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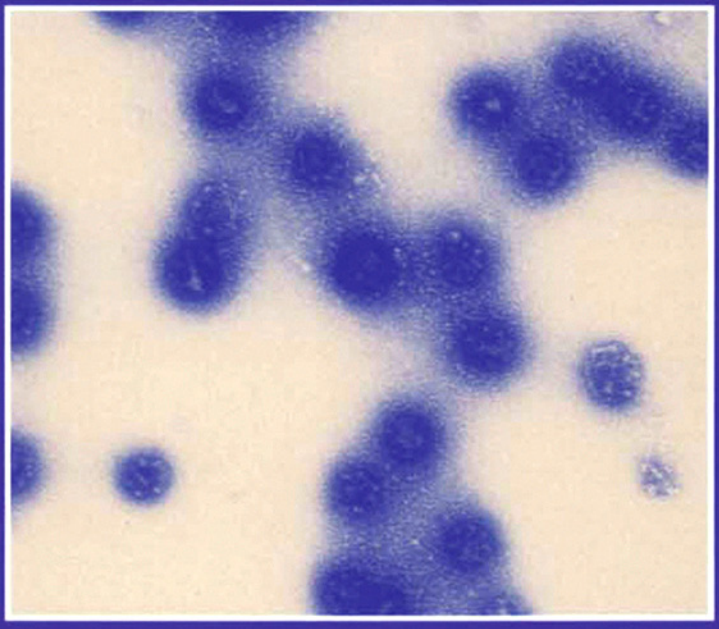
A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



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Front cover photograph: Electron micrograph of reassembled coats formed on dialysis of total pea clathrin-coated vesicle coat proteins showing distinct polygonal lattice structure. (See Chapter 1 for more details.)

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Clathrin-Coated Vesicles in Plants

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This chapter focuses on current knowledge of coated vesicles from plant systems. It is apparent that although the studies have been directed by the much greater volume of information from animal systems, there has been considerable progress in our understanding of the function and biochemical characterization of clathrin-coated vesicles from plants over the past decade. Ultrastructural studies have demonstrated coated vesicles in a variety of plant cells. Within the cells, the vesicles are involved in endocytosis, membrane recycling, and the intracellular transport of vacuolar proteins. Improved isolation procedures have facilitated the biochemical characterization of clathrin-coated vesicles. To date, coat components of 180-kDa clathrin have been identified; however, the identity of light chains remains enigmatic. Adaptor peptides have been isolated and potential receptor proteins for vacuolar targeted proteins identified. The functioning of coated vesicles requires removal of the clathrin coat and appropriate uncoating ATPase has been identified. The dissociation of the receptor(s) and targeting ligands of the transported protein appears to involve a proton-pumping vacuolar H^+-ATPase associated with the vesicle. It is suggested that the capacity to routinely isolate vesicles, combined with techniques of molecular biology, should lead to a more rapid accumulation of information on the function and biochemistry of clathrin-coated vesicles from plants.

KEY WORDS: Clathrin, Adaptors, Endocytosis, Receptors, Protein transport, Uncoating ATPase, Vacuolar H^+-ATPase, Protein body.

I. Historical Background

The term "coated vesicle" was coined in the mid-1960s to describe characteristic vesicles that participated in the endocytosis of exogenous ferritin in amphibian spinal ganglia (Rosenbluth and Wissig, 1963, 1964). Other

contemporary and earlier ultrastructural studies had identified complex vesicles, vesicles with amorphous coats, or alveolate vesicles in a wide array of animal cells (Ockleford and Whyte, 1980). Ultrastructural studies in both lower (algal) and higher plant systems, conducted over the same time period, contained frequent reports of alveolate coated vesicles. The majority of these studies were descriptive, merely identifying the vesicles, and provided little information in the way of definitive details.

The earliest detailed report of coated vesicles in higher plants appears to be that of Bonnett and Newcomb (1966), which described the occurrence of the organelles in root hairs of radish, *Raphanus sativus.* "The coated vesicles were 85–90 nm in diameter and consisted of a unit membrane structure surrounded on their cytoplasmic face by an alveolate or reticulate layer. In median section the coat exhibited radiating spokes of columnar projections about 25 nm long. In tangential sections the coat was seen to consist of pentagonal or hexagonal units." More recent studies have confirmed the occurrence of similar coated vesicles in many cell types from higher plants. They have been reported in cotton fibers (Ryser, 1979), developing pollen (Nakamura and Miki-Hirosige, 1982; Sheldon and Dickinson, 1983), *Rhizobium* infected root cