. Van der Mei, H. M. Uyen, and H. J. Busscher, *FEMS Microbiol. Lett.* **69,** 263 (1990).

[40] H. C. Van der Mei, S. D. Cox, G. I. Geertsema-Doornbusch, R. J. Doyle, and H. J. Busscher, *J. Gen. Microbiol.* **139,** 937 (1993).

[41] T. Dabros, *Colloids Surf.* **39,** 127 (1989).

[42] K. Małysa, T. Dabros, and T. G. M. Van de Ven, *J. Fluid Mech.* **162,** 157 (1986).

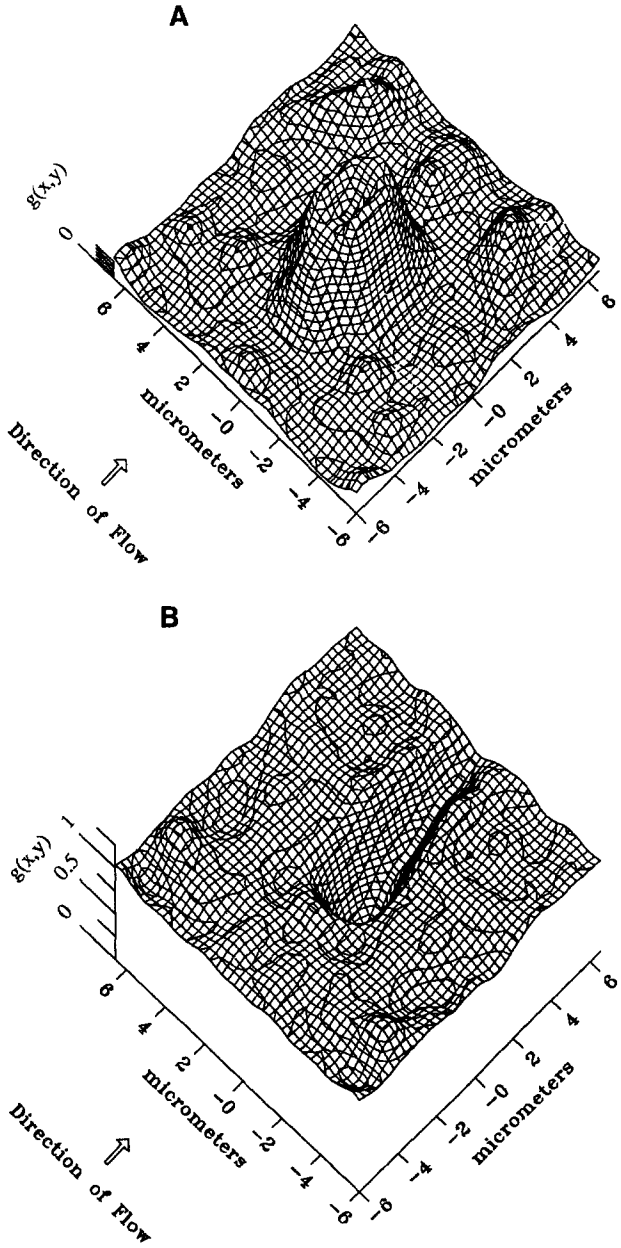


FIG. 9. Local pair distribution functions $g(x, y)$ of various microorganisms adhering to solid substrata. (A) *Streptococcus salivarius* HB adhering by cooperative mechanisms to glass (low ionic strength). (B) *Streptococcus salivarius* HB-C12 lacking the ability to adhere to glass by cooperative mechanisms. Note the asymmetrical depletion zone caused by electrostatic repulsion at low ionic strength. (C) *Streptococcus salivarius* HB-C12 adhering to glass from a high ionic strength solution.

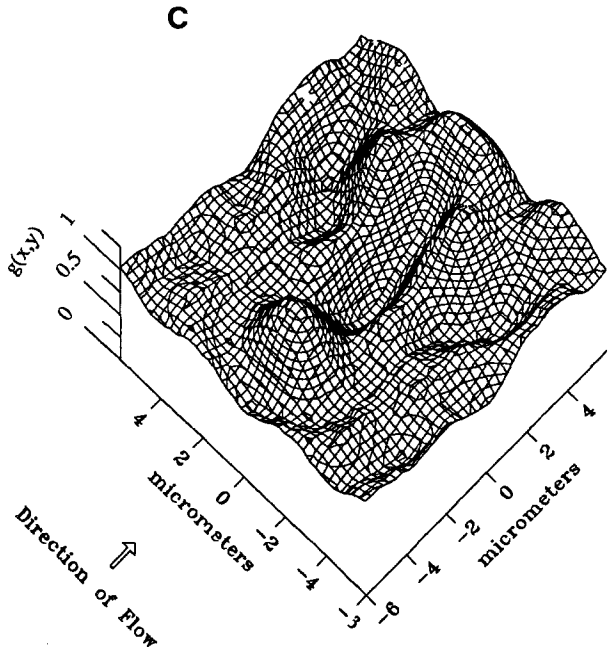


FIG. 9. (*continued*)

Example 5: Desorption and Bond Aging of Adhering Microorganisms under Flow

Bond aging results in a residence time dependence of the desorption rate coefficient β. Bond aging occurs through progressive removal of interfacial water, reorientation effects, extracellular polysaccharide production more firmly anchoring the organisms, etc., although inert polystyrene particles are also subject to bond aging. Desorption in a flow device involves real "thermodynamic" reversibility but, in addition, a contribution of collision-induced desorption.[42]

In the so-called history mode,[19] the analysis method developed enables one to measure bond aging, that is, $\beta(t - \tau)$. Figure 10 presents an example for an *Enterococcus faecalis* depositing from urine onto FEP (fluoroethylene propylene) during exposure to a shear of 15 sec^{-1}. Aging of the bond from an initial β_0 value of 11×10^{-3} sec^{-1} to a final β_∞ value of 5×10^{-6} sec^{-1} occurs over an average time of approximately 250 sec. As an interesting option in the flow chamber system, the flow can be switched to cell-free suspending fluid without passing an air–liquid interface, and desorption can

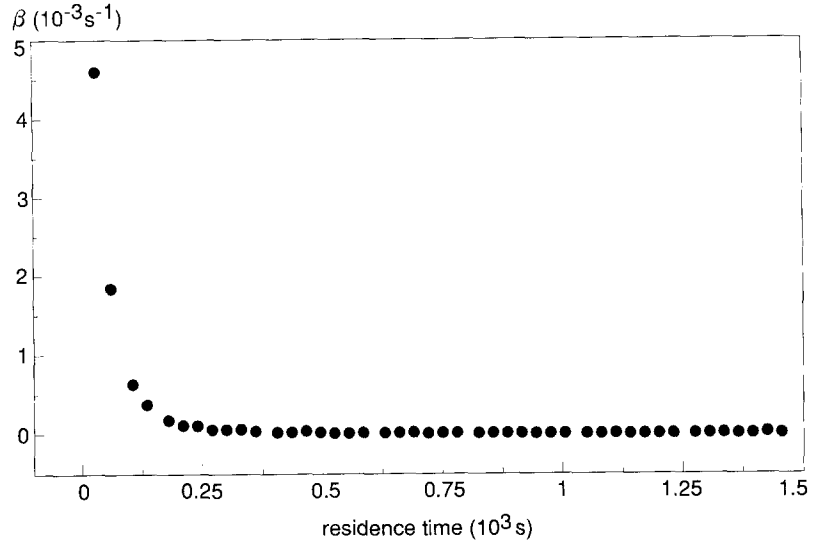


FIG. 10. Residence time dependence of the desorption rate coefficient $\beta(t - \tau)$ of *E. faecalis* 1131 depositing from urine onto FEP.

subsequently be measured excluding the contribution of collisions between flowing and adhering organisms.

The value of the desorption rate coefficient thus measured, β_{esc} (esc denotes escape) is always smaller than β_∞ and amounts 1×10^{-6} sec^{-1} for *E. faecalis* 1131 in the example given above. To our knowledge, these experiments constitute the first direct proof that microbial adhesion to surfaces is a reversible process, with the proof arising directly from the unlimited possibilities of the parallel plate flow chamber with *in situ* observation and image analysis techniques.

Advantages and Disadvantages of System

The major advantages of the system outlined are controlled shear and mass transport; a high data density in time; and no air–liquid interface passages over the adhering microorganisms. These experimental advantages translate into the theoretical advantage that every conceivable parameter involved in the deposition process can be quantified.

To get the system working, a huge amount of computer programming has to be done, as no image analysis software is commercially available

dedicated to measure the deposition parameters of interest. This is a disadvantage, making the technique available only to groups with access to adequate computer know-how. Another disadvantage, particular to our image handling, is that only one field of view per experiment is examined, yielding sufficient statistical samples only when at least 1000–1500 organisms adhere per image. This disadvantage is also directly associated with the history mode, in which connectivity between images has to be ascertained.

Faster computers, the use of 1024 × 1024 pixel image analyzers, and digital image analysis techniques will make the flow chamber method even more valuable and reliable. Another future option is to start working with two or more microbial strains in order to study competition effects in initial microbial adhesion to surfaces. A higher pixel resolution will allow one to distinguish between different organisms on the basis of size and shape; another method is to use fluorescence labels combined with fluorescence phase-contrast microscopy. Both methods are under development at present.

Relevance for Adhesion of Microbial Pathogens

The analyses described in this chapter may appear as technological highstands with little relevance to adhesion of microbial pathogens. Microbial pathogens, however, can tenaciously adhere to biomaterials implanted in the human body,[43] like artificial heart valves, hip implants, voice buttons, dentures, contact lenses, and catheters. The major relevance of the analyses outlined here is that they finally allow for a complete and correct evaluation of the adhesive properties of biomaterials: correct in the sense that artifacts arising from liquid–air interfaces are avoided, and complete in the sense that both the on and the off rate of microbial adhesion and the adhesion bond strength are evaluated against the standard kinetic parameters of the adhesion process. This last point is especially emphasized, because it is recognized that it may well be impossible to prepare biomaterial surfaces to which microbial pathogens do not adhere. Fortunately, the simultaneous notion is arising that "easy wipe off" biomaterials might be fabricated, that is, biomaterial surfaces to which microbial pathogens do adhere but from which they can easily be desorbed or detached. The analyses outlined may greatly assist the development of this new generation of easy wipe off biomaterials.

[43] A. G. Gristina, *Science* **237,** 1588 (1987).

Appendix: Nomenclature and Most Commonly Used Units

Symbol	Definition	Units
A_1	Blocked area	μm^2
a_h	Hydrodynamic microbial radius	μm
b	Half-depth of the chamber	cm
c	Microbial density in suspension	cm^{-3}
D_∞	Microbial diffusion coefficient	$cm^2\ sec^{-1}$
F	Force	dynes, N
$g(x, y)$	Local pair distribution function	—
$j(t)$	Deposition rate at time t	$cm^{-2}\ sec^{-1}$
j_0	Initial deposition rate	$cm^{-2}\ sec^{-1}$
$j_0{}^*$	Initial deposition rate according to Smoluchowski–Levich approximation	$cm^{-2}\ sec^{-1}$
$j_{ads}(t)$	Adsorption rate at t	$cm^{-2}\ sec^{-1}$
$j_{des}(t)$	Desorption rate at t	$cm^{-2}\ sec^{-1}$
Le	Establishment length for laminar flow	cm
N	Total number of images taken per experiment	—
$n(t)$	Number of adhering microorganisms at t	cm^{-2}
n_∞	Number of adhering microorganisms in a stationary state	cm^{-2}
Pe	Peclet number	—
Q	Volumetric flow rate	$cm^3\ sec^{-1}$
Re	Reynolds number	—
t	Time	sec
v	Velocity	$cm\ sec^{-1}$
w	Width of the chamber	cm
x	Distance from the entrance of the flow chamber (parallel to the flow)	cm
y	Distance transverse to the flow	cm
z	Height above the bottom plate of the chamber	cm
α_d	Deposition efficiency	
β	Desorption rate coefficient	sec^{-1}
$\beta(t - \tau)$	Desorption rate coefficient as a function of residence time	sec^{-1}
β_0	Initial desorption rate coefficient	sec^{-1}
β_∞	Final desorption rate coefficient	sec^{-1}
$\Delta n_{ads}(\tau_{i-j})$	Number of microorganisms adhering between time τ_{i-j-1} and τ_{i-j}	cm^{-2}
$\Delta n_{des}(t_i)$	Number of microorganisms desorbing between time t_{i-1} and t_i and adhering between τ_{i-j-1} and τ_{i-j}	cm^{-2}
δ	Reciprocal relaxation time for bond aging	sec^{-1}
μ	Absolute viscosity	$g\ cm^{-1}\ sec^{-1}$, $kg\ m^{-1}\ sec^{-1}$
ρ	Fluid density	$g\ cm^{-3}$, $kg\ m^{-3}$
ρ_0	Average density of adhering microorganisms	cm^{-2}
$\rho(x, \Delta x, y, \Delta y)$	Local density of adhering microorganisms	cm^{-2}
σ	Wall shear rate	sec^{-1}
τ	Time of arrival of adhering microorganisms	sec
τ_x	Wall shear stress	dynes cm^{-2} N m^{-2}

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The authors are grateful to Mrs. Marjon Schakenraad-Dolfing for manuscript preparation and furthermore thank various co-workers in the department for providing sections of data as examples.

[38] Methods for Studying Microbial Colonization of Plastics

By GORDON D. CHRISTENSEN, LUCILLA BALDASSARRI, and W. ANDREW SIMPSON

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Nearly all of the chapters in this book concern the attachment of microorganisms to living tissues, but considering the full breadth of the microbiological world it is obvious that most microorganisms colonize nonliving things rather than living things. To maintain themselves in their ecological niche, such surface-dwelling organisms must first find a suitable inanimate substratum, attach to the material, and multiply on the surface; following this colonization the organisms must then disperse to new sites to repeat the cycle of colonization. With one exception, this process rarely comes to the attention of investigators interested in the pathogenesis of human infections. The exception, of course, is the colonization and infection of implantable medical devices.

It is an unfortunate but true fact that for every appliance placed in humans there is a corresponding microbial infection. With the increasing number and variety of medical appliances, there is an increasing number and variety of microbial infections, a problem which will be with us for the foreseeable future. Although the coagulase-negative staphylococci (CoagNS) cause the majority of these infections,[1] the full range of infectious microorganisms includes virtually every saprophytic and pathogenic microbe that can come into contact with the device.[1] In all such cases, detailed scanning electron microscope studies of infected devices have found the microorganisms in intimate contact with the surface of the device, usually embedded in a matrix of amorphous material of uncertain origin.[1] The challenge is to understand the nature of this intimate association so that

[1] G. D. Christensen, L. M. Baddour, D. L. Hasty, J. H. Lowrance, and W. A. Simpson, *in* "Infections Associated with Indwelling Medical Devices" (A. L. Bisno and F. A. Waldvogel, eds.), p. 27. American Society for Microbiology, Washington, D.C., 1989.

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the infection can be prevented by interrupting colonization or eliminated by poisoning the attached microorganisms with antimicrobial agents. The experimental approach to exploring the relationship between microbe and substratum depends on the ability to count the number of microorganisms on the surface of the device.

This chapter presents the basic approaches to counting microorganisms on a plastic surface, which is the material of choice for the fabrication of most medical appliances. Much of the following discussion is also relevant to the study of the colonization of nonplastic materials by environmental organisms and nonplastic medical appliances by pathogenic bacteria. Focused comments regarding the colonization of similar materials by urogenital organisms are available in the chapters by Reid [40] and by Mobley *et al.* [28] in this volume. The interested reader should also consult the review by Ladd and Costerton.[2]

Colonization Process

Like the colonization of tissues, the colonization of medical devices is a multiphasic process. For both animate and inanimate substrata, colonization begins with either a directed or random encounter of the microbe with the substratum followed by the rapid binding of the microbe to the surface.[1,3] The rapid binding depends on a variety of nonspecific, relatively weak physicochemical forces, such as hydrophobic interactions, and electrostatic forces.[1,3] The effect of these forces is the immediate "reversible" attachment of the organism to the surface. "Reversible" applies only in a theoretical sense; if the attachment is due to a large number of weak bonds, the cumulative effect of the bonds (under constant environmental conditions) may essentially be a permanent attachment.[4] For medical devices, the physicochemical properties of the materials used in fabricating the device primarily govern this stage of colonization; because the properties of these materials are subject to modification, they are of primary interest to engineers and microbiologists.

The colonization of medical devices, however, does not stop at this stage, but is quickly followed by the permanent attachment of the loosely held organism to the surface. For animate tissues permanent attachment often involves the specific interaction of microbial structures (adhesins) with reciprocal substratum structures (receptors), usually through a lectin–saccharide interaction.[1,3] Such targeted attachment may take place for inani-

[2] T. I. Ladd and J. W. Costerton, *Methods Microbiol.* **22,** 285 (1990).

[3] L. M. Baddour, G. D. Christensen, W. A. Simpson, and E. H. Beachey, *in* "Principles and Practice of Infectious Diseases" (G. L. Mandell, R. G. Douglas, and J. E. Bennett, eds.), p. 9. Churchill Livingstone, New York, 1990.

[4] S. McEldowney and M. Fletcher, *Appl. Environ. Microbiol.* **52,** 460 (1986).

mate structures as well if the substratum has adsorbed receptors onto its surface. *Staphylococcus aureus*, for example, will bind specifically to fibronectin adsorbed onto the surface of intravascular catheters,[5] and this specific attachment promotes the *in vivo* colonization and infection of the catheters.[6] Inanimate materials, however, are more often nonspecifically colonized by microorganisms, particularly saprophytic bacteria like *Staphylococcus epidermidis*[1] and *Pseudomonas*,[7,8] that can permanently cement themselves to the surface by the production of adhesive extracellular polymers. The cumulative growth of these latter organisms usually leads to a viscid surface coating known as a "biofilm" or "slime."

Microbial proliferation, the penultimate phase of colonization, is subject to the concentration of nutrients in the bathing fluid and the presence of other microorganisms on the surface. When colonization takes place *in vivo*, microbial multiplication may also be subject to the humoral and cellular immune responses of the host as well as the administration of antimicrobial agents. Colonization is concluded by an often overlooked stage in which there is release of free-floating (planktonic) organisms from the sessile colony and dispersal of the planktonic forms to new colonization sites.[9] Release occurs when daughter cells stop expressing adhesive structures. This may be accomplished either by a cellular response to a change in environmental conditions (phenotypic modulation) or by a (usually reversible) spontaneous change in the genetic mechanisms that govern the expression of microbial adhesive structures (phase variation).

It is not possible to study the colonization process *in toto*; instead, investigators must break down the process into one or two constituent phases. A frequent source of confusion in describing the resulting experiments is imprecise terminology in describing the different stages of colonization. The term "adherence" can be particularly misleading as different authors use the term to refer to different phenomena. For example, some authors use the term "adherence" when referring exclusively to immediate attachment (e.g., incubation periods of 2–180 min[10–22]), whereas others

[5] P. Vaudaux, D. Pittet, A. Haeberli, E. Huggler, U. E. Nydegger, D. P. Lew, and F. A. Waldvogel, *J. Infect. Dis.* **160,** 865 (1989).

[6] P. Vaudaux, D. Pittet, A. Haeberli, P. G. Lerch, J.-J. Morganthaler, R. A. Proctor, F. A. Waldvogel, and D. P. Lew, *J. Infect. Dis.* **167,** 633 (1993).

[7] M. Fletcher and G. D. Floodgate, *J. Gen. Microbiol.* **74,** 325 (1973).

[8] M. Fletcher, *Can. J. Microbiol.* **23,** 1 (1977).

[9] G. D. Christensen, L. M. Baddour, B. M. Madison, J. T. Parisi, S. N. Abraham, D. L. Hasty, J. H. Lowrance, J. A. Josephs, and W. A. Simpson, *J. Infect. Dis.* **161,** 1153 (1990).

[10] N. K. Sheth, H. D. Rose, T. R. Franson, F. L. A. Buckmire, and P. G. Sohnle, *J. Surg. Res.* **34,** 213 (1983).

[11] M. A. Ishak, D. M. Groschel, G. L. Mandel, and R. P. Wenzel, *J. Clin. Microbiol.* **22,** 1025 (1985).

use the term to refer to immediate and permanent attachment (e.g., an incubation period of 5 hr[23]), and still other investigators use the term to refer to immediate and permanent attachment as well as microbial proliferation (with incubation periods of 18–96 hr[24-28]). For this reason the term should probably be avoided[29]; instead, we agree with the editors of this volume that "adhesion" is the preferred term for the immediate binding of a microbe to a surface, and this term should be used when referring to the results of experiments that follow colonization for up to the first 1–2 h following exposure. When the experiments use a longer time scale, such as 4–24 hr, the observations include adhesion as well as the subsequent multiplication of the microorganisms on the surface; better terms for this would be "microcolony formation," "biofilm formation," "slime production," etc.

Besides imprecise terminology, another common problem in interpreting colonization data is overlooking the contribution of the unstudied phases of colonization toward the complete phenomenon; this oversight can lead to erroneous, even absurd, conclusions. Investigators should keep

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[12] J. M. Harris and L. F. Martin, *Ann. Surg.* **206,** 612 (1987).
[13] K. G. Kristinsson, *J. Med. Microbiol.* **28,** 249 (1989).
[14] M. J. Harber, R. Mackenzie, and A. W. Asscher, *J. Gen. Microbiol.* **129,** 621 (1983).
[15] W. M. Dunne and E. M. Burd, *Appl. Environ. Microbiol.* **57,** 863 (1991).
[16] J. McCourtie and J. Douglas, *Infect. Immun.* **32,** 1234 (1981).
[17] J. Carballo, C. M. Ferreiros, and M. T. Criado, *Med. Microbiol. Immunol.* **180,** 149 (1991).
[18] B. Sugarman and D. Musher, *Proc. Soc. Exp. Biol. Med.* **167,** 156 (1981).
[19] D. Rotrosen, T. R. Gibson, and J. E. Edwards, *J. Infect. Dis.* **147,** 594 (1983).
[20] D. J. Gower, V. C. Gower, S. H. Richardson, and D. L. Kelly, *Pediatr. Neurosci.* **12,** 127 (1986).
[21] S. Ashkenazi, E. Weiss, and M. Drucker, *J. Lab. Clin. Med.* **107,** 136 (1986).
[22] S. Katz, M. Izhar, and D. Mirelman, *Ann. Surg.* **194,** 35 (1981).
[23] A. M. R. Mackenzie and R. L. Rivera-Calderon, *Appl. Environ. Microbiol.* **50,** 1322 (1985).
[24] C. C. P. M. Verheyen, W. J. A. Dhert, J. M. A. de Blieck-Hogervorst, T. J. K. van der Reijden, P. L. C. Petit, and K. de Groot, *Biomaterials* **14,** 383 (1993).
[25] T. R. Franson, N. K. Sheth, L. Menon, and P. G. Sohnle, *J. Clin. Microbiol.* **24,** 559 (1986).
[26] G. D. Christensen, W. A. Simpson, A. L. Bisno, and E. H. Beachey, *Infect. Immun.* **37,** 318 (1982).
[27] G. D. Christensen, W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey, *J. Clin. Microbiol.* **22,** 996 (1985).
[28] M. A. Deighton and B. Balkau, *J. Clin. Microbiol.* **28,** 2442 (1990).
[29] We have previously suggested using the term "adherence" to refer to mechanisms of specific attachment (Ref. 3); this usage, however, has not been generally accepted. As the term is not grammatically correct [K. C. Marshall, *in* "Microbial Adhesion" (K. C. Marshall, ed.), p. 1. Dahlem Konferenzen, Springer-Verlag, Berlin, 1984] and because there is such a disparity between authors in the meaning of the term, we agree with the editors of this volume that the term is outmoded.

in mind that the entire colonization process is cyclical, each phase depending on the preceding phase; in other words, slime production cannot occur without adhesion, adhesion without exposure, exposure without release from some other source, and so on.

Experimental Design

In considering the colonization of medical devices, investigators usually ask one of four basic questions: (1) What is the capacity of a standard strain of microorganisms under standard conditions to colonize different substrata or substrata treated in different manners (in order to change the physicochemical parameters of the material or to vary the variety of adsorbed molecules on the surface of the material)? These studies are designed to explore the physical and chemical characteristics of the substratum surface which promote or repel colonization.

(2) What is the capacity of a standard strain of microorganisms to colonize a standard substratum under varying conditions, such as inoculum of microorganisms, pH, buffers, temperature, and fluid flow rate? These studies are designed to determine the precise conditions required for promotion or inhibition of binding; the results provide information regarding the nature and avidity of the organism-to-surface bond.

(3) What is the relative capacity of different microorganisms to attach to and colonize a standard surface under standard conditions? These studies are designed to correlate the capacity of different organisms to colonize a material with the capacity of the microorganism to cause a medical device infection. Differences in colonization could reflect differences in pathogenic capacity and certainly reflects differences in colonization mechanisms.

(4) What is the rate of survival and proliferation of organisms on a surface, particularly when the organisms are exposed to noxious agents, such as antimicrobial agents or phagocytes? These studies are designed to determine whether surface microorganisms have enhanced (or restrained) capacities to resist the antimicrobial action of therapeutic agents and host defenses.

Before presenting the experimental protocols for addressing these questions, a few comments on the critical variables of these studies are in order, variables which must be considered and carefully controlled by the investigator in designing colonization studies of inanimate materials.

Critical Controls

The four questions regarding the microbial colonization of inanimate objects reflect the four basic elements of microbial colonization: substratum, bathing fluid, microorganism, and time. The systematic examination of any

one element requires the other three elements to be closely controlled. Failure to recognize and control adequately these elements is a frequent criticism of published reports.

Substratum

Important variables include the architecture of the object, surface microtopography, chemical composition, inclusion of leachable chemicals, surface contamination with foreign substances, surface treatment, and the physicochemical characteristics of the surface (electrostatic charge, hydrophobicity). Microbial colonization is a function of the surface area available for colonization; surface area in turn is a function of the dimensions of the device, its geometric shape, and its microtopography ("smoothness"). Comparable objects should have comparable surface areas. For example, some protocols examine microbial adhesion to small beads.[30,31] However, even minor differences in the diameter of the beads used in two different preparations can lead to significant differences in the surface area presented by the two materials, and under such conditions there will be greater adhesion to the material with the greater surface area even if the materials are identical in all other respects. Some material differences may not be apparent to the investigator; similar appearing objects may have deceivingly different surfaces at the microscopic level; if one surface is rougher than another, microbial colonization will be greater for the rough surface by virtue of the larger surface area[24] as well as the entrapment of microbes in the microscopic pits, grooves, and interstices of the material.[12] A common problem arises when investigators modify the shape of the device by cutting and trimming so that the object can be placed in the test chamber or compared to another similarly shaped object. In modifying the object the investigator may not allow for the effects on the experiment of cutting the material and exposing fresh, rough surfaces for microbial attachment.

Materials are simply but deceivingly labeled; plastics, for example, are known by their polymeric names: polystyrene, polyethylene, polypropylene, etc. These categories can mislead the investigator into regarding plastics of the same type to be the same material. In exact terms the chemical composition of plastics can vary from manufacturer to manufacturer (and even from lot to lot)[32]; likewise, plastics include plasticizers which make the material durable and pliable, but the precise composition and amount

[30] C. P. Timmerman, A. Fleer, J. M. Besnier, L. de Graaf, F. Cremers, and J. Verhoef, *Infect. Immun.* **59,** 4187 (1991).

[31] P. Vergeres and M. J. Blaser, *J. Infect. Dis.* **165,** 281 (1992).

[32] I. C. Shekarchi, J. L. Sever, Y. J. Lee, G. Castellano, and D. L. Madden, *J. Clin. Microbiol.* **19,** 89 (1984).

of the plasticizers in the material often varies from manufacturer to manufacturer. Details regarding the precise chemical composition of a particular plastic material are usually unavailable to the investigator; often this information is considered a trade secret or is simply not known. These compositional details, however, may have profound effects on the attachment of organisms and substances (like proteins[17,32]) to the plastic surface by perturbing material physicochemical properties such as surface charge and hydrophobicity.

Materials are also subject to postmanufacture modification of the surface. In some situations the manufacturer may purposefully treat the surface to modify the adhesion characteristics; a notable example of this is the difference between 96-well microtiter plates [for enzyme-linked immunosorbent assays (ELISAs)] and tissue culture plates. Although both plates have similar appearances and are made out of similar plastic, manufacturers irradiate tissue culture plates to increase surface charge and reduce hydrophobicity.[4,27] As a result, the adhesion of microorganisms to tissue culture plates is distinctly different from the adhesion of microorganisms to microtiter plates.[4,27] Microbial colonization of plastic materials may also be influenced by inapparent but significant accidental soiling and abrasion of the surface. Furthermore, as the plastic ages the surface may oxidize and plasticizers will evaporate, and additional surface changes may be caused by heat sterilization, chemical cleaning, and exposure to fluids. All of the changes have the potential for changing microbial adhesion.

Obviously, material variables can be difficult to control, a factor which must be considered in any comparison of the susceptibility of different objects or materials to colonization by a particular microorganism. The problem can be minimized by attention to detail regarding the manufacturer and lot of the materials, physicochemical characterization of the surface when indicated, care in the handling, storage, and cleaning of objects, and experimental adjustments for design and size differences. When material composition is a critical element in the study, the National Institutes of Health provides investigators with standard reference materials[33] with known composition that can be used as performance standards for materials of uncertain composition.

Bathing Fluid

There are two aspects of the bathing fluid which must be considered by the investigator, namely, the composition of the fluid and the fluid

[33] NHLBI–DTB primary reference materials (low-density polyethylene and silica-free polydimethylsiloxane) can be obtained from the Biomaterials Program, Devices and Technology Branch, Division of Heart and Vascular Diseases, National Heart, Lung, and Blood Institute, Federal Building, Room 312, 7550 Wisconsin Avenue, Bethesda, MD 20892.

flow over the test object. Important compositional variables include the concentration of dissolved and suspended materials, particularly ions[4] (including pH),[7,8,34] chelating agents,[14,16,34] proteins,[17,35] detergents,[4] and atmospheric gases.[36] Electrolytes, particularly multivalent cations, such as Ca^{2+} [16,34] and Mg^{2+},[34] may promote adhesion by cross-linking anionic groups on bacterial cells and surfaces.[4] The concentration of electrolytes can also influence hydrophobic interactions,[4] and bacterial adhesion may exhibit pH optima and minima.[4,14,34] The presence of detergents in water can diminish surface tension and interrupt attractive van der Waals forces to create a net repulsive force between particle and substratum, leading to desorption.[4,37] Unfortunately, because of the variety of operative mechanisms, with the present state of knowledge the precise effects of each of these variables on any given microorganism are virtually impossible to predict.[4] Dissolved and suspended materials affect not only the fluid but also the substratum. With exposure to a fluid, suspended and dissolved materials will bind in some proportion to all submerged surfaces and modify the surface chemistry. These modifications can profoundly influence microbial attachment. Some organisms, for example, are repelled by the adsorption of serum proteins onto plastic surfaces,[17,35] whereas the same proteins may paradoxically promote colonization for particular strains by providing unique sites for the targeted attachment of the organisms via adhesin-to-protein bindings.[6,7] Finally, experiments that include microbial growth as well as adhesion are also subject to the choice of culture media,[26] availability of nutrients such as glucose,[26,28] and essential nutrients such as iron,[38] and the concentration of gases such as oxygen and carbon dioxide.[36,39]

Common mistakes include the failure to recognize the presence of ions and detergents in the test chamber, failure to account for the effects of oxygenation and media on microbial growth, and failure to recognize the influence on colonization of organic materials, particularly proteins, that are carried over from the culture media or are present in the test chamber fluid. Appropriate controls include the use of reagent grade materials and the preparation of buffers and reagents in pure water. Washing bacterial cells and the avoidance of complex culture media and protein-enriched solutions will minimize the carryover of organic materials; when these effects cannot be eliminated, appropriate allowances should be made in the experimental design.

[34] W. M. Dunne and E. M. Burd, *Microbiol. Immunol.* **36,** 1019 (1992).
[35] M. Fletcher, *J. Gen. Microbiol.* **94,** 400 (1976).
[36] S. P. Denyer, M. C. Davies, and J. A. Evans, *J. Clin. Microbiol.* **28,** 1813 (1990).
[37] C. J. van Oss, C. K. Charny, D. R. Absolom, and T. D. Flanagan, *Bio/Technology* 194 (1983).
[38] M. Deighton and R. Borland, *Infect. Immun.* **61,** 4473 (1993).
[39] L. P. Barker, W. A. Simpson, and G. D. Christensen, *J. Clin. Microbiol.* **28,** 2578 (1990).

Investigators should also consider the dynamic aspects of the bathing fluid. Most assays are performed when the substratum is submerged under static (motionless) conditions; such systems are dominated by gravity and may not be a desirable model of a natural dynamic condition. When using dynamic models, however, the investigator should be sure that the flow of fluids over the test objects is equivalent and laminar. Bacterial adhesion under high (and particularly turbulent) flow rates requires higher energy bonds than adhesion under low flow rates or static conditions. Likewise, the temperature of the fluid will determine the thermodynamic stability of the bond as well as the subsequent microbial proliferation. These conditions can be controlled by attention to detail regarding the positioning and design of the test objects and maintaining standard conditions of temperature and fluid flow.

When protocols require testing the colonization of materials under flow conditions, investigators should consider a Robbins device.[2,40] This apparatus consists of a block of material pierced by precisely engineered tunnels through which the fluid flows. Test materials are inserted through side ports in the block so that the test surface is flush with the lumenal surface of the tunnel. The fluid flow and tunnel dimensions allow the investigator to determine whether surface attachment takes place under laminar or turbulent flow conditions (Reynolds number) and to calculate the wall shear rate and wall shear surface.[40]

Microorganisms

As for the substratum, the choice and preparation of the test organism can appear to be a simple manner, but it is really a complex and virtually impossible variable to control. For understandable convenience, protocols generally treat test cultures of microorganisms as single immutable objects. This convention, however, may lead one to overlook the fact that microbial cultures are actually asynchronous transient populations of short-lived life forms, and individual members of the population are likely to exhibit variations in phenotype.[41] Variability is particularly likely for organisms that colonize solid surfaces; because sessile life forms include a planktonic stage in their life cycles, cultures of surface growing organisms are likely to include a mixture of adhesive and nonadhesive phenotypes.[9,41] The precise proportions will be subject to genotypic drift, natural variation, and the deliberate or accidental enrichment of one form or the other by environmental, storage, and propagation conditions.[9] Because individual experiments are always separated by time and are likely to be different in terms

[40] W. F. McCoy, J. D. Bryers, J. Robbins, and J. W. Costerton, *Can. J. Microbiol.* **27,** 910 (1981).
[41] P. Gilbert, P. J. Collier, and M. R. W. Brown, *Antimicrob. Agents Chemother.* **34,** 1865 (1990).

of the number of generations separating the test cultures as well as the culture propagation conditions, culture suspension conditions, etc., it is virtually impossible to reproduce any two preparations of microorganisms. Although minute, these differences can have important effects on the overall capacity of the microbial culture to colonize a surface, and the cumulative impact of the effects only increases over a series of experiments and among different laboratories.[9] Fortunately, colonization capacities of different strains tend to be fairly predictable given similarity in test conditions. Nevertheless, the investigator interested in colonization of surfaces must be willing to tolerate a wide variability in test results and be vigilant for sources of experimental variation.

Investigators can limit the inherent variation of microbial preparations by attention to details regarding the propagation, harvesting, and storage of cultures so that equivalent experiments have similarly prepared test organisms. One approach is to prepare a large batch of organisms, divide the batch into samples, and store them at −70°. When thawed, different samples are likely to have equivalent colonization properties; however, the population of organisms that survive this treatment may not accurately reflect the original test population; furthermore, depending on survival under frozen conditions, samples of different ages may have different mixtures of adhesive and nonadhesive phenotypes. Finally, once the samples are exhausted the experiment can never be truly duplicated. An alternate approach is to use a minute starter inoculum to propagate a microbial strain under set culture conditions and then harvest the culture so that the preponderance of the organisms are products of known culture conditions and are at a similar stage in the life cycle (e.g., harvesting in mid-exponential phase or early stationary phase). This approach has the appeal that the culture conditions are readily duplicated, but for the previously stated reasons the products of the culture may not be the same from experiment to experiment.

Another common problem is for investigators to select one or more clinical strains that are convenient or interesting and proceed with detailed studies of the capacity of these organisms to colonize a material. The problem with this approach is that without access to the study organisms other investigators cannot replicate the experiments. To avoid this problem one does not have to limit studies to a few laboratory strains, but it is advisable to include a well-studied laboratory strain in the experiments as a performance standard and as a positive or negative control. Table I lists some organisms, available from international culture collections, whose capacities to attach to plastic surfaces have been well studied.[42–49] If an appropriate reference organism does not exist, then many investigators will

[42] R. J. Sherertz, I. I. Raad, and A. Belani, *J. Clin. Microbiol.* **28,** 76 (1990).

TABLE I
REFERENCE STRAINS OF MICROORGANISMS FOR STUDYING COLONIZATION OF PLASTIC DEVICES

Strain[a]	Species	Properties	Refs.
RP62A (ATCC 35984)	*Staphylococcus epidermidis*	Produces slime[b] in Trypticase soy broth (TSB), exhibits phase variation of slime production	9, 15, 27, 28, 34, 38, 42, 43
RP12 (ATCC 35983)	*S. epidermidis*	Produces slime in TSB but not in glucose-poor TSB	9, 26, 27, 28, 43, 44
ATCC 12228	*S. epidermidis*	Antimicrobial susceptibility reference	10, 12, 45
ATCC 14990	*S. epidermidis*	Type strain	12, 46
RP14 (ATCC 35981)	*Staphylococcus hominis*	Attaches but does not proliferate in glucose-poor TSB	9, 27
SP2 (ATCC 35982)	*S. hominis*	Does not produce slime in TSB	9, 15, 26–28
Cowan 1 (ATCC 12598, NCTC 8530)	*Staphylococcus aureus*	Binds immunoglobulin, fibronectin, and fibrinogen	5, 6
ATCC 25923	*S. aureus*	International reference strain for antimicrobial susceptibility	10, 45, 47
ATCC 25922	*Escherichia coli*	International reference strain for antimicrobial susceptibility	10
ATCC 8739	*E. coli*	Preservative testing reference strain	48
NCMB 2021	*Pseudomonas*	Marine organism, attaches to plastic	7, 8, 35
Medeiros (ATCC 27853, NCTC 10662)	*Pseudomonas aeruginosa*	International reference strain for antimicrobial susceptibility	31

[a] ATCC, American Type Culture Collection (Rockville, MD); NCTC, National Collection of Type Cultures (London, England); NCMB, National Collection of Marine Bacteria (Aberdeen, Scotland).
[b] Additional *S. epidermidis* strains KH6, KH11, KH12, and V2 have been widely used by investigators interested in slime production,[13,31,49] but these strains are only available from G. Peters (Institute of Medical Microbiology, Universitat Munster, Germany) or individual investigators.

include the type strain or an antimicrobial reference strain in studies as the performance standard; of course, published organisms with interesting or well-established colonization mechanisms should be submitted to an international culture collection for later reference.

[43] M. Hussain, J. G. M. Hastings, and P. J. White, *J. Med. Microbiol.* **34,** 143 (1991).
[44] D. J. Silverhus, D. D. Schmitt, and C. E. Edmiston, *Surgery (St. Louis)* **107,** 613 (1990).
[45] J. A. Roberts, E. N. Fussell, and M. B. Kaack, *J. Urol.* **144,** 264 (1990).
[46] M. Wengrovitz, S. Spangler, and L. F. Martin, *Am. Surg.* **57,** 161 (1991).
[47] O. V. Lyons, S. L. Henry, M. Faghri, and D. Seligson, *Clin. Orthop. Relat. Res.* **278,** 260 (1992).
[48] P. Gilbert, D. J. Evans, E. Evans, I. G. Duguid, and M. R. W. Brown, *J. Appl. Bacteriol.* **71,** 72 (1991).
[49] A. Ludwicka, B. Jansen, T. Wadstrom, and G. Pulverer, *Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A* **256,** 479 (1984).

Time

The distinction between early attachment (e.g., adhesion), and attachment followed by accumulation (e.g., colony formation or slime production) and the necessity for precision in referring to the two phenomena was discussed earlier. Experiments that focus on adhesion usually use dense microbial suspensions (10^6–10^8) and short incubation periods (5 min to 2 hr) under nonnutritive conditions (buffer and 0°–4°), whereas experiments that focus on microbial proliferation and survival usually use low inocula (10–10^6) and long incubation periods (6 to 24 hr) under nutritive conditions (liquid medium and 20°–37°).

Experimental Protocols

The basic approaches to studying the colonization of medical devices are listed in Table II along with the relative advantages and disadvantages of the methods and the applicability to the four basis research questions listed above. The following paragraphs provide expanded comments regarding application and technical details.

Direct Observation and Radiolabeling

Two procedures, namely direct observation (via an optical microscope or scanning electron microscope) and using radiolabeled microorganisms, are the classic approaches to counting the numbers of microorganisms on a particular surface. The procedures themselves are not inherently unique to plastic materials and are well presented elsewhere. Therefore, detailed procedures for applying these methods to plastic materials will not be discussed; instead the limitations and advantages of these assays when applied to inanimate materials are presented.

Optical Microscope. The optical microscope is probably the oldest and most versatile instrument for quantifying bacteria on a surface. It has, for example, been applied to counting *Candida albicans* on acrylic sheets,[16] *Escherichia coli* on various polymethacrylate films,[50] *Pseudomonas* on polystyrene petri dishes,[8] and *E. coli* and *S. epidermidis* on glass cover-slips.[48] The obvious limitation is the requirement for optically clear, planar material for the substratum. The procedure also involves cell fixation, destroying the organisms, and does not provide information on viability. The primary limitation to the procedure is the tedium of counting (or photographing and counting) large numbers of microscopic fields. Furthermore, individual cells in dense consortia of organisms cannot be reliably counted, and the

[50] G. Harkes, J. Feijen, and J. Dankert, *Biomaterials* **12,** 853 (1991).

TABLE II
EXPERIMENTAL APPROACHES TO COUNTING MICROORGANISMS ON SURFACES

Method	Advantages	Limitations	Applications[a]
Direct observation	Direct visualization, wide applicability, *in situ*	Labor-intensive, wide variability in data, destroys life forms, insensitive	
Optical microscope	Inexpensive, low technology, expedient	Tedious, insensitive, requires optically clear materials	Major: 1, 2
Scanning electron microscope	Visualization of fine structure details	Requires specialized equipment, tedious	Major: 1, 2, 4
Radiolabeling	Sensitive, wide applicability, *in situ* data	Indirect counting method, requires specialized equipment and hazardous materials, limited to adhesion	Major: 1, 2
Counts of living detached organisms	Direct counting method	Paradoxical approach to measuring colonization	
Roll method	Inexpensive, expedient, low technology	Limited to catheters and rods, imprecise, wide variability in data, ignores lumenal surface	Minor: 1, 2, 3, 4
Sonication	Precise, sensitive, wide applicability	Requires specialized equipment, tedious	Major: 1, 2, 3, 4
Stained bacterial films	Expedient, *in situ* data	Indirect, insensitive	
Tube method	Inexpensive, low technology	Insensitive, unreliable qualitative data, limited to test tubes	Major: 3 Minor: 1, 2, 4
Microtiter plate method	Expedient, inexpensive, reliable	Requires specialized equipment, limited to 96-well microtiter and tissue culture plates, insensitive	Major: 2, 3 Minor: 1, 4
Assay for biological product	*In situ* data, wide applicability, expedient	Indirect, insensitive, requires specialized equipment and specialized reagents	Major: 1, 2, 4 Minor: 3

[a] Applications refer to the following: (1) attachment of a standard organism under standard conditions to different substrates, (2) attachment of a standard organism to a standard substrate under different conditions, (3) attachment of different strains to a standard substrate under standard conditions, and (4) multiplication and survival (under adverse conditions) of a colonizing strain on a substrate.

procedure is relatively insensitive to detecting low numbers of organisms on a surface. If a microscope equipped for epifluorescence microscopy is available, the sensitivity can be increased 5- to 10-fold by the use of a fluorescent dye such as acridine orange. The bacterial counts can be widely variable from sample to sample and experiment to experiment, which disturbs many investigators. Specific protocols are available in the review by Ladd and Costerton.[2]

Scanning Electron Microscope. The advent of the scanning electron microscope has allowed investigators to observe in fine detail the attachment of microorganisms to surfaces. As with optical microscopy, the method can be converted from qualitative observations to quantitative observations by simply counting the number of organisms over a given surface area. The procedure has particular advantage in counting the number of organisms on opaque or highly textured surfaces; for example, it has been used to count bacteria on smooth and sandblasted materials,[51] metals,[51,52] plastics,[51,52] ceramics,[51,52] intravascular catheters,[10] surgical biomaterials,[52] polymethyacrylate beads,[47] and urethral catheters.[45] Obviously the procedure requires access to a scanning electron microscope, and because the field of view is so much smaller the procedure can be more tedious (consuming large amounts of instrument time) and less sensitive than similar observations made via an optical microscope. Because the instrument visualizes the specimen at an angle from the side, maintaining the same field size between different fields and determining the field dimensions (observed area) can be difficult. The scanning electron microscope has the same limitations of the optical microscope in that individual members of a consortia cannot be reliably counted, and bacterial counts tend to fluctuate widely from sample to sample and experiment to experiment. Specific protocols are described in the review by Ladd and Costerton.[2]

Radiolabeled Bacteria. Perhaps the most sensitive and versatile assay for microbial adhesion to surfaces involves the use of radiolabeled organisms. The utility of this approach is illustrated by the wide variety of radionucleotides, microorganisms, and substrata used in radiolabeling experiments. Most investigators label the organisms by propagation in liquid medium that includes a radiolabeled essential nutrient. For example, [^{3}H]thymidine has been used to label various gram-positive and gram-negative bacteria for adhesion to intravascular catheters,[6] suture materials,[18] and glass[31] and polystyrene[30] beads; likewise, [^{14}C]glucose has been used to measure the adhesion of *Candida* to intravascular catheters[19] and gram-positive and

[51] C. C. P. M. Verheyen, W. J. A. Dhert, J. M. A. de Blieck-Hogervorst, T. J. K. van der Reijden, P. L. C. Petit, and K. de Groot, *Biomaterials* **14,** 383 (1993).

[52] M. Oga, Y. Sugioka, C. D. Hobgood, A. G. Gristina, and Q. N. Myrvik, *Biomaterials* **9,** 285 (1988).

gram-negative bacteria to intravascular catheters,[21] needles,[21] and suture material.[22] Radiolabeled bacteria have also been harvested from agar containing [^{3}H]glucose for studies of adhesion to crushed silicone rubber[53] and passively labeled with ^{51}Cr for studies of adhesion to silicone plastic neurosurgical prostheses.[20]

The primary limitation to the use of radionucleotides is that the ratio of counts per minute (cpm) to microbe is unstable: with time the label is diluted by microbial replication and lost by metabolic processes. For this reason, under most conditions experiments with radiolabeled bacteria can reliably follow colonization for only the first few hours, limiting the technique to experiments concerning microbial adhesion. Radionucleotides are, of course, hazardous materials (as is the scintillation fluid), purchase and disposal are expensive, and counting requires specialized instruments. Radiolabeled organisms should be well washed prior to use (discarded supernatant from the washing procedure should be included in each experiment as a quality control), a standard curve comparing radioactive counts by colony-forming units to counts per minute of the radiolabeled culture should be constructed for each organism, a blank consisting of the sterile object should be run in parallel with the test samples, and investigators should report the specific activity of the radionucleotide when describing the methods.

Counts of Living Detached Organisms

One of the more gratifying approaches to enumerating surface microorganisms is to detach the organisms and count the number of colony-forming units recovered from the stripping procedure. The resulting data are numerical, direct, and an index of the number of living organisms on the surface. The procedures themselves are straightforward and generally do not involve special technology or materials. Nevertheless, the approach is inherently paradoxical: one ends up studying attached organisms by first detaching the organisms. There are also drawbacks, as the detachment procedure may not be complete or may be harmful to the cells. If the detached cells are present in packets rather than individual cells, results based on colony-forming units may not accurately reflect the true numbers of attached cells. Despite these limitations there are two basic approaches which have wide applicability to medical devices.

Roll Technique (Also Touch and in Situ Cultures). The roll technique is a variation on the clinical method Maki *et al.*[54] developed to assess the degree of bacterial colonization of intravascular catheters as an index of colonization and possible infection. Sheth, Franson, and co-workers applied

[53] S. Barrett, *Br. J. Clin. Pract.* **37** (Suppl. 25), 81 (1983).
[54] D. G. Maki, C. E. Weise, and H. W. Sarafin, *N. Engl. J. Med.* **296,** 1305 (1977).

the technique to determine the capacity of catheters of different materials to accept surface colonization.[10,25] The technique is simple, expedient, and requires little in supplies or equipment. The described technique is limited to small-gage cylindrical objects (like catheters or rods), but similar touch and *in situ* cultures have been applied to other materials such as vascular grafts[46] and plastic petri plates.[23] Although simple, the approach has its limitations as it does not measure intralumenal colonization for catheters. Because dislodged microscopic organisms must be separated from one another by macroscopic dimensions in order to be counted, the dislodged colony-forming units from objects with moderate to heavy colonization will be difficult if not impossible to count. Perhaps the major problem is that the technique depends on dislodging organisms by touching the object to the tacky surface of the agar; tightly held organisms are not likely to be displaced by the procedure.

For the procedure, cylindrical material is cut into standard lengths (e.g., 1–2 cm) and gas sterilized. The sterile test materials are placed in chambers (e.g., small 12 × 75 mm test tubes) containing the microbial suspension, and the chambers are incubated, usually under static conditions and at 37°, with the catheters (or rods) oriented in a perpendicular manner to ensure as well as possible uniform contact of the microbial suspension with the cylindrical surface. After a set time (from minutes to hours), the segments are gently removed from the chamber with a pair of sterile forceps, rinsed in some manner (Sheth, Franson, and co-workers[10,25] rinsed the catheters under running tap water for 2 min; some find it preferable to dip the segments serially in four changes of 50 ml sterile buffer), and then rolled over the surface of the agar plate. Experimental precision might require the segments to be rolled a set number of times over a set distance, but this is impractical. After incubation for 18 to 24 hr, the number of colony-forming units on the plate can be individually counted or the plates semi-quantitatively ranked by some preset criteria. The number of dislodged colony-forming units should be expressed as a function of cylinder surface area and the data expressed logarithmically for comparison of geometric means.

Sonication. Investigators have used a variety of means, such as vortexing and scraping, to remove microorganisms from the surface of an object. Cleaning with sonic energy is the culmination of this approach. Unlike vortexing,[46] this approach is efficient and easily standardized. Silverhaus *et al.*[44] used sonication to study bacterial colonization of two forms of Dacron vascular graft material, Sherertz *et al.* have refined the technique for assessing colonization of vascular catheters,[42] and Wengrovitz *et al.*[46] have demonstrated the superiority of this method over *in situ* cultures and vortexing. The approach has wide versatility, because it can be performed on a variety of objects with complex shapes,[46] it is quantitative, and it provides

TABLE III
SONICATION PROTOCOLS

Device (manufacturer)	Protocol	Application	Refs.
Artek Sonic Dismembrator, Model 301 (Imaging Products, Chantilly, VA)	In 5 ml for 30 sec at 0.6 power setting (60% of 300 W) (60 mHz), with medium horn, at temperature of 0°	Bacteria, small objects (catheter segments, plastic rods, screws)	*a*
Ultrasonic cleaner (Ultrasonic Industries, Plainview, NY)	In 10 ml for 1 min at 55 kHz, 125 W	Catheters, bacteria, and fungi	*b*
Benchtop Ultrasonic Cleaner (Mettler Electronic Co., Haights, NJ)	In 10 ml for 15 min	Bacteria, rods	*c*
Fisher Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA)	In 20–40 ml for 10 min at 20 kHz	Bacteria, vascular graft material	44
Unknown	In 30 ml for 40 min at 20 mHz	Bacteria, vascular graft material	46

[a] G. Christensen and W. A. Simpson, unpublished, (1994).
[b] From Ref. 52.
[c] C. C. Chang and K. Merritt, *J. Orthop. Res.* **9,** 284 (1991).

information on living organisms. The major limitations are the equipment requirements and tedium of performing serial dilutions to quantify released bacteria.

A source of sonic energy is required for performing the procedure, such as a sonic dismembrator or an ultrasonic cleaner. Because sonic energy can lead to cellular disruption, preliminary studies should be performed to determine the optimal conditions *vis à vis* stripping the object of microorganisms and promoting organism survival. Table III lists several experimental protocols. Because ultrasonic cleaning can heat the contents of the test chamber, we strip the materials in chilled buffer. Depending on the architecture of the object and placement in the cleaning chamber, all surfaces of the objects may not be uniformly cleaned of attached organisms. The cleaning efficiency can be checked on the cleaned object by either fixing and staining the organisms *in situ* or immersing the object in a clear nutrient agar. Both methods should show the macroscopic presence of any residual microbial deposits.

In the sonication procedure, sterile test objects are placed in the test chamber with the microbial suspension and incubated for a set time as described in other protocols. After incubation the test objects are individually extracted from the chamber with a pair of sterile forceps, rinsed in serial changes of sterile buffer, and placed in the sonic chamber. Because ultrasonic vibrations can damage hearing, the operator should take care

to wear noise dampening ear muffs and ear plugs before turning on the equipment. For sonication equipment requiring the use of a probe, the probe is first sterilized (dipping in ethanol followed by four serial rinses in sterile buffer will accomplish this), then positioned near the surface of the fluid to provide maximal disruptive force to the object (visible by a swirling motion and bubbles). After the surface is stripped, the sonication effluent is quantitatively evaluated, usually by culture of serial dilutions.[42,44,46] The number of dislodged colony-forming units should be expressed as a function of object surface area and the data expressed logarithmically for comparison of geometric means. As there are a variety of instruments available, when reporting studies the investigator should include the manufacturer and model number of the equipment, as well as the power (in watts) and oscillation frequency (in hertz). If a particular horn is used it should also be mentioned. Finally, the reaction volume, fluid temperature, and sonication time should be described.

Stained Bacterial Films

Firmly attached microbial colonies are easily stained and visualized, the appearance of the deposits can form the basis for qualitative and quantitative assays of microbial colonization of inanimate surfaces. Two approaches have evolved; the first, known as the test tube or tube method, is a simple qualitative statement of the macroscopic presence or absence of a stained bacterial film on test tubes that had contained broth cultures of bacteria; the second approach, known as the microtiter plate method, uses an automatic spectrophotometer to read the optical density (OD) of a similarly stained bacterial film on the floor of a microtiter plate.

Test Tube Method. The test tube method was introduced by Christensen *et al.*[26,27] as both a demonstration of and an assay for slime production among strains of CoagNS associated with intravascular catheter infection. When introduced, the procedure called for using either trypan blue or safranin as the staining agent; however, most investigators have used safranin.[55-57] As both reagents primarily stain the microbial cells and not the slimy matrix material, the OD of the stained residue is a rough approximation of the bacterial density on the test tube surface. Other investigators[58,59] have used Alcian blue, which stains the extracellular matrix[17,26,27] as well as the bacterial cells and therefore is more of an index of extracellular matrix

[55] D. S. Davenport, R. M. Massanari, and M. A. Pfaller, *J. Infect. Dis.* **153,** 332 (1986).
[56] G. A. Hebert, C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry, *J. Clin. Microbiol.* **26,** 1939 (1988).
[57] W. Alexander and D. Rimland, *Diagn. Microbiol. Infect. Dis.* **8,** 215 (1987).
[58] A. H. Hogt, J. Dankert, and J. Feijen, *FEMS Microbiol. Lett.* **18,** 211 (1983).
[59] P. Kotilainen, J. Nikoskelainen, and P. Huovinen, *Scand. J. Infect. Dis.* **23,** 325 (1991).

production. Because the procedure is simple, inexpensive, and expedient, it has been used by a number of investigators, primarily in the context of clinical isolates of CoagNS, although it has also been extended to *Staphylococcus aureus*.[56] The simplicity of the procedure is deceptive. Important variables include both the inoculum (because the phenomenon is dependent on surface proliferation, small inocula are more likely to lead to positive tests than large inocula) and the surface to volume ratio (under static conditions narrow test tubes are likely to have conditions of low oxygenation at the bottom of the tube, which for some slime-producing strains[39] discourages slime production).

The test is qualitative, and results are interpreted as either (strongly or weakly) positive or negative. For these reasons, unless care is taken, the test can be difficult to replicate among observers[27] and laboratories. The basic procedure has been converted to a quantitative assay by Alexander and Rimland,[57] who used a spectrophotometer (Junior Spectrophotometer, Coleman Instruments, Maywood, IL) at 550 nm to read the OD of the safranin stained bacterial film directly, and by Tsai *et al.*,[60] who measured the OD at 590 nm of solubilized (0.2 *M* NaOH at 85° for 1 hr) bacterial films stained with toluidine blue. The assay is obviously limited to culture tubes and is not easily expanded to other materials. Because the procedure is intended as a quick approximation of the surface colonization potential of a given strain of bacteria, the procedure presented here is the qualitative method of Christensen *et al.*[26,27]

In the procedure, a small inoculum (small loopful of plate bacteria, single bacterial colony, $\sim 10^6$ cfu) is placed in 10 ml of Trypticase soy broth (TSB) in a sterile plastic or glass test tube. TSB should be used, as some common laboratory media, like Mueller–Hinton, Todd–Hewitt, and nutrient broth, do not reliably support slime production.[26] (The procedure can be scaled down to smaller volumes, e.g., 1–2 ml of broth, by using a smaller inoculum; a final density of bacteria equal to a 1 : 100 dilution of a stationary broth culture appears to work well.) The loosely capped tube is incubated in a stationary position at 37° under aerobic conditions. After 18 to 24 hr (Freeman *et al.*[61] recommend an extended incubation period of 48 hr for tubes with indeterminate slime production), the contents of the tube are gently poured out, the tube is refilled with a safranin solution (identical to the solution used for gram staining), and then the tube is reemptied and placed upside down to dry. Hebert *et al.*[56] recommend the appropriate modifications of a 30-min dwell time for the safranin solution and gently rinsing the stained tube twice with phosphate-buffered saline (PBS) before setting aside to dry. The tubes are then read before a bright light, using *S.*

[60] C.-L. Tsai, D. J. Schurman, and R. L. Smith, *J. Orthop. Res.* **6,** 666 (1988).
[61] D. J. Freeman, F. R. Falkiner, and C. T. Keane, *J. Clin. Pathol.* **42,** 872 (1989).

epidermidis RP62A or RP12 (in TSB) as positive controls and RP12 (in TSB without glucose) or *S. hominis* SP2 as negative controls.

Most investigators find that is is easy to score strongly positive and negative strains, but weakly positive strains can present a problem. By convention strains that form a ring of growth at the air–fluid interface but otherwise appear negative are scored negative; however, now it is recognized that some strains require oxygen for slime production[39] and that CO_2 depresses slime production.[36] Therefore, it is possible that many of the "ring formers" may actually be slime producers. Group data are best compared by positive and negative proportions (χ^2); repetitive tests may be required to score accurately any given strain, particularly if weakly positive.

Microtiter Plate Method. The microtiter plate method was introduced to correct some of the deficiencies of the tube test. The actual approach is a modification of the methodology introduced by Fletcher, who used the OD of stained bacterial films on plastic petri dishes as a model for the colonization of marine surfaces by aquatic microorganisms.[35] After exposing plastic petri dishes to bacterial suspensions, Fletcher rinsed the dishes four times with buffer, then fixed the residual attached bacteria with Bouin's fixative and stained the cells with ammonium oxalate–crystal violet; the plates were then air-dried and the OD read at 590 nm with a spectrophotometer. Christensen *et al.*[27] adapted this approach to 96-well tissue culture plates, which increased data acquisition by allowing the use of an automatic spectrophotometer. The resulting method is inexpensive, easily performed, and produces reliable quantitative data.

The primary drawback to the procedure is the confinement to 96-well plates; therefore, most investigators rely on this approach when protocols examine attachment to a standardized substratum. Providing the material is available in optically clear sheets, it is possible, albeit cumbersome, to examine the colonization of other materials by cutting material into sheets or disks and placing the specimens on the floor of the test chamber. The material can then be processed as originally described by Fletcher.[35] Because 96-well plates have a higher surface-to-volume ratio than test tubes, the results of colonization experiments with this method can be at variance with those obtained with the test tube method, primarily by the identification of additional slime-producing strains which are dependent on higher oxygenation conditions.[39] Because the assay depends on the optical density of attached cells, the method is insensitive when the bacterial density on the surface is low.

In setting up experiments using microtiter plates, the investigator must consider the choices of spectrophotometer, 96-well plate, and fixative procedure. For example, the MicroELISA Auto Reader (Dynatech Laboratories, Chantilly, VA) automatic spectrophotometer has an OD ceiling of 1.500,[27]

whereas the Bio-Rad EIA reader (Bio-Rad Laboratories, Richmond, CA) records optical densities that approach 7.5.[62] Although the significance of optical densities of this magnitude is uncertain, considerable data in the OD range of 1.5 to 3.0 may be lost with the Dynatech instrument but recovered by the Bio-Rad instrument. Different 96-well plates exhibit different colonization characteristics. In particular, the surfaces of microtiter plates tend to be hydrophobic, whereas the surfaces of tissue culture plates are electrically charged to reduce hydrophobicity.[4,27] Bacteria generally attach to a greater extent to tissue culture plates,[4,27] which is the substratum of choice. The choice of fixative is the final decision. As the microtiter plate procedure has become more popular, different investigators have introduced a variety of fixatives,[62] probably because the original procedure as developed by Fletcher callled for Bouin's fixative. Bouin's fixative includes picric acid, a hazardous substance, the disposition of which is both difficult and expensive. Alternative fixatives, such as methanol, formalin, and Carnoy's fixative, however, do not work as well as Bouin's fixative and can lead to widely variable results, even with the same strain of bacteria.[62] Nevertheless, simple air-drying appears to work nearly as well as Bouin's fixative[62] and is the procedure offered here.

For determination of slime production, bacteria are first propagated overnight in TSB and then diluted 1 : 100 in fresh TSB. Individual wells of a flat-bottomed 96-well microtiter plate are then filled with a 0.2 ml of the inoculated broth, and the plates are incubated in a stationary position at 37° for 18–24 hr. For adhesion experiments, bacteria are suspended in buffer to a density of 10^7–10^8 cfu/ml, 0.2-ml portions of the suspension are then dispensed into individual wells, and the plate is incubated for 5–120 min, usually in a stationary position at 37°. At the conclusion of the incubation period the liquid contents of the wells are gently aspirated by tipping the plate forward, placing the tip of the aspirator (usually an 8- or 12-prong aspirator connected to low vacuum) on the lowest surface of the well wall (at the air–fluid meniscus), and, as the fluid is aspirated, slowly following the retreating meniscus into the corner created by the sides and floors of the well. (It is important to not disturb the film in the center of the well, which is used to measure the OD.) The wells are then refilled with 0.2 ml of PBS and reaspirated; the procedure is repeated for a total of four changes of PBS. The residual adhesive bacterial film is fixed by drying at 60° for 1 hr and stained by flooding the wells with Hucker crystal violet.[63] The stained

[62] L. Baldassarri, W. A. Simpson, G. Donelli, and G. D. Christensen, *Eur. J. Microbiol. Infect. Dis.* **12,** 866 (1993).

[63] Hucker's crystal violet solution is prepared as follows. Solution A contains crystal violet (2 g) in 95% (v/v) ethanol (20 ml), and solution B contains ammonium oxalate (0.8 g) in water (80 ml). Mix solutions A and B, and after 24 hr filter the mixture through filter paper.

plates are rinsed under running tap water, emptied by shaking the upside down plate, and set aside to dry. The dried plates are read in a Bio-Rad ELISA reader at a wavelength of 570 nm. Appropriate controls include *S. epidermidis* RP62A or RP12 (in TSB) as positive controls and RP12 (in TSB without glucose) or *S. hominis* SP2 as negative controls.

For interpretation of experiments, each plate should contain a medium blank, the OD of which is subtracted from the sample OD. The absolute contribution of the blank reading to the data, however, tends to be negligible and in many experiments can be safely ignored. Samples are usually run in multiples (usually 4–8 wells), and assays are repeated at least once. In experiments following the colonization of plates by one (or a few) strains (relative values), investigators have analyzed the data by using Student's *t* test of the averaged sample OD. For studies that compare the slime-producing capacity of different strains, an arcane procedure which combines the OD of bacterial films produced in TSB with the OD produced in TSB without glucose has been proposed.[27] Currently, however, most investigators simply average the OD of the bacterial films produced in TSB. For studies looking at group data, the average OD readings are compared by Student's *t* test; for determinations of the slime-producing capacity of a particular strain (absolute values), the organism is labeled either negative, weakly positive, or strongly positive on the basis of the averaged OD. The accepted convention for these categories[27,28] is to use the ceiling OD for negative strains three standard deviations above the mean value of a series of blank wells (usually an OD of 0.12 to 0.3); strains with an OD that exceeds this ceiling value but is less than double the value can be considered weakly positive (usually an OD of 0.12 to 0.6), and strains with an OD greater than double (usually 0.24 to 0.6) the ceiling value for negative strains can be considered strongly positive. The problem with this approach is that many strains have OD readings of 1.5 or greater, and the potentially important data are not captured by such a categorizing system.

Biological Assays

The previously listed procedures have the disadvantages of being either tedious and insensitive (direct microscopic counts) or hazardous with a limited observation window (radiolabeled organisms) or limited to optically clear materials (stained bacterial films) or awkward by requiring the detachment of the organism (roll and sonication methods). The final procedure to be presented avoids these problems by measuring the production of a microbial product as an indirect assay for the number of microorganisms on a surface.

Biological assays have the advantage of being sensitive, linear assays that are easily performed and allow investigators to follow microbial coloni-

zation over a wide variety of complex objects under many conditions. The disadvantage to these approaches is that the assays often rely on specialized equipment and reagents. Furthermore, they require a standardization step which correlates the amount of product to the number of microbes generating the product. Because this standardization is usually done with planktonic organisms, a serious theoretical limitation to the approach is the not necessarily correct assumption that the generation of biological products by planktonic organisms is similar to the generation of biological products by sessile organisms.[41] It may also be necessary to rerun the standardization curve for each test organism. Nevertheless, monitoring colonization by monitoring the production of a biological product has great utility, particularly for following the activities of well-established microbial communities. To perform these studies, investigators have monitored ATP production via light release from a solution of firefly luciferin and luciferase,[13,14,43,49] the number of attached bacterial cells via the production of cell-associated urease,[15,34] and electron transport via formazan production.[2,64] The procedure presented here is the bioluminescence method, which appears to have the greatest sensitivity, utility, and ease of use. Details regarding the formazan production method can be found in the review by Ladd and Costerton.[2]

ATP Bioluminescence Assay. Harber *et al.* introduced the use of the bioluminescence assay for ATP as a quantification procedure for monitoring microbial attachment by applying the technique to the colonization of plastic test tubes by *E. coli.*[14] The procedure has been used primarily to monitor adhesion,[13,14,49] but it has been successfully applied to study 18-hr biofilms.[43] The procedure correlates reasonably well with colony-forming units recovered by sonication.[13] The procedure offered here is the method of Ludwicka[49] as modified by Kristinsson[13] that is appropriate for variously shaped small objects (e.g., catheter sections). Hussain *et al.* have modified the protocol so that microtiter plates can be used for both the substratum and data acquisition.[43] The procedure requires bioluminescence reagents and a luminometer (available from Wallac Oy, Turku, Finland, or Wallac, Gaithersburg, MD; for a complete annotated list of suppliers, see the reviews by Stanley.[65,66]).

The procedure is run with all reagents at room temperature. Colonized objects are extensively rinsed in sterile buffer and then placed in small plastic containers to which is added 0.2 ml of trichloroacetic acid (2.5%, w/v, TCA). The containers are gently shaken to distribute the TCA and

[64] R. F. Gagnon, A. D. Harris, J. Prentis, and G. K. Richards, *in* "Advances in Peritoneal Dialysis" (R. Khanna, K. D. Nolph, B. F. Prowant, Z. J. Twardowski, and D. G. Oreopoulos, eds.), p. 138. Univ. Toronto Press, Toronto, 1989.

[65] P. E. Stanley, *J. Biolumin. Chemilumin.* **7,** 77 (1992).

[66] P. E. Stanley, *J. Biolumin. Chemilumin.* **8,** 51 (1993).

release bacterial ATP from the attached bacteria as well as destroy ATP-degrading enzymes. A blank control, consisting of a clean (uncoated) object, should be run in parallel to the test specimens, and duplicate or triplicate specimens should be run for each data point. To measure light output, a 50-μl sample of the TCA extract from each test object (and blank) is transferred to a plastic cuvette to which is added 1 ml of reconstituted firefly luciferin and luciferase reagent (ATP-monitoring reagent, Wallac; 1 vial reconstituted with 50 ml of Tris–EDTA buffer, buffer consisting of 0.1 *M* Tris and 2 m*M* EDTA adjusted to pH 7.75 with acetic acid). The cuvettes are placed in a luminometer for a preliminary sample reading and then supplemented by adding 20 μl of ATP standard (0.2 nmol) (Wallac; 1 vial reconstituted with 10 ml of Tris–EDTA buffer to a final ATP concentration of 10 μM) to each sample and measuring the resulting increase in light. Standard curves of ATP production versus number of viable organisms are constructed by performing the bioluminescence assay on serial dilutions of pelleted planktonic organisms in parallel with quantitative cultures.

For interpretation, the amount of released ATP in the sample (in nanomoles) is calculated by the following formula: ATP (nmol) = [light output from sample (mV) − light output from standard (mV) × amount of standard added (nmol)]. From this number the result obtained with the blank (calculated in the same way) is subtracted to obtain the true value, and the values are averaged for each data point. The number of microorganisms corresponding to the data point is determined from a standard curve and expressed as the number of cells per square centimeter of surface area. For complex-shaped objects with uncertain surface area, investigators can alternatively report the absolute ATP production per object or number of microorganisms per object.

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[39] Binding of Extracellular Matrix Proteins by Microbes

By ÅSA LJUNGH and TORKEL WADSTRÖM

Introduction

After the original discovery by Kuusela in 1978 that *Staphylococcus aureus* strains bind serum fibronectin (Fn, or cold-insoluble globulin), research on how Fn and other major extracellular matrix (ECM) proteins interact with pathogenic microbes has rapidly increased. In the 1980s emphasis was mainly put on two classes of microbes: group I, staphylococci and groups A, C, and G streptococci binding ECM in wounded tissue[1]; and group II, some tissue-invasive microbes such as *Treponema pallidum* and *Candida albicans* and parasites such as *Plasmodium falciparum, Leishmania,* and *Trypanosoma* binding Fn, laminin (Ln), and collagens (Cn).[1,2]

A number of bacterial species, such as the oral streptococci involved in the pathogenesis of periodontal disease and endocarditis,[2,3] *Mycobacterium tuberculosis,*[4] *Yersinia enterocolitica,*[5,6] *Escherichia coli,*[4,7,8] *Aeromonas* spp.,[9] and *Helicobacter pylori,*[10] have also been shown to bind specifically to various ECM components. Heparan sulfate glycosaminoglycans (GAGs) were identified as cell surface receptors for *Herpes simplex* virus[11] (Table

[1] T. Wadström, L. M. Switalski, P. Speziale, K. Rubin, C. Rydén, G. Fröman, A. Faris, M. Lindberg, and M. Höök, *in* "The Pathogenesis of Bacterial Infections," Bayer Symposium 8, p. 193. Springer-Verlag, Berlin and Heidelberg, 1986.

[2] M. Höök, L. M. Switalski, T. Wadström, and M. Lindberg, *in* "Fibronectin" (D. F. Mosher, ed.), p. 295. Academic Press, San Diego, 1989.

[3] J. H. Lowrance, L. M. Baddour, and W. A. Simpson, *J. Clin. Invest.* **86,** 7 (1990).

[4] B. Westerlund and T. K. Korhonen, *Mol. Microbiol.* **9,** 687 (1993).

[5] H. Schulze-Koops, H. Burkhardt, J. Heesemann, T. Kirsch, B. Swoboda, C. Bull, S. Goodman, and F. Emmrich, *Infect. Immun.* **61,** 2513 (1993).

[6] H. Schulze-Koops, H. Burdhardt, J. Heesemann, K. Van der Mark, and F. Emmrich, *Infect. Immun.* **60,** 2153 (1992).

[7] Å. Ljungh, L. Emödy, P. Aleljung, O. Olusanya, and T. Wadström, *Curr. Microbiol.* **22,** 97 (1991).

[8] G. Fröman, L. M. Switalski, A. Faris, T. Wadström, and M. Höök, *J. Biol. Chem.* **259,** 14899 (1984).

[9] F. Ascencio, P. Aleljung, and T. Wadström, *Appl. Environ. Microbiol.* **56,** 1926 (1990).

[10] T. Wadström, Å. Ljungh, and J.-I. Flock, *in* "Biology of Vitronectin and Their Receptors" (K. T. Preissner, S. Rosenblatt, C. Kost, J. Wegerhoff, and D. F. Mosher, eds.), p. 257. Elsevier, Amsterdam, 1993.

[11] P. G. Spear, M. T. Shieh, B. C. Herold, and D. Wudunn, *in* "Molecular Mechanisms of Microbial Adhesion II" (M. Höök and L. M. Switalski, eds.), p. 43. Springer-Verlag, Berlin, 1992.

I). Some ECM proteins, such as Fn and Vn, are also found in serum. It is likely that proteins expose different microbial binding domains when they are in soluble form and when they are immobilized in the ECM or on a biomaterial surface. This is confirmed by the finding that Yad A of *Y. enterocolitica* and P fimbriae of uropathogenic *E. coli* bind surface immobilized Fn but not serum Fn.[4,5] Binding of only immobilized or soluble forms of ECM proteins suggests that binding occurs to an epitope which is sensitive to conformational changes of the ECM molecules.

Extracellular Matrix Constituents

A number of ECM proteins have been purified and cloned, and several are commercially available. They all have a eukaryotic cell binding domain containing the Arg-Gly-Asp (RGD) sequence.[12] Some structures shown to bind to microbes are listed below.

Fibronectin. Fibronectin has been purified from human and animal plasma. Although Fn has a highly conserved structure, there are differences among proteins from different animal species. Fibronectin has two defined binding domains for heparin and fibrin, and one for collagen and gelatin.[13] *Staphylococcus aureus* and other bacterial species bind the N-terminal domain of Fn.[2] There are several indications that *S. aureus* also binds to a domain in the carboxyl terminus of Fn.[2,14]

Collagen. The Cn family now comprises more than 15 different types which all contain at least one triple-helical domain. They are major constituents of bone and cartilage, and Cn type IV is the dominating protein of basement membranes. Collagens form supramolecular aggregates with Cn or other ECM constituents, such as C1q and Fn. Collagens bind heparin and heparan sulfate, and Cn type IV also binds firmly to Ln, which is mediated by nidogen (entactin).

Vitronectin. Vitronectin (Vn) or S-protein is present in vascular walls, aging skin, and the ECM of certain tissues. In plasma, Vn circulates as a one-chain form (75 kDa). This form is easily reduced by heparin into two chains of 65 and 10 kDa. Vitronectin binds and inhibits heparin, the complement C5-9 complex, and plasminogen activator inhibitor 1. Although human and bovine Vn have the same molecular weights and cross-react immunologically, they appear to express minor structural differences as

[12] T. Kreis and R. Vale, "Guidebook to the Extracellular Matrix and Adhesion Proteins." Oxford Univ. Press, Oxford, New York, and Tokyo, 1993.

[13] K. M. Yamada, *in* "Cell Biology of Extracellular Matrix" (E. D. Hay, ed.), p. 111. Plenum, New York, 1991.

[14] S. Bozzini, L. Visai, P. Pignatti, T. E. Petersen, and P. Speziale, *Eur. J. Biochem.* **207,** 327 (1992).

TABLE I

EXTRACELLULAR MATRIX GLYCOPROTEINS SHOWN TO INTERACT WITH MICROBES

Glycoprotein	Microbial species	Soluble form	Immobilized form
Fibronectin	*Staphylococcus aureus*[a]	+	+
	CoagNS[b]	(+)[c]	+
	Streptococcal groups A, C, G	+	NT[d]
	Streptococcus sanguis	+	NT
	Escherichia coli	+	+
	Porphyromonas gingivalis	+	NT
	Mycobacterium tuberculosis	+	+
	Vibrio cholerae[e]	+	+
	Treponema pallidum	+	+
	Yersinia spp.[f]	—	+
	Candida albicans	+	+
	Leishmania	+	NT
	Plasmodium falciparum	+	NT
Collagen I, II	*S. aureus*	+	+
	E. coli	+	+
	Aeromonas hydrophila	NT	+
Collagen IV	*E. coli* Dr fimbriae	+	+
	Streptococcus pyogenes	+	NT
Vitronectin	*S. aureus*	+	+
	CoagNS	—	+
	Streptococcus pneumoniae	+	NT
	Streptococcus dysgalactiae	+	+
	Helicobacter pylori	+	NT
	E. coli	+	NT
	Pneumocystis carinii	+	+
	C. alibicans	+	NT
Laminin	*S. aureus*	+	+
	S. pyogenes	+	NT
	H. pylori	+	NT
	Treponema denticola	+	+
	C. albicans	+	NT
Thrombospondin	*S. aureus*	+	+
Bone sialoprotein II	*S. aureus*	+[g]	NT
	CoagNS	+	NT
Heparan sulfate/heparin	*S. aureus*	+	+
	CoagNS	—	+
	H. pylori	+	+
	S. pyogenes	+	NT
	Herpes simplex virus	+	NT

[a] A conserved acidic core sequence is necessary for binding. Staphylococci, *S. pyogenes,* and *S. equisimilis* bind Fn by similar mechanisms [S. Sela, A. Aviv, A. Tovi, I. Burstein, M. G. Caparon, and E. Handksi, *Mol. Microbiol.* **10,** 1049 (1993)].

[b] CoagNS, Coagulase-negative staphylococci.

[c] (+) denotes poor binding.

[d] NT, Not tested.

[e] Mediated by D-mannose and likely to involve lectin-like interactions [E. J. Wiersma, G. Fröman, S. Johansson, and T. Wadström, *FEMS Microbiol. Lett.* **44,** 365 (1987)].

[f] Mediated by Yad A, similar to binding of Cn.[5,6]

[g] Strains were shown to bind BSP II but not osteopontin and other bone-associated proteins.

strains of *Streptococcus dysgalactiae* could bind bovine and human Vn, whereas strains of other streptococcal species only bound human Vn.[15]

Laminin. Laminin is a highly glycosylated protein which is a major constituent of basement membranes. Laminin exerts profound effects on cell adhesion, migration, and differentiation, and it has two binding sites for eukaryotic cells. In addition, Ln binds to itself in a Ca^{2+}-dependent manner; it binds heparin and heparan sulfate, as well as Cn type IV, and forms a complex with entactin.

Bone Sialoprotein II. Bone sialoprotein II (BSP) is a heavily glycosylated ECM protein. In adult tissue, BSP is exclusively found in the skeleton and dentin.

Thrombospondin. Thrombospondin (Trp) is a major glycoprotein produced by platelets and developing tissues but also by adult tissue as a response to injury and inflammation. The current opinion is that Trp is an ECM protein which is produced during cell proliferation. Thrombospondin has a heparin binding domain and also interacts with Fn and fibrin.

Heparan Sulfate, Heparin, and Other Glycosaminoglycans. Glycosaminoglycans (GAGs: heparan sulfate, heparin, chondroitin, dermatan and keratan sulfate, and hyaluronic acid) are polysaccharides that form part of the ECM and are found on cell surfaces ubiquitously. Hyaluronic acid differs from the other GAGs in that it is unsulfated.

When using commercial ECM proteins, several points should be noted: (i) the source is important because the structure of human ECM proteins differs from those of different animal ECM proteins; (ii) the method of purification used in some methods may induce changes of configuration or denaturation; (iii) the degree of contamination with other proteins may be significant (e.g., commercial Cn type I usually contains a small portion of Cn type III, Vn is commonly copurified with some albumin, and laminin with nidogen); (iv) the delivery conditions (e.g., freeze-drying) may induce permanent structural changes of some ECM proteins such as Vn.

Microbes

Several of the identified ECM-binding structures on *S. aureus* and other bacteria are proteins,[2,16,17] and hence the use of heat-killed bacteria may render false low or negative results. Proteases are produced by many micro-

[15] L. F. Filippsen, P. Valentin-Weigand, H. Blobel, K. T. Preissner, and G. S. Chhatwal, *Am. J. Vet. Res.* **51,** 861 (1990).

[16] L. M. Switalski, J. M. Patti, W. Butcher, A. G. Gristina, P. Speziale, and M. Höök, *Mol. Microbiol.* **7,** 99 (1993).

[17] T. Wadström, M. Paulsson, and Å. Ljungh, *in* "Staphylococcal Infections" (R. Möllby, J. I. Flock, C. E. Nord, and B. Christensson, eds.), p. 343. Gustav Fischer Verlag, Stuttgart, 1994.

bial pathogens, and fibronectinolytic activity has been detected in strains of *S. aureus, Staphylococcus epidermidis, Propionibacterium acnes,* and a number of other anaerobic bacterial species.[18] Binding of Fn by proteolytic bacterial strains may thus permit subsequent analysis of binding domains if the Fn is analyzed after proteolytic degradation in parallel.

Most bacteria seem to express binding of ECM proteins during the exponential growth phase.[7,19] Binding of Vn by *S. aureus,* however, was best expressed after 20 hr of growth.[7,10] The growth medium and temperature profoundly influence expression of binding of ECM proteins.[20,21] Binding of Fn, Vn, Ln, Cn, and heparan sulfate by *S. aureus* was best expressed after growth under nutrient starvation.[22] In *E. coli* strains, expression of binding of Fn, Cn, and Ln was suppressed in culture media with a molarity over 0.5% NaCl, whereas addition of up to 10 m*M* $CaCl_2$ to a minimal medium enhanced expression of Fn but not of Ln or Cn binding.[7] To study the role of adhesion to ECM components in relation to tissue tropism, the construction of mutant bacterial strains which do not express ECM binding has proved very useful. Using transposon inactivation mutagenesis, Lowrance *et al.*[3] obtained a low Fn-binding mutant of *S. sanguis* which was less virulent than the parent high Fn-binding strain in a rat endocarditis model. Likewise, the construction of mutant strains of *Streptococcus pyogenes* has revealed that two domains on the protein F molecule are necessary for expression of Fn binding.[23]

Binding of Soluble Proteins and Glycosaminoglycans

Binding of soluble proteins in iodinated form has been studied extensively. The chloramine-T method has been applied on numerous proteins.[8] Iodobeads (Pierce, Rockford, IL) have been introduced which provide a standardized procedure to iodinate proteins and GAGs. The specific activity of labeled ECM proteins is about 1×10^6 counts/min (cpm)/μg. The ^{125}I-labeled Vn is stored at −20°, whereas other labeled ECM proteins and GAGs can be stored for a few weeks at 4°.

[18] M. S. Lantz, R. D. Allen, L. W. Duck, J. L. Blume, L. M. Switalski, and M. Höök, *J. Bacteriol.* **173,** 4263 (1991).

[19] C. Rydén, A. Yacoub, I. Maxe, D. Heinegård, Å. Oldberg, A. Franzén, Å. Ljungh, and K. Rubin, *Eur. J. Biochem.* **270,** 331 (1989).

[20] T. Wadström, *in* "Microbial Cell Surface Hydrophobicity" (R. J. Doyle and M. Rosenberg, eds.), p. 315. American Society for Microbiology, Washington, D.C., 1990.

[21] I. Ofek and R. J. Doyle, "Bacterial Adhesion to Cells and Tissues," p. 23. Chapman & Hall, New York and London, 1994.

[22] O. D. Liang, F. Ascencio, R. Vazquez-Juarez, and T. Wadström, *Zentralbl. Bakteriol.* **279,** 180 (1993).

[23] S. Sela, A. Aviv, A. Tovi, I. Burstein, M. G. Caparon, and E. Hanski, *Mol. Microbiol.* **10,** 1049 (1993).

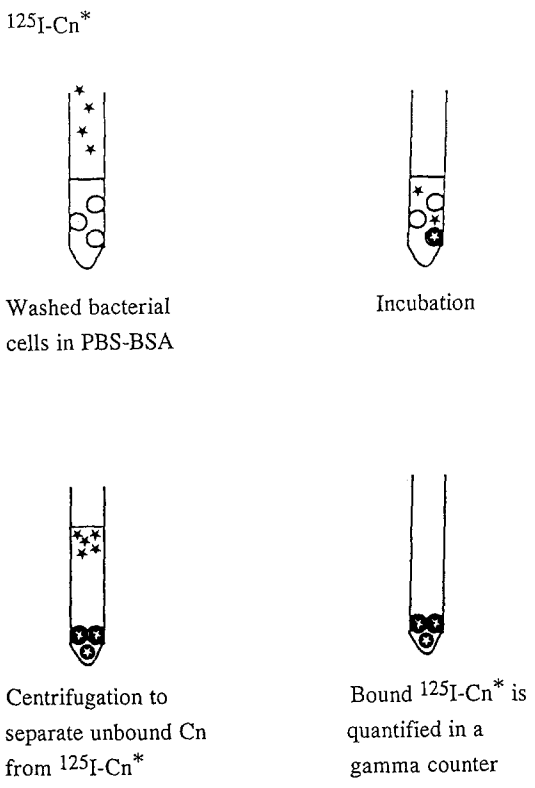

FIG. 1. Binding Assay of Soluble ECM Protein.

A standard assay for determination of binding of soluble proteins is shown in Fig. 1. A suspension of washed microbes in 0.14 *M* NaCl with 10 m*M* sodium phosphate, pH 7.2 (phosphate-buffered saline, PBS), at a density of 5×10^9 to 1×10^{10} cells/ml is mixed with iodinated protein for 1 or 2 hr at 20°. In several studies the test tubes are precoated with 5% (w/v) bovine serum albumin (BSA) to prevent nonspecific binding. However, inclusion of 0.1% BSA in the incubation buffer has been shown to be sufficient to block nonspecific binding.[24] Sodium azide (0.02%) is included in the buffer to inhibit microbial proliferation. The suspensions are centrifuged (1300 *g*, 20° 15 min), and the supernatants are aspirated (Fig. 1). The incubation mixture (100 μl) can be mixed with 200 μl NaCl/P_i and layered on top of 1.5 ml Percoll in NaCl/P_i (density 1.020 g/ml) before centrifugation.[1,19] Comparing the two methods, no significant difference was noted.[7] The radioactivity associated with the pellet is counted, and binding is ex-

[24] R. A. Proctor, D. F. Mosher, and P. J. Olbrantz, *J. Biol. Chem.* **254,** 14788 (1982).

pressed as the percentage of total added radioactivity. One strain expressing high binding and a nonbinding strain should be included as controls, as well as tubes containing buffers without bacteria.

Supplementation of the incubation buffer with Ca^{2+} and Mg^{2+} (1.2 and 0.7 m*M*, respectively) was shown to increase binding of BSP by *S. aureus* strains,[19] whereas binding of heparan sulfate was not affected.[25] It is advantageous that microbes can be grown under conditions to promote or suppress expression of binding properties, for example, on different solid or liquid media in different environments (aerobic/anaerobic, temperature, etc). One disadvantage is that coupling of proteins to iodine may modify a microbe-binding domain.

Binding of Immobilized Proteins

Immobilization in Microtiter Plate Wells

Polystyrene plates can be used for passive coating of proteins.[26-28] Solutions of proteins or peptides (0.01–0.1 mg/ml) in 100-μl volumes are placed in each well. Coating with Fn is performed at room temperature for 2–3 hr, whereas coating with Cn is performed at 4° overnight.[26,29] The plates can also be coated with fragments of proteins and the Arg-Gly-Asp (RGD) peptide (Sigma, St. Louis, MO). The coating efficiency is analyzed by coating with ^{125}I-labeled protein,[26] and the intactness of the protein layer is assessed by incubation with antiprotein antibodies.[28] The procedure is given in Table II. As an alternative to fetuin, 0.5% hen ovalbumin (Sigma) can be used as the negative control, immobilized in microtiter plates as described above.

Quantitation of microbial binding immobilized ECM proteins can be done by different methods. (i) [^{3}H]thymidine labeling of bacteria is frequently employed. Most microbes incorporate thymidine during growth. Alternatively, other tritiated metabolic compounds, such as [^{35}S]methionine can be used.[8] Quantitation is made in a liquid scintillation counter. Soft detachable microtiter plates are easily cut, and wells are transferred to separate vials. (ii) Microbes contain ATP, and sensitive methods have been developed to measure ATP by bioluminescence (BioOrbit Oy, Turku,

[25] F. Ascencio, O. D. Liang, K. H. Schmidt, L.-Å. Fransson, and T. Wadström, *in* "Lectins" (E. van Driessche, H. Franz, S. Beeckmans, U. Pfüller, A. Kallikorm, and T. C. Bög-Hansen, eds.), p. 235. Textop, Hellerup, Denmark, 1993.

[26] B. Westerlund, P. Kuusela, J. Risteli, T. Vartio, H. Rauvala, R. Virkola, and T. K. Korhonen, *Mol. Microbiol.* **3,** 329 (1989).

[27] W. Shen and Å. Ljungh, *Curr. Microbiol.* **27,** 311 (1993).

[28] M. Haapasalo, U. Singh, B. C. McBride, and V.-J. Uitto, *Infect. Immun.* **60,** 2058 (1992).

[29] I. Maxe, C. Rydén, T. Wadström, and K. Rubin, *Infect. Immun.* **54,** 695 (1986).

TABLE II
BINDING OF IMMOBILIZED COLLAGEN TYPE IV BY *Escherichia coli*[a]

1. Microtiter plates (96 wells, Costar, Cambridge, MA) are coated with 10 μg/ml Cn type IV in 100-μl volumes for 18 hr at 4°. Fetuin (10 μg/ml) is used as negative control.
2. Nonspecific binding sites are blocked by incubation with PBS–BSA [10 m*M* sodium phosphate, 0.14 *M* NaCl, 1% (w/v) bovine serum albumin, pH 7.2] for 1 hr at 20° and then washed three times in PBS–BSA.
3. *Escherichia coli* is grown on colonization factor antigen (CFA) agar with 1 μCi/ml of methyl-[1′,2′-^{3}H]thymidine for 18 hr at 32°.
4. Washed *E. coli* cells (7×10^7) are added to each well in triplicate. The plates are incubated at 20° for 5 hr with slow agitation. The disintegrations per minute (dpm) of the inoculated suspension is measured, and a serial dilution is made to determine dpm per colony-forming unit (cfu).
5. The plates are washed three times with PBS containing 0.1% Tween 20.
6. Bound radioactivity (dpm/min) is measured in a liquid scintillation counter. Background radioactivity is subtracted, and results (mean values dpm/min) are multiplied with the coefficient obtained for the inoculated suspension, giving the number of cfu bound.

[a] From Shen and Ljungh.[27]

Finland). In brief, microbes are washed in Tris–acetate (0.1 *M* with 2 m*M* EDTA, pH 7.75, TAE), and ATP is extracted in 2.5% trichloroacetic acid (TCA). After shaking, 50 μl of the extraction fluid is transferred to 200 μl ATP Monitoring Reagent (BioOrbit) with 750 μl TAE. The amount of ATP is recorded in a luminometer, and the amount of ATP in each sample is calculated by a formula.[30] The number of microbial cells in the sample is calculated from a species-specific standard curve.[30] (iii) Measurement of absorbance at 540 nm may be used.[31] (iv) Labeling of bacteria with fluorescein isothiocyanate allows quantitation of bound bacteria photometrically.[15] (v) An enzyme-linked immunosorbent assay (ELISA) uses antibodies to whole-cell microbes.[28] (vi) Finally, quantitation of bound parasites is usually done by counting in light microscopy after staining with Wright–Giemsa stain.[1]

Immobilization on Particles

Immobilization of proteins on particles such as latex beads (1–10 μm) by passive adsorption is a well-established method. Proteins immobilized on beads are usually stable for weeks at 4°. The method has been used to determine binding of immobilized Fn, fragments of Fn, Vn, Cn, Ln, and, more recently, heparin by staphylococci in a semiquantitative way.[9,17,32]

[30] M. Paulsson, M. Kober, C. Freij-Larsson, M. Stollenwerk, B. Wesslén, and Å. Ljungh, *Biomaterials* **14,** 845 (1993).
[31] T. L. Ratliff, R. McCarthy, W. B. Telle, and E. J. Brown, *Infect. Immun.* **61,** 1889 (1993).
[32] M. Paulsson, I. Gouda, O. Larm, and Å. Ljungh, *J. Biomed. Mater. Res.* **28,** 311 (1994).

Bacteria are washed twice and suspended in 20 m*M* potassium buffer, pH 6.8, at a density of 10^{10} cells/ml. Before testing, the cells are assayed for autoagglutination by mixing equal volumes of bacterial suspension with 20 m*M* potassium buffer on a glass slide. The slides are tilted and observed for agglutination during 1 min.

The development of carboxyl-, amino-, or amido-modified latex beads (0.8 μm diameter, Seradyn, Particle Technology Division, Indianapolis, IN) has permitted covalent coupling of proteins in a defined way. The choice of beads determines which domain of the protein to be bound and hence determines domains exposed to bind microbial agents.[33] For coupling of carboxyl-modified latex where amine groups of the glycoprotein are coupled to carboxylate groups, the particles are washed twice in 0.1 *M* phosphate buffer (pH 8.1) and incubated for 18 hr at 4° in phosphate buffer with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2 mg/ml). After centrifugation, the particles are suspended in phosphate buffer with protein or GAG (1 mg/ml) and incubated for another 18 hr at 4°. The suspensions are centrifuged (12,000 *g*, 4°, 10 min), and the pelleted coated particles (about 50 μg/ml protein) are washed four times in 100 m*M* Tris-HCl buffer with 50 m*M* $CaCl_2$ (pH 7.0) and suspended in the same buffer at a final density of 1% total solids. The coated particles can be stored at 4° for at least 3 months. Although BSA coupled in the same way can be used as control particles, we have found horse myoglobin (Sigma) coupled to carboxyl-modified particles to be the best negative control.[34]

Affinity Blotting

Extracellular matrix proteins can be electrophoretically transferred to nitrocellulose membranes. After blocking of nonspecific binding sites with 1% BSA in PBS (2 hr, 20°), the filters are incubated with unlabeled or ^{125}I-labeled microbes. Binding is visualized after incubation with antimicrobial antibodies and enzyme-labeled secondary antibodies, or by autoradiography. Using this method, Schulze-Koops *et al.*[4,5] could show that Yad A-positive *Y. enterocolitica* strains (3×10^9 bacteria/ml in PBS) specifically bound Fn and Cn types I–V and XI, but not Cn type VI.

Artificial Bacteria

Staphylococcus aureus-purified Cn receptor protein or recombinant *S. aureus* Fn-binding protein–protein A ZZ–fusion protein[2] (10^3 ng protein)

[33] J. Lelwala-Guruge, F. Ascencio, A. S. Kreger, Å. Ljungh, and T. Wadström, *Zentralbl. Bakteriol.* **280,** 93 (1993).

[34] J. Lelwala-Guruge, F. Ascencio, Å. Ljungh, and T. Wadström, *APMIS* **101,** 695 (1993).

are covalently coupled to polystyrene particles (1.2 μm diameter, 10^8 beads, Polysciences, Warrington, PA) using the carbodiimide method according to the manufacturer's recommendations, giving 6.8×10^4 molecules Cn receptor per bead.[16] Uncoupled reactive sites on the beads are saturated by incubation with 1% BSA. These "artificial bacteria" are further labeled with ^{125}I and used to demonstrate that the Cn receptor, but not the Fn binding protein, mediates attachment to cartilage.[16] This is an elegant method which can be used to study other purified ECM-binding surface structures as well.

Binding of Extracellular Matrix Proteins Immobilized in Tissue *in Vitro*

Tissue culture cells can be used to study microbial interaction with immobilized ECM proteins.[35-37] The advantage is that the configuration of the proteins is more *in vivo* like. Microtiter wells are used without prior coating with Fn or other adhesive glycoproteins to ensure that all exposed ECM proteins emanate from the tissue culture cells. The protocol for assaying binding of *Streptococcus defectivus* to baby hamster kidney (BHK) cells is outlined in Table III.[35] After the ammonium hydroxide treatment, the cells can be stored at −20°.

Characterization of Binding of Extracellular Matrix Proteins

General Characterization

To study if the binding of an ECM protein is mediated by a microbial cell surface protein, washed suspensions of microbes are heated at 80° or 100° for 30 min and rapidly cooled, then incubated with proteolytic enzymes with broad and narrow specificity (e.g., 10 μg/ml trypsin, 37°, 60 min, pH 7.0).[27] The reaction is stopped by adding 1 m*M* phenylmethylsulfonyl fluoride (PMSF). Thereafter, binding of soluble or immobilized ECM protein is assayed. Likewise, treatment of proteins as well as of microbes with periodate (100 m*M* sodium periodate in 0.1 *M* sodium acetate, pH 5.5, at 4°) and endoglucosidase H (2.5 mU/ml in the same buffer, at 20°) for 16 hr before the standard binding assay is performed is a useful screening method to determine possible protein–carbohydrate involvement in binding to ECM components. Using this approach Carneiro *et al.*[38] showed

[35] R. Creech Tart and I. van de Rijn, *Infect. Immun.* **59,** 857 (1991).
[36] R. Calderone and E. Wadsworth, *J. Med. Microbiol.* **18,** 197 (1993).
[37] A. Szcepanski, M. B. Furie, J. L. Benach, B. P. Lane, and H. B. Fleit, *J. Clin. Invest.* **85,** 1637 (1990).
[38] C. R. W. Carneiro, E. Postol, C. Boilesen, and R. R. Brentani, *Braz. J. Med. Biol. Res.* **26,** 689 (1993).

TABLE III
BINDING OF *Streptococcus defectivus* TO EXTRACELLULAR MATRIX PROTEINS OF BABY HAMSTER KIDNEY CELLS[a]

1. Uncoated 96-well microtiter plates are seeded with BHK cells (5×10^4 cells/well) in Dulbecco's modified Eagle's medium (DMEM) with 10% (w/v) fetal bovine serum, penicillin, and streptomycin.
2. When confluent monolayers are obtained, the cells are washed twice with Earle's balanced salt solution with calcium and magnesium (EBSS) and incubated with 0.5% (v/v) Triton X-100 for 30 min at 37° to expose ECM proteins.
3. The cells are treated with 25 m*M* ammonium hydroxide for 10 min at 37° and washed three times with EBSS to remove nuclei and cytoskeletal structures.
4. The removal of cells is confirmed by light microscopy, and removal of cytoskeleton is verified by incubation with antiactin antibodies and fluorescein isothiocyanate–phallocidin staining. The exposure of Ln, Fn, and Cn type IV is confirmed by enzyme-linked immunosorbent assay (ELISA) with antibodies to Ln, Fn, and Cn type IV.
5. Late exponential phase cells of *S. defectivus* are washed twice in 20 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.4 (PBS), and suspended in PBS to an optical density of 0.5–0.6 at 530 nm. To each well is added 50 μl cell suspension and an equal volume of 3% casein–0.05% Tween 20 to block nonspecific binding, in quadruplicate. After 30 min of incubation at 37° with gentle agitation, the cells are washed three to five times with PBS–0.05% Tween 20 to remove nonadherent bacteria.
6. Adherent bacteria are fixed by incubation at 60° for 15 min.
7. The cells are incubated with polyclonal rabbit antiserum to *S. defectivus* diluted in 3% (w/v) casein (1:250) for 1 hr at 37°. Unbound antibody is removed by washing the cells with PBS–0.05% Tween 20. The number of bound bacteria is quantified after incubation with an enzyme-labeled anti-rabbit antibody.
8. Bacterial binding to uncoated wells is subtracted from the mean of ECM binding.
9. One ECM-binding bacterial strain and one nonbinding strain are included as controls. Rabbit antiserum raised against an unrelated bacterial species is used to ensure specificity of the final assay.

[a] From Creech Tart and van de Rijn.[35]

that trypsin treatment of *S. aureus* cells, but not oxidation with periodate, abolished subsequent binding of Ln, and that treatment of immobilized Ln with *N*-glycosidase F (Genzyme, Boston, MA; 5 mU/μg protein in 200 m*M* sodium phosphate buffer, pH 8.6) for 18 hr at 37° abolished binding, indicating that lectin-like interactions form part of the binding.

Competitive binding, saturability and reversibility of binding, and quantitation of binding sites according to Scatchard can be analyzed using both soluble radiolabeled ECM proteins and proteins immobilized in microtiter plates.[8,10,18,24,27,39,40] The specificity of binding can be analyzed after preincubation of the microbial suspension with ECM proteins (100 μg/ml final

[39] E. J. Wiersma, G. Fröman, S. Johansson, and T. Wadström, *FEMS Microbiol. Lett.* **44,** 365 (1987).

[40] F. Ascencio, O. D. Liang, L.-Å. Fransson, and T. Wadström, *J. Med. Microbiol.* **38,** 240 (1993).

concentration, 1 hr, 4°) before the standard binding assay is continued.[19] Experiments in which bacterial cells are incubated with ECM proteins before the standard binding assay is performed have to be interpreted with caution owing to protein–protein interactions.[12] To circumvent this, isolated microbial binding structures and cell surface extracts can be electrophoretically transferred to nitrocellulose membranes. After blocking nonspecific protein binding sites with 1% BSA (in PBS, 1 hr, 20°), the membranes are incubated with ^{125}I-labeled or enzyme-labeled ECM molecules, washed, and developed according to standard procedures; fragments of these molecules can be used to study the specificity of binding.[16,28]

Studies with Fragments of Extracellular Matrix Proteins

To localize the binding domain on the ECM molecule, fragments of ECM proteins can be used in inhibition assays with soluble ECM proteins, in affinity blots, or immobilized in microtiter trays.[6,17,28,41] The N terminus of Fn (29 kDa) is obtained by incubation of Fn with plasmin-Sepharose (37° for 20 hr).[42] Proteolytic cleavage of Fn (0.1 μg in PBS with 0.1% BSA) with trypsin [12,000 units/mg, 1 μg, 1 hr, 20°, followed by addition of 2.5 μl freshly prepared 0.1 *M* PMSF in 95% (v/v) ethanol] yields degradation products which can be used for binding studies. Fröman and co-workers.[43] used trypsin-digested ^{125}I-labeled Fn and incubated the labeled degradation products with an equal amount of *E. coli* cells (4×10^8 cells, 1 hr, 20°). After centrifugation the pellets were washed once in PBS with 0.1% BSA. Proteins bound to bacterial cells were released by boiling (1 min) in electrophoresis sampling buffer, pH 8.8, and centrifuged. Electrophoresis was conducted in a 5–15% polyacrylamide gradient gel. By autoradiography, *E. coli* cells were shown to bind two fragments, one 150-kDa fragment and the N-terminal fragment.[43]

Aumailley *et al.*[44] have presented protocols for proteolytic digestion of Ln using elastase (fragments E1–4, E8), pepsin (P1 fragment), and chymotrypsin A (C1 fragment). Several ECM proteins have been subjected to cyanobromide (CNBr) cleavage.[6,19] The CNBr cleavage products can be further purified by FPLC (fast protein liquid chromatography) on a Superose 12 column in 0.1 *M* ammonium acetate (pH 5). The purity of isolated

[41] E. Jakab, M. Paulsson, F. Ascencio, and Å. Ljungh, *APMIS* **101,** 187 (1993).
[42] K. Rubin, S. Johansson, I. Pettersson, K. Ocklind, B. Öbrink, and M. Höök, *Biochem. Biophys. Res. Commun.* **91,** 86 (1979).
[43] J. I. Flock, G. Fröman, K. Jönsson, B. Guss, C. Signäs, B. Nilsson, G. Raucci, M. Höök, T. Wadström, and M. Lindberg, *EMBO J.* **6,** 2351 (1987).
[44] M. Aumailley, V. Nurcombe, D. Edgar, M. Paulsson, and R. Timpl, *J. Biol. Chem.* **262,** 11532 (1987).

fragments is analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with buffer systems according to Laemmli.[6] Synthetic peptides having defined sequences of ECM molecules are commercially available, for example the Cn peptides poly(L-lysine) (10 kDa) and poly(L-proline) (8 and 36 kDa).[7]

Eukaryotic Cell-Binding Domain

To analyze microbial binding to the cell-binding domain (the RGD sequence), microbes are preincubated with RGD peptides and related peptides (1 mg/ml final concentration, 1 hr, 21°; RGD, RGDS, GRGDS, RGES, Bachem, Basel, Switzerland) before the standard binding assay is performed.[41]

An alternative approach is to preincubate microbes with antibodies to different integrin receptors (1 hr, 20°) before incubation with immobilized ECM proteins.[45] Rabbit polyclonal antibodies against the human Fn receptor, $\alpha_5\beta_1$, and human Vn receptor, $\alpha_v\beta_3$, are commercially available (Telios Pharmaceutical, San Diego, CA). The integrin–receptor binding requires the presence of Ca^{2+}. As a control, microbial cells are preincubated with 20 m*M* EDTA (1 hr, 21°) before standard binding assay is performed.[45]

Heparin-Binding Domain

The Fn, Cn, Vn, Ln, and Trp molecules have a heparin-binding domain (see above). To determine if microbes bind to the domain, microbial suspensions are incubated with (i) heparin (1–5 IU, KABI, Stockholm, Sweden) for 1 hr at 21°[41] or with (ii) heparitinase (heparan sulfate lyase, 0.05 U/ml in PBS with 10^{-5} *M* calcium) for 1 hr at 37°[46,47] before the standard binding assay. The microbes can further be preincubated with heparan sulfate, hyaluronic acid, or other GAGs before analysis of binding.[40,48]

Importance

Expression of binding of ECM proteins has been proposed as a mechanism of tissue adhesion for a number of microbial agents. For some, like *S. aureus,* expression of Cn binding was shown to home *S. aureus* to cartilage tissue,[16] and Vn binding of *P. carinii* enhanced adhesion to lung cells.[46]

[45] S. P. Rao, K. R. Gehlsen, and A. Catanzaro, *Infect. Immun.* **60,** 3652 (1992).

[46] A. H. Limper, J. E. Standing, O. A. Hoffman, M. Castro, and L. W. Neese, *Infect. Immun.* **61,** 4302 (1993).

[47] J. C.-R. Chen and R. S. Stephens, *Mol. Microbiol.* **11,** 501 (1994).

[48] O. D. Liang, F. Ascencio, L.-Å. Fransson, and T. Wadström, *Infect. Immun.* **60,** 899 (1992).

Expression of Fn binding was shown to increase the virulence of *S. aureus* and *S. sanguis* in animal endocarditis models.[3,49] The biological importance of binding of ECM proteins was most conclusively shown for Fn binding of *M. tuberculosis*,[4] which has led to the clinical use of instillation of *M. tuberculosis* BCG cells in the bladder to block Fn binding and the subsequent immune response and tumor activity of human superficial bladder cancer. It has been proposed that microbial binding of ECM proteins may induce structural and functional alterations of the proteins which in turn may activate other cellular mechanisms and enhance tissue invasion of bacterial as well as parasite pathogens.[4,11,50]

Acknowledgments

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[49] J. M. Kuypers and R. A. Proctor, *Infect. Immun.* **57,** 2306 (1989).
[50] T. K. Korhonen, R. Virkola, K. Lähteenmäki, Y. Björkman, M. Kukkonen, T. Raunio, A.-M. Tarkkanen, and B. Westerlund, *FEMS Microbiol. Immunol.* **100,** 3072 (1992).

[40] Adhesion of Urogenital Organisms to Polymers and Prosthetic Devices

By GREGOR REID

Introduction

The medical device market in North America has been growing at a rate of around 9% per year, indicating the enormous number of biomaterials being applied to the human host. For example, over 50×10^9 diapers and 16×10^6 urinary catheters were sold in 1992.[1] An important problem with inserted open devices (e.g., catheters, pads, lines) and closed implanted prostheses (hips, heart valves, joints) is the risk of microbial colonization providing a nidus for infection.[1,2] Once adherent, the organisms can form dense biofilms which appear capable of resisting host and antimicrobial attack.[3]

[1] G. Reid, *Colloids Surf. B: Biointerfaces* **2,** 377–385 (1994).
[2] A. G. Gristina, *Science* **237,** 1588 (1987).
[3] J. W. Costerton, K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie, *Annu. Rev. Microbiol.* **41,** 435 (1987).

Expression of Fn binding was shown to increase the virulence of *S. aureus* and *S. sanguis* in animal endocarditis models.[3,49] The biological importance of binding of ECM proteins was most conclusively shown for Fn binding of *M. tuberculosis*,[4] which has led to the clinical use of instillation of *M. tuberculosis* BCG cells in the bladder to block Fn binding and the subsequent immune response and tumor activity of human superficial bladder cancer. It has been proposed that microbial binding of ECM proteins may induce structural and functional alterations of the proteins which in turn may activate other cellular mechanisms and enhance tissue invasion of bacterial as well as parasite pathogens.[4,11,50]

Acknowledgments

The authors' studies were supported by grants from the Swedish Medical Research Council, the Technical Science Research Council of Sweden, and the Board for Technical Development (NUTEK).

[49] J. M. Kuypers and R. A. Proctor, *Infect. Immun.* **57,** 2306 (1989).
[50] T. K. Korhonen, R. Virkola, K. Lähteenmäki, Y. Björkman, M. Kukkonen, T. Raunio, A.-M. Tarkkanen, and B. Westerlund, *FEMS Microbiol. Immunol.* **100,** 3072 (1992).

[40] Adhesion of Urogenital Organisms to Polymers and Prosthetic Devices

By GREGOR REID

Introduction

The medical device market in North America has been growing at a rate of around 9% per year, indicating the enormous number of biomaterials being applied to the human host. For example, over 50×10^9 diapers and 16×10^6 urinary catheters were sold in 1992.[1] An important problem with inserted open devices (e.g., catheters, pads, lines) and closed implanted prostheses (hips, heart valves, joints) is the risk of microbial colonization providing a nidus for infection.[1,2] Once adherent, the organisms can form dense biofilms which appear capable of resisting host and antimicrobial attack.[3]

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The ability to prevent microbial adhesion and kill attached organisms continues to be the subject of substantial investigation. The procedures involve examining the mechanisms of initial adhesion as well as the development and nature of biofilms. The intent of this chapter is to provide researchers with an awareness of the parameters which must be considered in performing microbial experiments on biomaterials, and also to describe procedures to undertake such studies.

Substratum

The selection of the substratum is important and not always obvious. Should a commercial device be used over a pure polymer? Should the surface tension be measured by water contact angle and the surface examined by scanning electron microscopy (SEM) and characterized by energy-dispersive X-ray analysis, static secondary ion mass spectrometry, X-ray photoelectron spectroscopy, or other techniques? How will a substratum be altered by exposure to antibiotics, host proteins, and other substances such as hydrogel and ozone coating? It is clear that the surfaces of substrata do change when exposed to host and other factors, and some of the changes can be demonstrated by sophisticated experimental techniques.[4,5] The decision as to which material to use, and to what extent its content and surface properties should be analyzed, will in part depend on the objectives of the adhesion experiment.

Another point in the selection of a substratum is whether to use a section of a device or polymer. It should be appreciated, for example, that by sectioning a hydrogel-coated catheter one exposes uncoated sections at the cut edge, and in theory this could influence the adhesion data obtained. In addition, how many sides of the substratum will be exposed to bacteria: is the material fixed or free-floating in suspension?

Microorganism

The adhesion of organisms to metal, polymer, and other surfaces can be applied to industrial, environmental, and medical areas. However, this chapter deals with adhesion related to infectious diseases, and as most cases relate to bacterial adhesion, only these organisms are discussed. As fungal infections can involve adhesion and as the role of viruses in adhesion to biomaterials is relatively unknown, readers should be aware of other possible scenarios relevant to their area of interest.

[4] G. Reid and C. Tieszer, *Colloids Surf. B: Biointerfaces* **1,** 9 (1992).

[5] G. Reid, H. J.Busscher, S. Sharma, M. Mittelman, and S. McIntyre, *Surf. Sci. Reports* **7,** 251 (1995).

There are a few key decisions to be made regarding the selection and preparation of bacteria for study. First, should the organism be well defined, genetically and morphologically, or should it be freshly isolated from or passaged in the host? Second, is it important to use exponential, stationary, or lag phase cultures, special media (e.g., agar, broth, human urine, artificial urine), or suspension of the bacteria in buffer, media, or other fluids? It is clear that growth conditions and the presence of certain genetic characteristics will influence surface hydrophobicity, zeta (ζ) potentials, adhesin expression, and other factors which affect adhesiveness. The final point to consider is the duration of the experiment, which will be selected depending on whether the objective is to examine the initial adhesion, aggregation, biofilm formation, or detachment caused by sloughing or shear forces.

Suspending Fluid

The temptation in experimentation is to use a suspending fluid which can be defined and which has minimal impact on bacterial growth; thus, phosphate buffers are often used. On the other hand, the argument has been made that this does not simulate the *in vivo* condition, and thus urine or artificial urine should be employed. Each of these fluids has limitations, including the latter which does not contain host urinary proteins, for example, Tamm–Horsfall protein, known to affect adhesion of uropathogens.[6] Thereafter, selection of an appropriate pH (usually pH 7.0, although urine tends to range from pH 5.5 to 6.0), electrolyte content, experimental temperature, and shaking speed and duration is needed.

Methods to Determine Extent of Adhesion

There are many methods which can be utilized to determine the extent of bacterial adhesion to biomaterials. It is outside the scope of this chapter to describe each of these in detail; however, brief mention of a few techniques is warranted (some of these are discussed elsewhere in this volume).

Scanning electron microscopy (SEM) can be useful in visualizing adherent organisms, but relatively small sections tend to be examined and the preparation of material may skew the results. For example, in the case of a hydrophilic coated material, the surface may come off along with adherent organisms on fixation and washing. The use of fluorescent antibody, acridine orange, crystal violet, and gram stain can allow enumeration if the material is flat and smooth. Using radiolabeled bacteria is also feasible, but that approach is not often used in the study of adhesion to biomaterials. We have utilized several of these methods, along with image analysis to quantitate the

[6] L. Hawthorn and G. Reid, *J. Biomed. Mater. Res.* **24,** 1325 (1990).

data. However, the techniques do not indicate if the adherent bacteria are viable, and this is often important to know in clinically relevant experiments such as in documentation of biofilms on ureteral stents of patients treated with antibiotics.[7] Thus, a sonication technique has been developed and employed.

Adhesion Assay

The experimental technique described has consistently provided reproducible and usable data.

Bacteria. A *Lactobacillus* isolate, *L. casei* 36 from the human vagina, is found by water contact angle to be hydrophilic (19°). For experimentation, it is taken from storage at −70°, grown overnight in MRS broth (Difco, Detroit MI), checked by colony morphology and gram stain for purity, then cultured for 18 hr in MRS broth at 37° in 5% (v/v) CO_2. The organisms are centrifuged at 1800 *g* at room temperature for 10 min, the supernatant is removed, sodium phosphate-buffered saline (PBS, pH 7.1) is added, and the cells are again washed three times and then suspended in PBS to a density of 1×10^8 cells/ml.

Substratum. A 1-cm-long segment of silicone-impregnated latex Foley catheter (Rusch, Toronto, Canada) is cut 3 cm above the tip of the device. If using a hydrogel-coated device, cut a section which is coated: these products are often made by dipping the end into the gel, and thus sections above a certain point (around 30 cm) are not coated. If using a pure flat polymer, the dimensions will not be the same as the commercial cylindrical catheter.

Suspending Fluid. It is preferable to use PBS or human urine centrifuged at room temperature at 500 *g* for 10 min, then filter-sterilized through 0.45- and 0.22-μm filters (Falcon, Becton Dickinson Co., Oxnard, CA). Urine often crystallizes if stored at 4°; thus, if storage is necessary, for example, to pool samples, the urine should be brought to 37° prior to use. Also, if a urinary suspending fluid is to be used, it should be added to resuspend the bacteria after the final wash of the organisms.

Assay. A bacterial suspension of 1 ml is added to a plastic test tube (glass is not used here as *L. casei* 36 is a hydrophilic organism and would adhere to the glass), along with the segment of catheter. If the organisms are known to aggregate, they are sonicated from 1 min prior to addition to the tubes. The test tube is placed at 37° in a gyratory water bath (Precision, US) to ensure exposure of all parts of the device to the organisms. If possible, the bath should be an incubator with 5% CO_2. The shaking speed will depend on the instrument but should be gentle (around 50 rpm) and not vibrant.

[7] G. Reid, J. D. Denstedt, Y. S. Kang, D. Lam, and C. Nause, *J. Urol.* **148,** 1592 (1992).

After a time (0.5, 1, 12, 24, 48 hr, etc.), the test tube is taken from the shaker. To remove the unattached bacteria and enumerate the adherent organisms, several factors must be taken into consideration. It has been shown that bacteria are present at the liquid–air interface, and thus if the device is simply lifted out, the organisms could, in theory, be retained (as distinct from being firmly adherent) onto the surface. Alternatively, because of shear forces at the liquid–air interface, adherent organisms could be sheared off[8] on the way out. The ideal way to resolve this problem is to use the flow cell chamber utilized by H. J. Busscher and described in [37] in this volume. Another method for washing might be to have an exit tap at the bottom of the tube, which could drain the suspending fluid as fresh fluid is added at the top.

For routine purposes, the catheter segment is removed from the tube with tweezers and PBS gently passed over the surface for 1 min. The segment is then immersed in 5 ml PBS in a sonicator bath. The sample is sonicated for about 5 min (this varies for each organism tested) to remove adherent organisms, which are dilution plated onto MRS agar, cultured for 24–48 hr, and enumerated. The removal of bacteria is confirmed by scanning electron microscopy and is checked periodically by gram staining the segment (cut open to view internal and external surfaces) and examining it at a magnification of $\times 1000$ under oil immersion.

The controls consist of catheter segments which are untreated and suspended in PBS for the duration of the experiment. It is also customary to check the number of viable organisms in the suspending fluid (with no catheter added) at the beginning and end of the experiments. Each run is carried out in duplicate or triplicate, and each dilution plate is duplicated.

Expected Results

In this example, the lactobacilli adhere well to the material within the first 1 hr, with perhaps 4×10^4 organisms attached per centimeter of catheter. However, if PBS is used as the suspending fluid and the bacteria are incubated for 48 hr to examine biofilm formation, very few viable lactobacilli will be recovered, owing to cell death rather than lack of adhesion. If the objective is to study biofilm formation for lactobacilli, the suspending fluid must contain nutrients or a buffer which provides longer term stability for the cells.

If the organisms tested for adhesion are uropathogens such as *Proteus mirabilis, Escherichia coli, Enterococcus faecalis,* or *Staphylococcus epidermidis,* it is likely that more adhesion will occur (up to 10^7 bacteria per 1-cm

[8] W. G. Pitt, M. O. McBride, A. J. Barton, and R. D. Sagers, *Biomaterials* **14,** 605 (1993).

section). It will be necessary to grow the organisms, after 3 min of sonication, on brain heart infusion–yeast extract agar, nutrient broth, or MacConkey agar (Difco). In the case of experiments over a longer duration of 24–48 hr, no evidence of cell death has been found, and biofilm formation is evident.

In practice, the results of adhesion experiments with lactobacilli and other members of the urethral population are very reproducible, with standard deviations of less than 20%.[9]

Acknowledgments

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[9] G. Reid and C. Tieszer, *Cells Mater.* **3,** 171 (1993).

[41] Bacterial Adhesion Measured by Growth of Adherent Organisms

By EVGENI V. SOKURENKO, VANESSA A. MCMACKIN, and DAVID L. HASTY

Introduction

One of the ways to study the mechanism of microbial adhesion to cells or tissues of the host is to test the adhesion of bacteria to purified host components directly. Many host components have been tested including a variety of proteins and glycoproteins, polysaccharides, lipids, and glycolipids originating from host fluids, cell membranes, or extracellular matrices.[1-3] To test microbial adhesion, the purified molecules are usually immobilized on one of a variety of surfaces, followed by incubation with the suspension of bacteria, washing away the unbound organisms, and, finally, determining the relative or absolute number of bacteria bound. The surfaces most often used to immobilize host components are glass or plastic slides or tubes, 96-well, microtiter-type assay plates, and various beads, such as spheroidal

[1] R. J. Gibbons and D. I. Hay, *Infect. Immun.* **56,** 439 (1988).
[2] C. S. Giampapa, S. N. Abraham, T. M. Chiang, and E. H. Beachey, *J. Biol. Chem.* **263,** 5362 (1988).
[3] P. Kuusela, T. Vartio, M. Vuento, and E. B. Myhre, *Infect. Immun.* **50,** 77 (1985).

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[3] P. Kuusela, T. Vartio, M. Vuento, and E. B. Myhre, *Infect. Immun.* **50,** 77 (1985).

hydroxylapatite. The use of different systems has enabled a great deal of progress in the analysis of the molecular mechanisms of microbial adhesion.

The methods most often used to enumerate bound bacteria are determination of absolute numbers of bacteria by direct counting when use of a microscope is feasible or estimation of relative numbers of bacteria using enzyme-linked immunosorbent assay (ELISA) types when specific antibodies are available.[4-6] Bacteria can also be labeled metabolically using radioisotopes and absolute numbers of bound bacteria determined by liquid scintillation or gamma spectrometers.[7] Another approach has been to label bacterial surfaces with biotin, followed by use of avidin–peroxidase to obtain an estimate of relative levels of bacterial adhesion.[8] However, each of these procedures has certain drawbacks. Microscopic counting is tedious and is not amenable to large numbers of samples; specific antibodies are not always available for ELISAs; use of radioactive labels is limited by safety issues; and labeling of the bacterial surfaces with biotin or other compounds could have deleterious effects that are difficult to account for.

A method has now been developed for measuring adherent bacteria based on the subsequent growth of the bacteria in nutrient broth. The assay is simple and relatively fast. Most importantly, however, it does not require special reagents, such as specific antibodies, nor does it require labeling the bacteria with molecules that could have undetected effects on surface components. The assay can be performed most efficiently using microtiter plates and a microplate reader, but it could be easily adapted to use with tubes and a simple spectrophotometer.

Methods

Assay Method

Principle. The principle is relatively simple: the optical density (OD) of nutrient broth increases during the growth of bacteria, and the OD, at least in the exponential phase of growth, will be directly related to the initial number of bacteria bound (the entity to be determined), time, conditions of incubation, and viability and growth rate of particular strains. Time and conditions of incubation can easily be made standard, and the growth rate

[4] E. V. Sokurenko, H. S. Courtney, S. N. Abraham, P. Klemm, and D. L. Hasty, *Infect. Immun.* **60,** 4709 (1992).

[5] J. H. Lowrance, D. L. Hasty, and W. A. Simpson, *Infect. Immun.* **56,** 2279 (1988).

[6] B. Westerlund, P. Kuusela, T. Vartio, I. van Die, and T. K. Korhonen, *FEBS Lett.* **243,** 199 (1989).

[7] S. K. Tylewska and R. J. Gibbons, *Curr. Microbiol.* **16,** 129 (1987).

[8] I. Ofek, H. S. Courtney, D. M. Schifferli, and E. H. Beachey, *J. Clin. Microbiol.* **24,** 512 (1986).

of strains can be determined experimentally. Therefore, the initial number of bound bacteria should be calculable, as described below.

Materials. It is assumed that the assay will be applicable to essentially any bacteria. In the examples presented here, wild-type and recombinant *Escherichia coli* strains, CI #4 and KB53, respectively, are used. Strain CI #4 was obtained from human urine and expresses type 1 fimbriae which are capable of binding to yeast mannan and to human plasma fibronectin. Strain KB53 is a recombinant strain expressing the adhesin subunit, FimH, obtained from CI #4. These strains have been described previously.[9]

Equipment. Necessary equipment include microtiter well strips (Nunc, Naperville, IN) an ELISA plate reader (Molecular Devices, Menlo Park, CA), and a horizontal shaker.

Reagents. Yeast mannan (YM), bovine serum albumin (BSA), α-methyl-mannoside, and α-methylglucoside, are obtained from Sigma (St. Louis, MO). Human plasma fibronectin (Fn) is purified from human plasma as described previously.[4,9] Brain–heart infusion broth (BHI) is purchased from Difco (Detroit, MI). The buffer is 10 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.0 (phosphate-buffered saline; PBS).

General Procedures. Microtiter plate wells are coated with 100 μl YM (10 μg/ml), Fn (20 μg/ml), or BSA (50 μg/ml) in 0.02 *M* $NaHCO_3$ for a minimum of 1 hr. Wells are washed twice with PBS and quenched with 170 μl of 0.1% (w/v) BSA in PBS for 30 min. The BSA-coated wells serve as controls. While the plate is being blocked by the 30-min incubation with BSA, the bacterial suspension should be prepared.

After overnight culture, bacteria are collected by centrifugation and washed twice with PBS, and the OD (540 nm) is adjusted to 1.0. The quenching solution of BSA is discarded, and the wells are filled with 50 μl of 0.2% (w/v) BSA solution with or without α-methylmannoside. Then 50 μl of the bacterial suspension is added and mixed with the BSA solution by gentle shaking of the plate. It is important to add buffer alone to at least five wells (no bacteria) for a background control. Wells are incubated with bacteria for an appropriate interval (30 min) without shaking, and the wells are then washed six to eight times with 150 μl PBS to remove unbound bacteria. After washing, 150 μl of BHI broth is added to every well, and the plate is covered with a lid or with Parafilm, placed on a horizontal shaker, and incubated at 150 rpm at 37° for 2 to 3 hr.

The broth in the wells where growth occurs will become turbid, but, because of the effects of shaking, the bacterial suspension will not be distributed evenly throughout the well. It is important before reading the

[9] E. V. Sokurenko, H. S. Courtney, D. E. Ohman, P. Klemm, and D. L. Hasty, *J. Bacteriol.* **176,** 748 (1994).

OD to make sure that the bacteria are uniformly suspended. This can usually be easily accomplished by tapping the plate at corners 90° to one another. Of course, be careful not to splash the broth out. The plate can then be placed into the microplate reader and the bacterial density recorded at 405 nm or other visible wavelength. The background level of BHI should be subtracted from all readings. The background reading is usually 0.25 to 0.35 and remains at this OD for at least 7 to 10 hr because any contamination that may occur is usually very light. This is true even though no special effort has been made to sterilize solutions, other than the BHI broth, or to use stringent sterile technique. At least for the organisms tested, this is well within the time frame needed to obtain growth in wells containing at least 25 adherent organisms.

The raw data for the binding of *E. coli* CI #4 to YM, Fn, and BSA are presented in Fig. 1A. For comparison, results obtained in a parallel experiment where an ELISA was used to detect bound bacteria are shown in Fig. 1B. It is only logical that the densities of the bacterial suspensions measured reflect the number of bacteria bound to the different host molecules after washing the wells with PBS. The fact that this is true and that, using standard curves, the absolute number of bacteria bound can be calculated is demonstrated below.

Calculation of Number of Bacteria Bound

Data presented in Fig. 1A are given in OD units and can be used directly to study relative levels of bacterial adhesion in the same way as can ELISAs. To convert OD units to absolute numbers of living bacteria bound, a

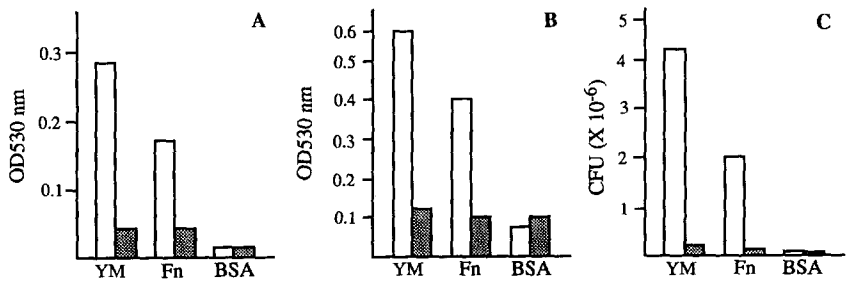

FIG. 1. Binding of *E. coli* CI #4 to YM-, Fn-, and BSA-coated wells in the absence (open columns) and presence (shaded columns) of 1% α-methylmannoside, detected by (A) turbidity of growth medium or (B) ELISA using anti-*E. coli* antiserum. (C) Conversion of optical units to absolute number of colony-forming units based on growth rate curve shown in Fig. 2.

"standard curve" of the growth yield for each strain, relating colony-forming units (cfu) to OD, should be constructed. The principle is to inoculate broth-filled wells with known numbers of bacteria (usually calculated by plating dilutions of various ODs) and incubating them in parallel with experimental wells. The standard curves thus created can be used for extrapolation of the OD values of the experimental wells to number of bacteria.

For preparation of the standard curve, the following procedure is recommended. The wells for the standard curve should be quenched with 170 μl of 0.1% (w/v) BSA–PBS, and optimally they should be in the same plate as the experimental wells. Immediately before the bacterial suspension is added to experimental wells, 0.2 ml of the same suspension is transferred into microcentrifuge tubes (also quenched with BSA to eliminate or at least reduce binding) with 0.2 ml of 0.2% (w/v) BSA–PBS. The tube is incubated under the same conditions as the plate (30 min, 37°, without shaking). While the wells of the plate are being washed with PBS, the bacterial suspension in the standard curve tube should be collected by centrifugation. The supernatant must be carefully removed as completely as possible and the pellet suspended to 1.0 ml in BHI. The wells for the standard curve are filled with 150 μl BHI, as for the other wells, and then 150 μl of the suspension from the standard curve tube should be serially diluted 7- or 8-fold in the BHI-filled wells. These and all other measures should be done in triplicate. Using the standard curve tube, dilutions should be made (e.g., $1:10^6$ and $1:10^7$) and plated in triplicate on BHI agar plates for determining colony-forming units per OD unit. If a particular strain is harvested at the same growth phase and the assay conditions are standard, the viability of the cells is substantially the same from experiment to experiment.

On completion of the microplate incubation, the ODs can be measured. When the colony-forming units are determined after overnight culture of the plates, the standard curve can be constructed by plotting OD against the number of colony-forming units inoculated. A variety of methods could be used to plot the curve, but semilogarithmic plots are convenient (Fig. 2). The number of bacteria bound to immobilized receptor molecules can be obtained by extrapolation of experimental OD to standard curve OD. The results of *E. coli* CI #4 binding to YM, Fn, and BSA shown in Fig. 1A are converted to colony-forming units in Fig. 1C, on the basis of the curve shown in Fig. 2.

The sensitivity of the assay is obviously dependent on the standard curve, and the useful portion of the standard curve will obviously change depending on the length of time the organisms are grown. From a practical standpoint, the organisms can be grown until growth of background contamination begins to overcome growth of the test organisms. It is usually

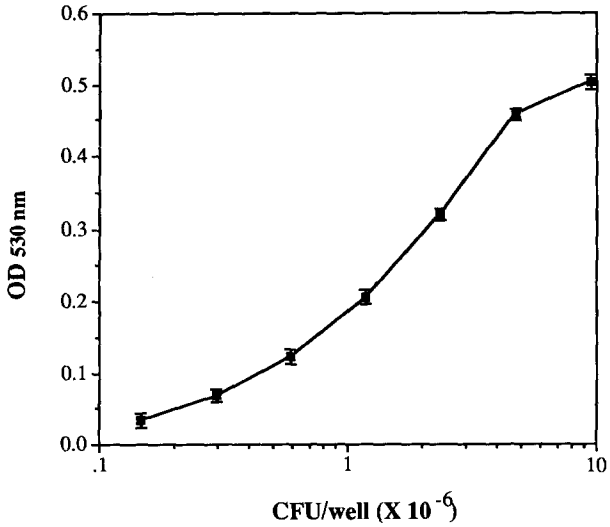

FIG. 2. Standard curve measuring growth yield of *E. coli* CI #4. The curve provides comparison of optical density and number of colony-forming units added to wells. Values given are means ± S.D. (n = 4).

unnecessary to extend the assay for more than about 7 hr. If the plates are read at intervals, it is obvious that the sensitive area of the standard curve varies, as one would expect (Fig. 3). The ultimate sensitivity of the assay has not been determined, but the data presented in Fig. 3 yield a sensitivity to distinguish between 25 and 50 cfu per well. Based on experience, the most reliable results are obtained in the OD range 0.02 to 0.4 above background.

Comparison of Growth Rates of Immobilized and Suspended Bacteria

The method described for determining the absolute number of bacteria bound is based on the assumption that any given number of bacteria will have the same growth yield whether bound or in suspension. To prove that the growth curve based on numbers derived from suspended *E. coli* can be applied to the determination of the number of bound bacteria, the following approach was used. *Escherichia coli* KB53 bound to YM-coated microtiter wells were eluted by 1% α-methylmannoside in 0.1% (w/v) BSA–PBS. One hundred microliters of the mannoside solution was added to wells containing adherent bacteria (washed wells); the wells were sealed with Parafilm and then rotated at 12 rpm for 30 minutes. As controls, additional wells were treated in the same way with α-methylglucoside, which does not inhibit type 1 fimbrial binding to YM and thus does not

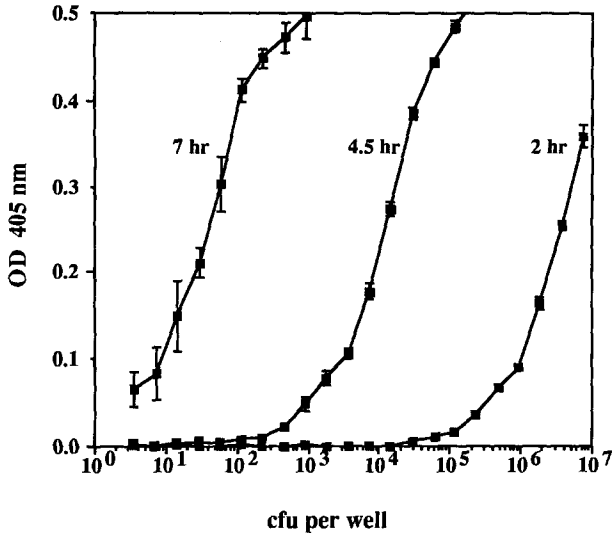

FIG. 3. Standard curve measuring growth yield of *E. coli* CI #4 after 2, 4.5, and 7 hr of growth. Background ODs were subtracted from the values reported and varied from 0.261 ± 0.001 at 1 hr to 0.255 ± 0.002 at 7 hr. Values given are means ± S.D. (n = 4).

elute KB53 cells. The recombinant strain was utilized in this case because use of antibiotics reduced any concerns about the presence of contaminants obtained in the elution procedure.

Eluted bacteria were enumerated by plating on BHI agar and counting colony-forming units, whereas noneluted bacteria were estimated from the growth curve as described above. The premise is that, although the fraction eluted will differ, the total number of bacteria should be identical when comparing mannoside- and glucoside-treated wells. The number of bacteria eluted can be assumed to be a reliable number, because it is determined by counting colony-forming units. The questionable number is the number of bacteria bound. If the total number of bacteria determined for mannoside- and glucoside-treated wells is the same, that is, if $Ym + Xm = Yg + Xg$ (Y, eluted; X, bound; m, mannoside wells; g, glucoside wells), then one should be able to assume that the number of bound bacteria has been determined correctly. According to the results obtained (Fig. 4), these assumptions appear to be true, at least for *E. coli*. Similar results were obtained in experiments of *E. coli* KB53 adhesion to Fn.

Using Growth Assay for Study of Adhesion of Other Species

The growth assay has been used to study adhesion of *Staphylococcus aureus*, several different streptococci, and enterobacteria. The method

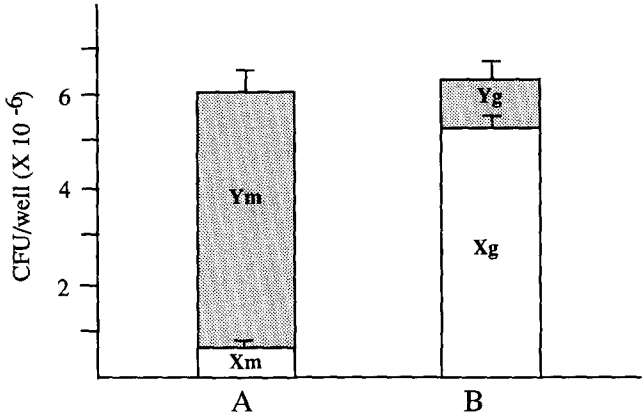

FIG. 4. Calculation of absolute numbers of colony-forming units of *E. coli* KB54 bound to immobilized YM, utilizing partial elution with 1% α-methylmannoside (A) and α-methylglucoside (B). *Ym*, Number of colony-forming units eluted with α-methylmannoside and calculated by plating; *Yg*, same for α-methylglucoside; *Xm*, number of colony-forming units remaining attached to YM after α-methylmannoside elution and calculated with growth assay curve; *Xg*, same for α-methylglucoside. Values given are means ± S.D. (n = 4).

worked well with all of the species, but bacteria with slower growth rates required a longer period of incubation before the plates could be read, up to 5 hr for some species of streptococci. Another factor complicating the numbers obtained using staphylococci or streptococci, as compared to *E. coli*, is the propensity of the strains to form chains or aggregates. In these cases, colony-forming units cannot be so precisely equated with absolute numbers of bacteria. This is not dissimilar to the unavoidable problems inherent in trying to determine the numbers of streptococci or staphylococci that are actually bound to epithelial cells or other substrata. For instance, if a chain of six *Streptococcus pyogenes* is seen to adhere to an epithelial cell in a light microscope-based assay, there is always a question whether one, some, or all of the cocci were actually bound. Measurements based on ELISAs could skew the data in the same way.

Study of Bacteria–Bacteria Interaction

The growth assay can be a convenient method for studying bacteria–bacteria interactions. It is possible to immobilize certain microorganisms on the plastic assay plates and test adhesion of another organism presented in suspension. In such a system, it is important to be able to keep the coating bacteria from growing and adding to the OD of the nutrient broth.

This is most easily accomplished if the bacteria added in suspension carry an antibiotic resistance marker, as essentially all recombinant strains do. Even if they do not grow, the coating bacteria do add slightly to the background simply by the fact that they coat the bottom of the wells. Even relatively heavy coats of bacteria usually increase readings by less than 0.1 OD.

Comments

Advantages

The assay is simple, relatively fast, and quantitative. Bacteria are not modified by incorporation of radioactive precursors or by biotinylation, etc. It should be an almost universal assay for any microorganism, except those having growth rates so slow that environmental contaminants would overgrow. As already mentioned, inordinate effort has not been made to maintain sterility, and yet the OD of BHI broth added to control wells does not change perceptibly over at least 7 or 10 hr.

Some of the advantage of the assay is merely that it does not have certain disadvantages of other assays. Enumeration of adherent bacteria by direct counting using a light microscope is very time-consuming and tedious, and although the relative values may be similar, absolute numbers are operator-dependent. Thus, among a series of assays, it is usually important that a single person perform the actual counting. The use of radiolabeled bacteria to quantitate adhesion is also popular and very useful. However, regulatory agencies are, with reasonable justification, making use of radioactivity more and more problematic. Furthermore, one must not ignore the potential effects of the radioactivity on the bacteria. Some authors have utilized chemiluminescent components (e.g., luciferase) to determine bound bacteria,[10] but this requires additional expensive equipment. Probably the most used method to quantitate microbial adhesion to purified molecules in microtiter plates is the ELISA. Specific antibodies bind to bacteria and then are quantitated using an anti-immunoglobulin labeled with enzymes, chemiluminescent components, or radiolabels. That method is quite effective for determining relative adhesion values, but it is not always easy to obtain quantitative data. Probably the principal problem is that specific antibodies are not always available, and, even when available, they do not always react equally with different strains within a species.

[10] W. Paranchych, personal communication (1993).

Limitations

When using the growth assay, certain precautions should be taken. The viability of the experimental bacteria must be preserved as much as possible throughout the experiment. For instance, when small amounts of Tween 20 were utilized in place of BSA in the incubation buffer to limit nonspecific binding, it was found that it inhibited the growth rate of the bacteria. Further, although expression of type 1 fimbriae has been said to be maximally expressed in 48-hr cultures, this also yields a sizable fraction of nonviable cells that still are adherent. Also, the effects of other factors, including the material used to coat the wells, must be taken into consideration. For instance, the nonmetabolizable α-methyl derivatives instead of mannose and glucose may be utilized as eluting agents to eliminate possible effects on bacterial growth.

Acknowledgments

The authors thank Jane Hurley for technical assistance. Research was supported by National Institutes of Health Grant DE-07218 (D.L.H.), by U.S. Veterans Administration Medical Research funds (D.L.H.), and by an NIH Medical Student Research Fellowship Grant DK 07405 (V.A.M.). Dr. Sokurenko is on leave from the Laboratory of Biomedical Technologies, Moscow Sechenov Medical Academy, Moscow, Russia.

[42] Enzyme-Linked Immunosorbent-Based Adhesion Assays

By ITZHAK OFEK

Introduction

Among the various adhesion assays that require some type of bacterial labeling to estimate the extent of bacterial adhesion to animal cells, the enzyme-linked immunosorbent assay (ELISA)-based system has been introduced as a convenient and sensitive means to enumerate adherent microorganisms. It is especially useful in systems employing animal cells immobilized on microtiter plates, such as tissue culture cells and phagocytic cells, which tend to adhere naturally to plastic surfaces. Even cells in suspension can be immobilized on plastic surfaces either before or after performing the adhesion assay without affecting their ability to bind bacteria, at the

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same time retaining the same receptor specificity as that of the nonimmobilized cells. Table I exemplifies a number of studies using ELISA-based systems to estimate adhesion of microorganisms to various substrata.[1-13]

In principle, monolayers of animal cells immobilized or grown to confluence onto the bottoms of microtiter wells are exposed to various bacterial densities in an appropriate buffer solution supplemented or not supplemented with a potential inhibitor. After washing off the nonadherent bacteria, the monolayers containing the adherent bacteria are fixed with gentle heating, with methanol, or simply by air-drying in order to minimize any detachment of cells from the plastic surfaces. When the adhesion assay is performed with cells in suspension, the nonadherent bacteria are washed off by differential centrifugation, and the animal cells containing the adherent bacteria are immobilized and fixed on the bottoms of the wells. An ELISA-based system is used for detection to enumerate the adherent bacteria. For this purpose, the fixed monolayers are treated with a quench solution containing an antibody against the bacteria (first antibody) and then reacted with a solution of a second antibody derivatized with horseradish peroxidase or alkaline phosphatase. The development of color after adding an appropriate chromogenic substratum reflects the amount of bound second antibody, an amount proportional to the amount of bound first antibody. The bound first antibody represents the number of bacteria adherent to the cells in the bottoms of the microtiter wells. In the following, an ELISA-based procedure for the enumeration of adhesion of type 1 fimbriated *Klebsiella pneumoniae* to mouse peritoneal macrophages in suspension is described.

[1] L. Stanislawski, W. A. Simpson, D. Hasty, N. Sharon, E. H. Beachey, and I. Ofek, *Infect. Immun.* **48,** 257 (1985).
[2] I. Ofek, H. S. Courtney, D. Schifferli, and E. H. Beachey, *J. Clin. Microbiol.* **24,** 512 (1986).
[3] R. Marre, B. Kreft, and J. Hacker, *Infect. Immun.* **58,** 3434 (1990).
[4] J. Goldhar, M. Yavzori, Y. Keisari, and I. Ofek, *Microb. Pathogen.* **11,** 171 (1991).
[5] S. G. Filler, L. G. Der, C. L. Mayer, P. D. Christenson, and J. E. Edwards, Jr., *J. Infect. Dis.* **156,** 561 (1987).
[6] A. Athamna and I. Ofek, *J. Clin. Microbiol.* **26,** 62 (1988).
[7] A. Athamna, I. Ofek, Y. Keisari, S. Markowitz, G. S. Dutton, and N. Sharon, *Infect. Immun.* **59,** 1673 (1991).
[8] S. B. Olmsted and N. L. Norcross, *Infect. Immun.* **60,** 249 (1992).
[9] A. R. Sloan and T. G. Pistole, *J. Immunol. Methods* **154,** 217 (1992).
[10] M. Sexton and D. J. Reen, *Infect. Immun.* **60,** 3332 (1992).
[11] L. J. Forney, J. R. Gilsdorf, and D. C. L. Wong, *J. Infect Dis.* **165,** 464 (1992).
[12] Y. Kurono, K. Shimamura, and G. Mogi, *in* "Recent Advances in Otitis Media" (D. J. Lim, C. D. Bluestone, J. O. Klein, J. D. Nelson, and P. L. Ogra, eds. p. 182. Decker Periodicals, Philadelphia, Pennsylvania, 1993.
[13] L. O. Bakaletz and D. J. Lim, *in* (D. J. Lim, C. D. Bluestone, J. O. Klein, J. D. Nelson, and P. L. Ogra, eds. p. 206. Decker Periodicals, Philadelphia, Pennsylvania, 1993.

Materials

Bovine serum albumin (BSA) is from Sigma (St. Louis, MO). Human immunoglobulin G (IgG) is from Travenol (Lessines, Belgium), and anti-rabbit IgG developed in donkeys and linked to horseradish peroxidase is from BDH Chemicals (Poole, UK). Brain–heart infusion broth (Difco Laboratories, Detroit, MI) is used for cell cultures, and assays are conducted in 96-well, flat-bottomed microtiter plates or Falcon 96-well polystyrene plates (Becton Dickinson Labware, Lincoln Park, NJ). Peroxidase-labeled horseradish anti-rabbit IgG and 2,2′-azino-di(3-ethylbenzthiazoline sulfate) are from Amersham (Chicago, IL). All other chemicals are of the highest purity available commercially. Rabbit polyclonal antibody to *Klebsiella pneumoniae* is prepared by intravenous injection of whole bacteria as described elsewhere.[14] The serum obtained after immunization is stored, and only sera that gave a titer of at least 1 : 600, as determined by slide agglutination of a bacterial suspension (10^9 bacteria/ml), are used in the experiments.

Bacteria

A urinary isolate (kanamycin- and cephalosporin-resistant) of unencapsulated *Klebsiella pneumoniae* Kbl is employed. This strain possesses two phenotypes: one expresses mannose-specific type 1 fimbriae and the other is nonfimbriated.[15] To obtain a bacterial suspension expressing mannose-specific type 1 fimbrial adhesin, the organisms are grown in brain–heart infusion broth under static conditions. After 48 hr at 37°, the organisms are harvested, washed in phosphate-buffered saline (PBS) (0.15 *M* NaCl in 50 m*M* phosphate buffer, pH 7.4) and suspended in Dulbecco's modified Eagle's medium to a density of 10^9 bacteria/ml, as determined by counting in a Petroff–Hauser chamber and corresponding to an optical density (OD) of 1.0 at 550 nm. The mannose-binding activity of the bacteria is determined by the yeast aggregation assay,[16] and only suspensions exhibiting a yeast aggregation rate of 5 to 10 U/min are used for further study. To avoid day-to-day variations in the adhesion activity, the bacterial suspensions are routinely stored at −70° in PBS containing 10% (v/v) glycerol in small batches. Before use, the suspensions are thawed, washed in PBS, and suspended to the desired density in PBS. To obtain the nonfimbriated phenotype lacking mannose binding activity, nonfimbriated clones are selected from MacConkey agar as described elsewhere.[15]

[14] A. Perry, I. Ofek, and F. J. Silverblatt, *Infect. Immun.* **39,** 1334 (1983).
[15] M. Maayan, I. Ofek, O. Medalia, and M. Aronson, *Infect. Immun.* **49,** 785 (1985).
[16] I. Ofek and E. H. Beachey, *Infect. Immun.* **22,** 247 (1978).

Isolation of Mouse Peritoneal Macrophages

Macrophages are collected from ICR strain male mice weighing 20 to 25 g by washing the peritoneums of the mice with cold PBS.[14] The cells are centrifuged (400 *g* for 5 min) and then suspended in PBS, and samples are counted in 2% (v/v) acetic acid in a hemacytometer. After the cells are washed three times with cold PBS, they are suspended in Dulbecco's modified Eagle's medium and adjusted to a density of 2×10^6 cells/ml.

Determination of Bacterial Adhesion to Macrophages

A 300-μl macrophage suspension is mixed in a tube with 300 μl of bacterial suspension derived from fimbriated or nonfimbriated clones and supplemented or not supplemented with a potential inhibitory sugar (methyl-α-D-mannoside or methylglucoside at 0.1 *M*). At the least, triplicates of the 600 μl mixture are prepared to ascertain the reproducibility of the results as well as to permit statistical evaluation. The mixture is rotated end-over-end at 4° (25 rpm) for 30 min, washed three times by centrifugation at 300 *g* for 5 min to remove nonadherent bacteria, and suspended in 600 μl buffer. A portion of the mixture (300 μl) is shifted to 4°, and the remaining 300 μl is incubated at 37° for 1 hr. Determination of the binding to and ingestion by the macrophages is performed on 100-μl samples. If determination of these parameters at various intervals during 1-hr incubation is desired, it is necessary to start the experiment by mixing large volumes of each of the cell suspensions to obtain a 100-μl sample for each extra determination in the protocol.

To estimate numbers of bacteria bound to the macrophages, one of the 100-μl is distributed into each well of a microtiter plate. The microtiter plate is centrifuged, the supernatant is discarded, and the sedimented phagocyte–bacterium pellet is fixed with 50 μl methanol for 10 min, followed by washing three times of the fixed monolayer with cold PBS. Alternatively, the monolayers may be fixed by heating the microtiter plates at 65° for 10 min or by exposing the monolayers to 100 μl of 0.1% glutaraldehyde for 10 min at 4° and then treating with 100 μl solution containing 0.2 *M* glycine and 1% BSA (w/v) for 30 min at ambient temperature. Bacteria attached extracellularly to the macrophages are quantitated by the ELISA-based system as follows:

1. To each well is added 100 μl of anti-*Klebsiella* serum as the first antibody diluted 1:2000 in 5% (w/v) BSA containing 10 μg/ml human IgG to block Fc receptors of the macrophages.
2. After incubation for 30 min at 37°, the monolayers are washed five times with PBS followed by the addition of 100 μl of peroxidase-labeled

horseradish anti-rabbit IgG as the second antibody diluted 1:5000 in PBS containing 5% BSA for 30 min at 37°.

3. After five washes with PBS, 100 μl of a substrate prepared according to the manufacturer's instruction is added to each well.

4. The color is allowed to develop for 15 min at 37° and is then read at 405 nm with a microtiter plate reader (usually, any automated reader is suitable).

5. The following controls are included in triplicate by preparing mixtures containing buffer instead of (a) macrophage and bacterial suspensions, (b) macrophage suspension, (c) bacterial suspension, (d) second antibody, (e) first antibody, and (f) first and second antibodies. Usually, the ELISA reader is zeroed using control wells containing buffer instead of first and second antibodies. The values obtained with control wells containing buffer instead of first antibody are subtracted from values obtained in all other experimental wells to obtain net OD values. Usually, the values of the control wells do not exceed 20% of the values obtained with the experimental wells, but if they do the experiment should be reevaluated.

The difference between the net ELISA values obtained in the experimental wells incubated at 4° and the values obtained in wells incubated at 37° reflect amounts of bacteria ingested because (a) the process of ingestion requires energy and takes place only at 37° and (b) neither the first nor the second antibodies can penetrate the macrophages to bind the ingested bacteria.[7]

Estimation of Number of Bacteria Bound per Macrophage

Because the ELISA values obtained reflect adherent bacteria, it is possible to convert those values to numbers of bacteria by preparing a standard curve in a separate set of wells for each experiment as follows:

1. A bacterial suspension in distilled water is prepared and diluted to obtain a density of 10^8 bacteria/ml.

2. A sample (100 μl) of serial 2-fold dilutions of the bacterial suspension is added to a separate set of wells in the microtiter plate.

3. The bacterial suspension is allowed to dry overnight in wells of the microtiter plates.

4. After washing the wells with distilled water, the ELISA is performed on the immobilized bacteria as described above.

The net ELISA values in OD_{405} units can be plotted as a function of the log number of bacteria in each well. A linear regression curve to include only values that increase proportionally to the number of bacteria can be

used. Such a standard curve is used to calculate the number of bacteria adherent to the monolayer of macrophages from the ELISA values obtained in the test experiment.

To determine the number of bacteria per macrophage it is necessary to estimate the number of macrophages remaining adherent after performing the adhesion assay. For this purpose the same wells used to determine the number of bacteria as described above are used to estimate numbers of macrophages remaining in the bottom of the well by staining the phagocytic nucleus:

1. Monolayers are stained with 100 μl of 1% methylene blue solution for 10 min.
2. Following washing with boric acid buffer (20 m*M*, pH 8.6), the stain is extracted by adding 100 μl of 0.1 *N* HCl solution.
3. The OD_{620} values of the extracted stain are read by using a microtiter plate reader.

These values reflect numbers of macrophages in the well. To convert the OD_{620} units to number of macrophages it is necessary to prepare a standard curve:

1. Various densities of macrophages starting with 10^6 cells/ml in a 100-μl volume are added to a separate set of wells.
2. After centrifugation, the macrophages are fixed with 100 μl methanol and stained as described above.
3. The stain is extracted as described above and a standard curve generated by plotting the OD_{620} values of the extracted stain as a function of the log number of macrophages in each well.
4. The linear regression portion of the curve is used to estimate the numbers of macrophages in each experimental well after extracting and reading the methylene blue stain for each test monolayer.

Comments

Although the procedure described above employs mouse peritoneal macrophages as target animal cells for adhesion, it can be applied for any type of cell. The only requirement is that the target cells for adhesion must be immobilized on the bottoms of wells of a microtiter plate before applying the ELISA system to detect adherent bacteria. This can be achieved by seeding the wells of the microtiter plates with the cell lines[1,3] or primary cell cultures[5,8] to obtain a confluent monolayer of cells which are strongly bound to the polystyrene surfaces or with phagocytic cells which naturally adhere to plastic surfaces.[7,9] Certain cell lines are loosely bound to the

TABLE I
ADHESION ASSAYS EMPLOYING ELISA-BASED SYSTEMS TO ENUMERATE MICROORGANISMS BOUND TO VARIOUS SUBSTRATA

Microbial Cell	Type of Substrata	Investigators
Streptococcus pyogenes	Tissue culture cells	Stanisławski *et al.*[1]
	Immobilized buccal cells	Ofek *et al.*[2]
Escherichia coli	Immobilized buccal cells	Ofek *et al.*[2]
	Immobilized enterocytes	Ofek *et al.*[2]
	Renal tubular cells	Marre *et al.*[3]
	Polymorphonuclear cells	Goldhar *et al.*,[4] Grunberg *et al.*[2]
Candida albicans	Human vascular endothelium	Filler *et al.*[5]
Klebsiella pneumoniae	Peritoneal macrophages	Athamna and Ofek,[6] Athamna *et al.*[7]
Staphylococcus aureus	Mammary epithelial cells	Olmsted and Norcross[8]
Streptococcus agalactiae	Peritoneal macrophages	Sloan and Pistole[9]
Pseudomonas aeruginosa	Immobilized buccal cells	Sexton and Reen[10]
Haemophilus influenzae	Immobilized buccal cells	Forney *et al.*[11]
	Immobilized pharyngeal cells	Kurono *et al.*[12]
Streptococcus pneumoniae	Immobilized buccal cells	Bakaletz and Lim[13]

polystyrene surface, and in this case gentle prefixation with glutaraldehyde (0.1%) of the cellular monolayer is recommended,[1] with the knowledge that such pretreatment may damage a potential receptor on the target cells. If cell suspensions are employed, the adhesion assay may be performed before or after the immobilization of the target cells onto the bottoms of microtiter plates.

In the procedure described above and elsewhere, the adhesion assay was performed before immobilization of the target cells.[4,6,17] This was possible because the phagocytic cells have a natural tendency to adhere to plastic. However, in a number of studies (Table I) the target cells, especially epithelial cells, were immobilized on the plastic surface before performing the adhesion assay,[2,10–13,18] In all procedures described, the target cells were immobilized onto glutaraldehyde–lysine-activated plastic surfaces without affecting the ability of the cells to bind bacteria. In one study, the epithelial cells were coupled to the glutaraldehyde-activated poly(L-lysine) plastic surface of 24-well polystyrene plates and attachment of the bacteria was evaluated microscopically.[19] It should be noted that, whenever examined,

[17] J. Grunberg, I. Ofek, R. Perry, M. Wiselka, G. Boulnois, and J. Goldhar, *Immunol. Infect. Dis.* **4,** 28 (1994).
[18] D. M. Schifferli, E. H. Beachey, and R. K. Taylor, *J. Bacteriol.* **173,** 1230 (1991).
[19] D. W. McEachran and R. T. Irvin, *J. Microbiol. Methods* **5,** 99 (1986).

the evaluation of bacterial adhesion by the ELISA-based method was in good correlation to that determined by direct microscopic counts.[2,6,9]

As shown in Table I, diverse microbial species can be employed when using specific antibacterial sera as first antibody. Usually, polyclonal anti-whole bacteria sera can be used at 1:2000 dilution, providing that the sera give an agglutinating titer of at least 1:600. Dilutions of the antisera of less than 1:2000 may be required for antisera possessing lower agglutinating titers.

If mixed populations of bacterial species are employed in the adhesion assay, the adhesion of each bacterial species can be evaluated by using different animals as a source of antisera against each bacterial species. The adhesion of one bacterial species is evaluated as described above; after washing the plates, the adhesion of the other bacterial species can be determined using appropriate antibacterial sera and corresponding second antibody. This allows one to study competition in the adhesion between two (or more) bacterial species for the same substratum.

Because the ELISA-based system employs cells immobilized on solid surfaces, the control wells devoid of target cells are not the best controls to account for nonspecific adhesion of bacteria to plastic surfaces. It has been suggested to use immobilized cells which poorly bind the test bacterial species instead of blocking agents so that nonspecific binding to polystyrene surfaces is made comparable.[9]

Other enzyme-linked assay procedures which involve the same principles as the ELISA-based system in obtaining quantitative adhesion data have been suggested. They include the enzyme-linked lectinosorbent assay (ELLA) and the enzyme-linked biotin–avidin assay (ELBA). In the ELLA-based system, an enzyme-linked lectin is used instead of both first and second antibodies, providing that the lectin binds only to the test bacterial strain and not to the target animal cells. A list of lectins that bind specific bacterial species has been described.[20] In the ELBA-based procedure, the microorganisms were biotinylated before they were applied onto the animal cell monolayer. After washing off the nonadherent microbial cells and fixation of the monolayer as described above, the number of adherent microorganisms was determined by adding enzyme-linked avidin. An ELBA-based system has been applied to enumerate adhesion of *Escherichia coli* to immobilized epithelial cells.[2,18] It is probably more sensitive than the ELISA and eliminates the problem of nonspecific binding of the first and second antibodies in the ELISA-based system, but it requires labeling of the bacteria with biotin before the adhesion assay is performed. Any potential effect of such labeling on adhesin activity of the labeled microor-

[20] M. Slifkin and R. J. Doyle, *Clin. Microbiol. Rev.* **3,** 218 (1990).

ganisms must be ruled out before performing the ELBA-based adhesion assay.

Finally, the ELISA-based system determines total adhesion to a given population of animal cells, whereas visual counting allows one to quantitate microbial adhesion to individual cells. Otherwise, the ELISA-based adhesion assays offers a number of advantages over other methods. All manipulations involved in the enumeration of microbial adhesion follow the actual adhesion process, whereas all other microbial labeling procedures are applied before the adhesion assay. It allows one to screen with a high sensitivity large numbers of potential inhibitors and diverse types of microbial species and target tissue cells. For example, as few as 5×10^4 bacteria per assay well could be detected. Because the bottom surface of the wells may contain as many as 10^5 (confluent monolayer) to 5×10^4 (immobilized cells) cells per well, an average of as few as 0.5–1 bacteria per cell can be detected. In addition, when the target cells (e.g., phagocytic cells) are capable of internalizing the adherent bacteria, the ELISA-based system allows discrimination between the attachment and ingestion (or internalization) stages of the endocytosis process.[6]

[43] Bacterial Adhesion to Hydroxylapatite

By KURT M. SCHILLING and RON J. DOYLE

Introduction

Oral bacteria, particularly members of the genera *Streptococcus* and *Actinomyces,* have a tendency to adhere to the hard tissues of the oral cavity. This adhesion is a prerequisite for plaque formation and may ultimately result in the development of dental decay or gingivitis. Numerous laboratories are studying the mechanism(s) of oral microbial adhesion, with the view in mind of learning how to prevent the adhesion.

There are now several model systems for the study of streptococcal and actinomyces adhesion to saliva-coated surfaces (reviewed by Schilling *et al.*[1]). Some of the model systems include metal slabs or wires, glass, pulverized enamel, and sections of teeth. The most common model systems, however, appear to be various forms of calcium phosphate, such as hydrox-

[1] K. M. Schilling, R. G. Carson, C. A. Bosko, G. D. Galikeri, A. Bruinooge, K. Hoyberg, A. M. Waller, and N. P. Hughes, *Colloids Surf. B* **3,** 31 (1994).

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ylapatite and whitlockite.[2-5] These are popular because they seem to bind the same proteins of saliva as enamel surfaces. Furthermore, these forms of calcium phosphate are dense and can be conveniently separated from suspended bacteria by their rapid rate of sedimentation. Although the rapid sedimentation is useful in separating bound (*B*) from unbound (*U*) bacteria, several washes are necessary to remove adventitiously bound cells. If radioactive bacteria are employed, it is also necessary to transfer the bacteria to a scintillation vial which has not come in contact with the labeled cells as suspended bacteria will adhere to the walls of the vial (with the degree of adhesion depending on the bacterium and/or conditioning film). In the methods described below, recommended procedures for the adhesion of radioactive streptococci or actinomyces to saliva-coated whitlockite beads and to saliva-coated hydroxylapatite (SHA) surfaces are presented.

Preparation of Bacteria

Various streptococci and actinomyces have been used in adhesion assays. It is recommended to use radioactive bacteria, as reasonably high levels of labeling can be achieved when the cells are grown in defined or rich medium. Most researchers find it useful to grow the cells in at least 10 μCi/ml [^{3}H]thymidine or 1–2 μCi/ml ^{14}C-labeled amino acid mix (algal hydrolysate). If the bacteria are retrieved from rich medium, the medium must first be treated with invertase (to remove trace levels of sucrose) and fungal dextranase (to remove trace levels of α-1, 6-glucan). Cells from treated medium form even suspensions, free of aggregates. When the cells form chains, as determined by light microscopy, they must first be subjected to brief bursts of sonic energy to effect dechaining. Cultures (exponential, stationary, etc.) are centrifuged and washed three times in 10 m*M* potassium bicarbonate, 1 m*M* inorganic phosphate, 0.5 m*M* calcium chloride, adjusted to pH 7.0 with potassium hydroxide or dilute hydrochloric acid (adhesion buffer). In some cases, the calcium salt may be deleted. Standard curves are made of radioactivity versus cell density. Bacterial density can be deter-

[2] R. J. Gibbons, *in* "Molecular Basis of Oral Microbial Adhesion" (S. Mergenhagen and B. Rosan, eds.), p. 77. American Society for Microbiology, Washington, D.C., 1985.

[3] W. B. Clark, L. L. Bammann, and R. J. Gibbons, *Infect. Immun.* **19,** 846 (1978).

[4] R. J. Doyle, *in* "Microbial Cell Surface Analysis: Structural and Physicochemical Methods" (N. Mozes, P. S. Handley, H. J. Busscher, and P. Rouxhet, eds.), p. 289. VCH Publ., Brooklyn, New York, 1991.

[5] I. Ofek and R. J. Doyle, "Bacterial Adhesion to Cells and Tissues," Chap. 2. Chapman & Hall, New York, 1994.

mined by turbidity measurements and by light microscopy.[6] For most adhesion assays, various densities of organisms are required.

Preparation of Hydroxylapatite and Whitlockite

For the preparation of hydroxylapatite (HA) substrata, it is recommended to coat the surfaces of microtiter plates with the HA. Best results are obtained with "tissue culture-treated" 96-well, polystyrene plates (No. 25860, Corning Glass Works, Corning, NY). The plates are filled with a calcifying solution (2.5 m*M* $CaCl_2$, 7.5 m*M* KH_2PO_4, 250 m*M* triethanolamine, pH 7.4). The plates are then incubated for 90 min at 75°, after which they are emptied and subjected to another incubation in fresh calcifying solution. A total of four 90-min calcification cycles are required to effect complete mineralization of the surfaces. After the final cycle, the wells are rinsed with distilled water and air-dried. Any well that does not appear to have even deposits of HA (it is convenient to use a 4× dissecting microscope) is marked and not used in adhesion experiments. That the surface coating on the wells is indeed HA has been determined by X-ray diffraction. The HA-coated surfaces, when compared to whitlockite bead (WB) surfaces, are much more even and do not contain deep pits or fissures.[1]

Whitlockite beads (BDH Chemicals, Poole, UK) are prepared by weighing bead samples directly into plastic or glass vials. A convenient weight is 100 mg. This amount can be distributed rapidly into vials by use of a calibrated scoop or ladle (use of the calibrated ladle usually provides ±5% reproducibility). Lower amounts require individual weighings. The beads are washed in adhesion buffer several times. It is difficult to remove "fines," as even the smallest beads rapidly settle to the bottom. The sizes of the commercial beads range from 50 to 300 μm, with the average size being about 150 μm.

The HA-coated wells or the WB can be used as substrata for the coating of saliva, glucosyltransferases, glucans, bacteria (to form substrata for other bacteria), or purified proteins (serum albumins, proteases, glucanohydrolases, etc.). Details for coating of the HA and or WB surfaces can be found elsewhere.[7]

Adhesion to Hydroxylapatite-Coated Plates

The wells are conditioned by the addition of adhesion buffer. The buffer is aspirated or decanted before addition of cells. It is convenient to use

[6] A. L. Koch, *in* "Manual of Methods for General Bacteriology" (P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips, eds.), p. 179. American Society for Microbiology, Washington, D.C., 1981.

[7] K. M. Schilling and W. H. Bowen, *Infect. Immun.* **60,** 284 (1992).

250-μl quantities of radioactive cells. To the cell suspensions can be added inhibitors or enzymes to give no more than a 350-μl volume in each well. Usually, the inhibitors are added prior to the distribution of cells into the wells. Some experiments, however, may require mixing inhibitors with cells prior to the addition of cells to the HA wells. The wells are covered with plastic wrapping (stretched Parafilm is convenient) and incubated on a rotary shaker for appropriate times at a constant temperature (4°, 21°, or 37°). Following incubation, the wells are aspirated and washed three times in adhesion buffer. Finally, the wells (and bacteria) are digested with 350 μl of 10 m*M* ethylenediaminetetraacetic acid (EDTA)–1.0 *M* sodium hydroxide. Samples (usually 100–200 μl) are transferred to scintillation vials and the radioactivity determined. The method is not amenable to viable cell determination, but the method can be adapted to enzyme-linked immunosorbent assay (ELISA) techniques (see [42] in this volume). The method is convenient for studies on inhibition of adhesion or to observe the effects of various agents on dissociation of adherent bacteria from the HA surfaces (or HA coated with enzymes, glucans, saliva, etc.).

Adhesion of cells to WB is similar in experimental setup as adhesion to HA. The beads are placed in 4.6- to 5.0-ml plastic vials to which can be added (without regard to order) bacterial suspensions, inhibitors, proteins, enzymes, antibodies, and chaotropes. The WB–cell suspension is rotated at a constant rate and a constant temperature. The Fisher Scientific (St. Louis, MO) Hematology Chemistry Mixer (No. 346) makes a convenient end-over-end rotator. The time of interaction must be predetermined by adhesion versus time plots at various cell densities. The vials with beads are aspirated and three sequential buffer washes performed using 4 ml buffer each time. The vials are dried overnight at 50°–60° and the dried beads transferred to unused scintillation vials. It is imperative that the vials employed to mix the radioactive bacteria with the WB substrata not be used in radioactivity determinations, as they may contain enough adherent bacteria to mask the counts on the beads. Care must be taken to transfer the cells quantitatively.[8] An alternative procedure is to use a fourth wash, by rinsing the contents of the original vial into a clean vial. The washing procedure normally does not remove radioactivity from the surfaces of the vials containing the original mix. The buffer in the vials should be evaporated before the addition of scintillation fluids.

Treatment of Adhesion Data

The proper presentation of results from adhesion experiments is often instructive about the mechanism of adhesive events. The most simple

[8] M. M. Cowan, K. G. Taylor, and R. J. Doyle, *J. Bacteriol.* **169,** 2995 (1987).

method for treating adhesion data is to plot bound (B) bacteria (or fungi) versus unbound (U) or total (T) cells added. Plots of B versus U (or T or log T, etc.) may give linear curves. The units for B, U, or T may be counts per minute (cpm), colony-forming units (cfu), or others as dictated by the experiment or particular organism.[5] In most cases, B versus $U(T)$ plots are not linear. Saturation of sites usually occurs at higher added cell densities. In many cases, adhesion is not proportional to added cells. A "threshold" of added cells may be required before significant adhesion occurs.

Figure 1 shows the results of adhesion of *Streptcococcus sanguis* to hydroxylapatite coated onto plastic wells. The adhesion curve is somewhat S-shaped, suggestive of cooperative adhesion. Also, the curve does not suggest that saturation of sites had been achieved. Changes in adhesion with respect to added cell numbers can best be determined by use of the now standard Scatchard plot of B/U versus B (Fig. 2). When positive slopes are observed, as in Fig. 2, there is reason to believe that the adhesion is cooperative, in which the adhesion of one cell enhances the adhesion of another. The mechanism of "positive cooperativity" is unknown, although data from numerous laboratories suggest that various bacteria adhere to surfaces via positive cooperativity.[5,8]

There are other means to plot adhesion data, including the Langmuir form of the law of mass action (U/B versus U). This plot is not sensitive to changes in slopes and is not recommended. Treatment of any adhesion data according to various equilibria plots must be carefully considered. Some researchers may question the validity of performing mass action plots

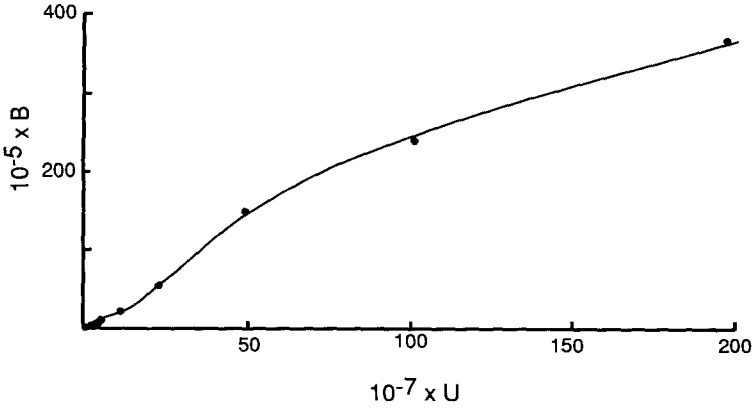

FIG. 1. Binding isotherm for the adhesion of *Streptococcus sanguis* to hydroxylapatite plates. (Data replotted from Schilling *et al.*[1])

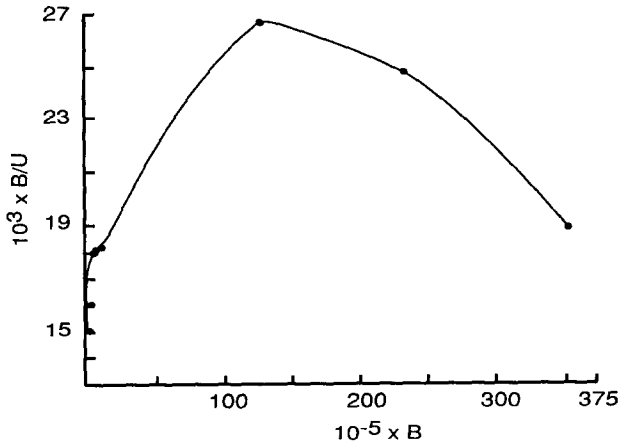

FIG. 2. Scatchard plot for the adhesion of *Streptococcus sanguis* to hydroxylapatite. (Data from Fig. 1.)

on adhesion results, because adhesion may be a composite of interactions involving different kinds of binding sites. If adhesion, as determined by radioactivity measurements, etc., obeys equilibria, then the plots are valid, regardless of the complexity of the adhesive reactions.

The following practical considerations for plotting data and experimental design are proposed in adhesion to hydroxylapatite as well as other systems. First, perform adhesion experiments at several cell densities, ranging from very low to saturating. Usually, thick suspensions of cells can be easily diluted to yield appropriate densities. Plot B versus U or B versus log U to determine saturation levels (where B does not increase with cell density). Plots of B versus U can frequently give a 50% saturation level, a cell density useful for developing inhibition of adhesion experiments. It is necessary to make preliminary runs to determine if equilibrium has been achieved at the various cell densities. Scatchard plots and other plots based on mass action are not valid unless conditions of equilibria have been employed. Typical adhesion experiments may require 10–15 separate cell densities.

Second, plot B/U versus B and note inflections, positive slopes, and negative slopes. If the results give linear curves, then it is valid to determine the association constant (K) and the number of adhesion sites (N) from the Scatchard equation:

$$B/U = K(N - B)$$

Third, adhesion experiments must be performed at constant temperatures, as only a few degrees variation may cause wide variations in the extent of adhesion.[8]

Finally, when studying inhibition of adhesion, it is required that saturating cell densities not be employed. Densities should be chosen that are sensitive to change. Plot percent inhibition versus log inhibitors so as to make it easy to estimate 50% inhibition. When comparing numerous inhibitors, it is convenient to determine 50% inhibition concentrations.

Proper analyses of adhesion results make it possible to study the mechanism(s) of adhesion and to study the nature of potential inhibitors. Cowan ([16] in this volume) describes how adhesion results can be analyzed by time-dependent or kinetic methods. Kinetics are important in defining binding or desorption events in adhesion.

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[44] Measurement of Microbial Adhesion to Hydrophobic Substrata

By RON J. DOYLE and MEL ROSENBERG

Introduction

Seven decades ago Mudd and Mudd[1,2] observed that some bacteria have a tendency to partition at oil–water interfaces. Their observations ultimately led to studies on the role of microbial adhesion in hydrocarbon degradation, the role of cell surface hydrophobicity in mucosal adhesion and pathogenesis, and the role of hydrophobicity in biofouling. The hydrophobic properties of microorganisms are known to contribute to their adhesion to various surfaces, including catheters, implants, teeth, red cells, mucosal cells, and plants. Hydrophobicity is best defined as the greater tendency of a microorganism to adhere to hydrocarbon or nonpolar substrata (hydrophobins) than to water. Numerous microbial pathogens seem to adhere totally or partially via the hydrophobic effect. These include members of the genera

[1] S. Mudd and E. B. H. Mudd, *J. Exp. Med.* **40,** 633 (1924).
[2] S. Mudd and E. B. H. Mudd, *J. Exp. Med.* **40,** 647 (1924).

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TABLE I
METHODS COMMONLY EMPLOYED TO MEASURE MICROBIAL CELL SURFACE HYDROPHOBICITY AND ADHESION TO HYDROPHOBINS

Method	Features
Microbial adhesion to hydrocarbon (MATH)	Microorganisms may adhere to hydrocarbon–aqueous interface; removal of cells from aqueous phase can be quantitated by spectrophotometry
Hydrophobic-interaction chromatography (HIC)	Octyl-Sepharose columns serve to trap hydrophobic microorganisms, whereas nonhydrophobic variants or strains do not adhere
Salt aggregation test (SAT)	Hydrophobic microorganisms, when mixed with ammonium sulfate, tend to aggregate; rates of aggregation can be monitored by turbidity readings
Contact angle measurements (CAM)	When water droplets are placed on dried lawns of microorganisms, the droplets form contact angles which are approximately proportional to surface hydrophobicity
Two-phase partitioning (TPP)	When microorganisms are mixed with two immiscible aqueous phases containing dextran and polyethylene glycol (PEG), hydrophilic cells tend to bind to the lower dextran phase, whereas hydrophobic cells partition into the more hydrophobic PEG upper phase
Direct binding	Small molecules, such as dodecanoic acid, can bind to hydrophobic cells, the binding of which can be determined by radioactivity assays
Adhesion to hydrophobic microspheres	Some hydrophobic microorganisms bind to polysytrene microspheres, the binding of which can be determined by light microscopy
Imprints of colonies	Microbial colonies, when pressed gently on the surface of polystyrene, are lifted off the agar substratum; washing the polystyrene leaves an "imprint" of the hydrophobic colonies

Bacillus (spores), *Candida, Escherichia, Staphylococcus,* and *Streptococcus* among others. A general review of microbial cell surface hydrophobicity can be found in Doyle and Rosenberg.[3] Although there are exceptions, microorganisms which have a high cell surface hydrophobicity exhibit a high tendency to adhere to hydrocarbons.

There are now numerous methods to measure microbial adhesion to hydrophobins and cell surface hydrophobicity. Each appears to have certain advantages. It is unlikely a single method will satisfy all requirements, however. Table I offers an outline of the most commonly used methods. Usually, the methods correlate quite well when care has been taken to

[3] R. J. Doyle and M. Rosenberg (eds.), "Microbial Cell Surface Hydrophobicity." American Society for Microbiology, Washington, D.C., 1990.

employ common buffers, cells of the same age, constant temperature, consistent levels of salts, etc. When salt concentrations are too low, microbial suspensions frequently do not readily bind hydrocarbons or plastics. In addition, cold room temperatures (2°–5°) give much lower binding to hydrophobic surfaces or molecules than temperatures in the 30°–40° range. Experience with the systems described in Table I suggests that optimal measurements of hydrophobicity are made at pH 5–6 and at least 0.2 *M* sodium chloride and 37°. The following section details use of the microbial adhesion to hydrocarbon (MATH) assay.

Microbial Adhesion to Hydrocarbon

The MATH assay is based on the tendency of certain microorganisms to adhere to the surfaces of liquid hydrocarbon droplets, during a brief mixing period.[4,5,6,7]

Utensils

Glassware should be clean and preferably acid-washed, as surfactants and other impurities can interfere with results. Disposable plastic pipettes and cuvettes can be used in most instances (one notable exception being use of a test hydrocarbon which is incompatible, e.g., *p*-xylene).

Preparation of Microorganisms

In general, microorganisms which have been grown in liquid medium are employed in the MATH assay. However, in certain cases cells may be scraped from agar plates. In other cases (e.g., dental plaque, feces), cells may be taken directly from their sources. Following microbial growth or direct isolation, cells are washed at least twice in buffer. The recommended buffer is 50 m*M* acetate containing 200 m*M* sodium chloride (pH 5.6). This buffer is often replaced by phosphate buffer (50 m*M* phosphate buffer plus 150 m*M* sodium chloride, pH 7.2). The assay may be attempted in other buffers, but ionic strength and pH may have an effect on results obtained. Following washing, the optical density of the microbial suspension is usually adjusted to approximately 0.8–1.0 at 450 nm. This is arbitrary, and the results obtained may depend on the initial cell density employed.

[4] M. Rosenberg and R. J. Doyle, *in* "Microbial Cell Surface Hydrophobicity" (R. J. Doyle and M. Rosenberg, eds.), p. 1. American Society for Microbiology, Washington, D.C., 1990.
[5] M. Rosenberg, *FEMS Microbiol. Lett.* **22,** 289 (1984).
[6] M. Rosenberg, *FEMS Microbiol. Lett.* **25,** 41 (1984).
[7] M. Rosenberg, M. Barki, R. Bar-Ness, S. Goldberg, and R. J. Doyle, *Biofouling* **4,** 121 (1991).

Test Hydrocarbon

In the original assay, three test hydrocarbons were chosen for the assay: *n*-hexadecane, *n*-octane, and *p*-xylene. In subsequent studies, hexadecane was used more frequently, although other test hydrocarbons sometimes provide important results which are not evident employing hexadecane. Hexadecane, obtained commercially, may on occasion contain contaminants. For example, hexadecane may be oxidized to yield components which can interfere with the assay. Hexadecane can be protected from oxidation by storage under nitrogen gas. A simple procedure was reported for determining whether the hydrocarbon and other assay components are relatively surfactant-free. If the buffer and test hydrocarbon are mixed together (with no microbial suspension) under the assay conditions and quickly coalesce following the mixing procedure, no significant contamination is evident. If, however, droplets do not coalesce quickly, or an emulsion is formed, then an interfering contaminant may be present. It is convenient to mix hexadecane with a mixed-bed ion-exchange resin to remove interfering components. The resin MB-3 is ideal for such use. Care must be taken when using aromatic hydrocarbons, because of possible toxicity to humans, damage to the microbial cell wall and membrane, and incompatibility with polystyrene and other plastics.

Assay

The original assay was performed with 1.2 ml microbial suspensions, in test tubes overlaid with samples of test hydrocarbon ranging from 0.01 to 0.2 ml. Obviously, volume differences as well as differences in the ratio of the aqueous and hydrocarbon phases affect results. The test tubes containing the microbial suspension and test hydrocarbon are vortexed for a set 2-min period. Following vortexing, the phases are allowed to separate. If no adhesion takes place, the phases should coalesce and separate completely. In the case of adhesion, a "creamy" upper layer is formed, consisting of cell-coated hydrocarbon droplets. Adhesion should always be confirmed by microscopic observation of the droplets. Adhesion is most readily quantified by measuring the drop in turbidity in the aqueous phase following the mixing procedure. The lower aqueous phase may be withdrawn using a Pasteur pipette and transferred to a cuvette for an optical density measurement. However, the assay can be performed directly within a cuvette or tube that is compatible with a spectrophotometer, in which case the decrease in turbidity can be determined directly, provided that (i) the hydrocarbon droplets have risen completely from the aqueous phase; (ii) care is taken to ensure that the upper phase does not interfere with the light beam; and

(iii) the walls of the vessel are free of interfering droplets (they can usually be detached by shaking the vessel once or twice sharply by hand).

The decrease in optical density should always be compared with that of the suspension prior to mixing. It is erroneous to incorporate as a control a vortexed test tube or cuvette with no hydrocarbon, because in the absence of the test hydrocarbon microorganisms may attach in large numbers to the walls of the glass or plastic. It is also unacceptable to measure turbidity following solidification of the liquid hydrocarbon phase at low temperatures, because this causes desorption of the cells from the oil–water interface. Microorganisms which do not adhere to hydrocarbons under one set of conditions cannot be inferred as being "nonadherent." Rather, several attempts should be made at altering various aspects of the assay (pH, type of hydrocarbon, etc.).

Kinetic Approach

Based on the observation that adhesion to hydrocarbons is time-dependent,[8] several investigators have developed kinetic approaches in which adhesion to different hydrocarbon volumes is followed as a function of time. This is most readily performed in 4-ml cuvettes, containing 1 ml of aqueous phase and hydrocarbon volumes ranging from approximately 5 to 50 μl, and taking time points of several seconds. Following each brief vortex, the phases are allowed to separate, the turbidity of the lower phase measured, and the mixture subjected to further mixing/measurement cycles. For a given aqueous-to-hydrocarbon volume ratio, the decrease in turbidity over time follows pseudo-first-order kinetics, yielding a removal rate. Plotting the removal rates as a function of the hydrocarbon-to-water volume ratio usually yields a straight line, the slope of which is termed the "removal coefficient."

Several aspects of the kinetic analysis require further clarification. For example, back extrapolation of the removal rate to zero hydrocarbon volume yields a finite number, rather than zero adhesion rate as one would predict. Furthermore, the removal coefficient may be a function of the initial cell density, suggesting some type of cooperative interaction between the cells and the hydrocarbon. Figure 1 shows a set of data for a typical MATH assay.

Modifications

Addition of various compounds may have a profound effect on MATH. For example, the presence of low concentrations of amphipathic cationic

[8] D. Lichtenberg, M. Rosenberg, N. Scharfman, and I. Ofek, *J. Microbiol. Methods* **4,** 141 (1985).

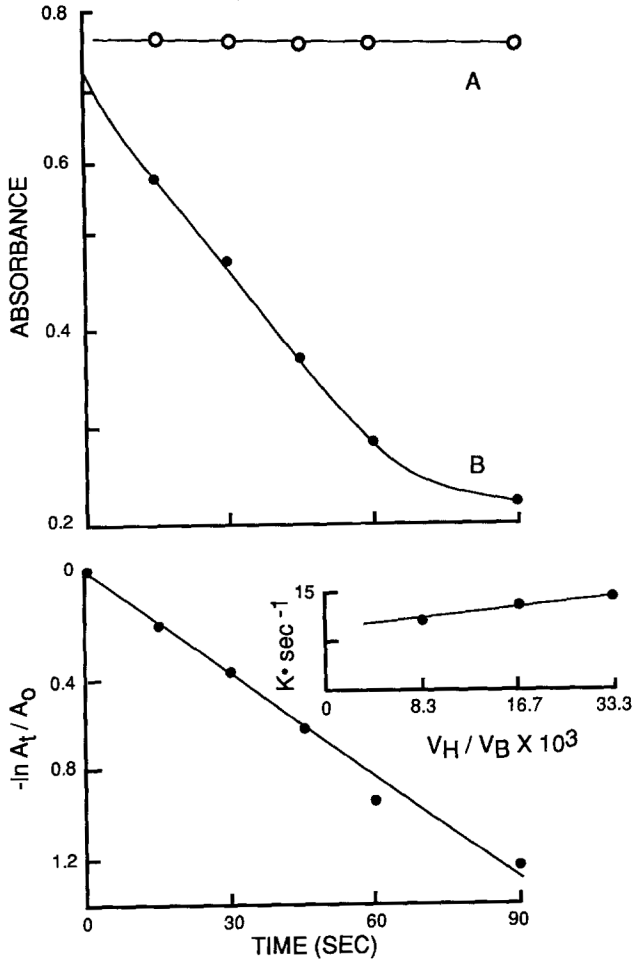

FIG. 1. Adhesion of *Streptococcus cricetus* AHT and *Bacillus subtilis* 168 to hexadecane. (*Top*) Curve A shows adhesion of *B. subtilis* to the hydrocarbon as a function of mixing time; curve B, adhesion of *S. cricetus* to hexadecane. (*Bottom*) First-order rate plot for the binding of *S. cricetus* to hexadecane, using data derived from the top curve. (*Inset*) The slope of the first-order rate plot (K min^{-1}) versus the ratio of hexadecane to buffer (V_H and V_B, respectively). The volume of cells in buffer was 3.0 ml for curves A and B (top), whereas the amount of hexadecane was 100 μl. The results show that *B. subtilis* 168 did not exhibit any tendency to adhere to the hydrocarbon, whereas *S. cricetus* was highly adherent. Independent assays have shown that *B. subtilis* has no ability to bind plastics or other hydrophobic surfaces.

agents, such as cetylpyridinium chloride and chlorhexidine, as well as cationic biopolymers [lysozyme, chitosan, poly(L-lysine)] can potentiate microbial adhesion to hydrocarbons.[9] The assay can also be modified for enrichment of mutants with altered surface properties. Microorganisms bound to hexadecane droplets can serve as a substratum for subsequent attachment of coadhering partners.[10]

Advantages and Disadvantages

Advantages of the MATH assay include (i) low cost of disposable items and materials, and ubiquitousness of instrumentation (vortex, photometer); (ii) its rapid nature, both in operation and in generation of data; (iii) the ability to observe the adhering cells by light microscopy; and (iv) measurement of the adhesion characteristics of the entire microbial suspension, rather than a small fraction of cells which usually comes in contact with a given surface.

The main disadvantages of the assay are that (i) the surface of attachment is not constant, precluding steady-state analysis; (ii) assay conditions may vary from laboratory to laboratory, making it difficult to compare results; and (iii) some bacteria autoagglutinate, making spectrophotometric readings unreliable.

Salt Aggregation Test

The salt aggregation-test (SAT), like the MATH assay, is inherently simple and requires no specialized equipment.[11,12] Basically, a suspension of bacteria is mixed with an equal volume of an ammonium sulfate (AMS) solution and observed for aggregation. The AMS solutions usually are 1–4 *M*, but could be lower if the bacteria are highly hydrophobic. In some cases, the AMS can cause the bacteria (or spores) to adhere avidly to polystyrene wells, the adhesion of which can be quantitated by enzyme-linked immunosorbent assay (ELISA)-like assays. In one case, very low densities of spores of *Bacillus anthracis* were quantitated by use of AMS-promoted adhesion of the spores to plastic surfaces. The spores were assayed on the plates by use of soybean agglutinin conjugated to horseradish

[9] S. Goldberg, R. J. Doyle, and M. Rosenberg, *J. Bacteriol.* **172,** 5650 (1990).

[10] H. C. Van der Mei, M. Rosenberg, and H. J. Busscher, *in* "Microbial Cell Surface Analysis" (N. Mozes, P. S. Handley, H. T. Busscher, and P. G. Rouxhet, eds.), p. 263. VCH Publ., Brooklyn, New York, 1991.

[11] C. J. Smyth, P. Johnsson, E. Olsson, O. Soderlind, J. Rosengren, S. Hjertén, and T. Wadström, *Infect. Immun.* **22,** 462 (1978).

[12] M. Lindahl, A. Faris, T. Wadström, and S. Hjertén, *Biochim. Biophys. Acta* **677,** 471 (1991).

peroxidase (ELLA, or lectinosorbent assay).[13] In other studies, employing more dense suspensions of microorganisms, it has been found convenient to measure the rate (as determined by plots governing first-order kinetics) of aggregation induced by AMS. If AMS concentrations are too high, the cells may flocculate, thereby rendering rate assays useless. When AMS concentrations are too low, aggregation may occur, but there could be a significant lag time before the rate of aggregation follows first-order kinetics. Usually, hydrophobic organisms yield much shorter lag times than nonhydropohobic variants.

Contact Angles and Cellular Hydrophobicity

Van der Mei *et al.*[10] and Busscher *et al.*[14] have reviewed the procedures needed to obtain reproducible contact angles. Basically, when a droplet of water is placed on a hydrophobic surface (a lawn of bacteria), the contact angle is proportional to the hydrophobicity of the surface. For bacteria, water contact angles are usually made by use of a system to determine the change in angle as a function of time. A requirement for contact angle measurements is that the surface must be dry. A further compounding factor is that the droplet may rapidly hydrate a partially hydrophobic bacterial lawn. In spite of these difficulties, the method clearly distinguishes between bacterial lawns, and it is useful in studying the differences in hydrophobicity between certain pathogens and avirulent mutants. Contact angles do not, of course, measure adhesion to hydrocarbons. Nevertheless, contact angles are useful in predicting adhesion of certain pathogens to hydrophobic surfaces.

Hydrophobic-Interaction Chromatography

Hydrophobic-interaction chromatography (HIC) is useful in separating hydrophobic from hydrophilic organisms and in estimating the proportion of a population capable of adhering to hydrocarbons. Agarose beads conjugated with phenyl or octyl groups are commercially available. The beads are washed with phosphate-buffered saline (PBS) or acetate buffer and poured into a column (Pasteur pipettes plugged with glass wool make convenient columns). A small volume of cells is poured onto the column and eluted with buffer. Fractions are then collected for analysis. It is conve-

[13] K. Graham, K. F. Keller, J. Ezzell, and R. J. Doyle, *Eur. J. Clin. Microbiol.* **3,** 210 (1984).

[14] H. J. Busscher, J. Sjollema, and H. C. Van der Mei, *in* "Microbial Cell Surface Hydrophobicity" (R. J. Doyle and M. Rosenberg, eds.), p. 335. American Society for Microbiology, Washington, D.C., 1990.

nient to grow cells in the presence of a tritiated amino acid mix or tritiated thymidine in order to obtain radioactive suspensions. Conversely, the fractions may be diluted and assayed for viable counts on suitable agar plates. An alternative and rapid method involves the suspension of cells with the conjugated beads. The beads are allowed to settle and samples of the supernatant assayed for viable counts, radioactivity, turbidity, etc.

The HIC method does not work well when bacteria capable of binding galactose are employed, as agarose contains galactosyl groups. Members of the genera *Pseudomonas, Escherichia,* and *Streptococcus* have been reported to bind galactose. In some cases, cross-linked dextrans (Sephadex) may be substituted for agarose. In addition, another control is usually run employing a nonconjugated agarose. This control is useful in assessing the trapping of cells in the bead suspension. Finally, the HIC method is not useful when cells tend to autoagglutinate. In some cases cell aggregates (as well as chains found in streptococci) may be disrupted by mild sonication. Sonication, however, may have an effect on surface hydrophobins.

Conclusion

The foregoing summarizes the method most commonly employed in determining adhesion to hydrophobins. In general, there is a good correlation between MATH and the other assays.[4] For this reason and the fact that the method is simple and reproducible, MATH has become the assay of choice for many laboratories. There are numerous factors involved in adhesion to hydrophobins, such as pH, ionic strength, temperature, age of cells, growth medium for the cells, time of incubation (or vortexing), antibiotics, and others.

Acknowledgments

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Contributors to Volume 253

Article numbers are in parentheses following the names of contributors.
Affiliations listed are current.

SOMAN N. ABRAHAM (3), *Department of Pathology, Jewish Hospital of St. Louis, Washington University School of Medicine, St. Louis, Missouri 63110*

WILLIAM AGACE (18), *Department of Medical Microbiology, Division of Clinical Immunology, Lund University, S-223 62 Lund, Sweden*

JOHN F. ALDERETE (33), *Department of Microbiology, The University of Texas Health Science Center, San Antonio, Texas 78284*

ROSSANA ARROYO (33), *Department of Microbiology, The University of Texas Health Science Center, San Antonio, Texas 78284*

LUCILLA BALDASSARRI (38), *Laboratorie di Ultrastrutture, Istituto Superiere di Sanita, 00161 Rome, Italy*

ISRAEL BURSTEIN (23), *Department of Clinical Microbiology, Hebrew University, Hadassah Medical School, Jerusalem 91010, Israel*

H. J. BUSSCHER (37), *Laboratory for Materia Technica, University of Groningen, 9712 KZ Groningen, The Netherlands*

MICHAEL CAPARON (23), *Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110*

FREDERICK J. CASSELS (7), *Department of Gastroenterology, Division of Medicine, Walter Reed Army Institute of Research, Washington, District of Columbia 20307*

GWYNN R. CHIPPENDALE (28), *Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, Maryland 21201*

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JANINA GOLDHAR (4), *Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*

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ITZHAK OFEK (8, 42), *Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Ramat-Aviv 69978, Israel*

NOBUHIKO OKADA (23), *Department of Bacteriology, Institute of Medical Sciences, University of Tokyo, Tokyo, Japan*

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Preface

In the past two decades there have been thousands of papers dealing with microbial adhesion phenomena. Adhesion seems to confer an advantage to the microorganism in that it gains resistance to serum factors, antibiotics, and deleterious agents. The discovery that microbial colonization and infection can be prevented by blocking adhesion has stimulated research on the identification of adhesins, their molecular biology, and their specificities. Numerous studies have been conducted to develop vaccines based on adhesin properties. New and sensitive methods have evolved to study various aspects of microbial adhesion.

This is the first volume devoted solely to adhesion of pathogens, including bacteria, fungi, and protozoa. The topics range from strategies involved in studying adhesion, to genetic manipulation of adhesins, to adhesin and receptor isolation and characterization, to distinguishing between adhesion and invasion, to various assays for adhesion, and to the kinetic and epidemiological considerations of adhesion and infection. Attempts have been made to include as many approaches to the adhesion of pathogens as possible. One notable exception is adhesion to glycolipids on silica gels, a topic covered in an earlier volume of *Methods in Enzymology.* Included in this volume are methods related to adhesion of enteric pathogens, respiratory pathogens, oral bacteria, *Candida,* protozoa, and to adhesion to plastics, hydrocarbons, hydroxylapatite, and various biosurfaces. A goal was to provide a volume containing strategies as well as specific methods.

We thank Atha Carter for superb administrative assistance in the development of this volume. We also thank the prominent scientists who provided papers for this first volume on methods for studying adhesion of pathogens.

RON J. DOYLE
ITZHAK OFEK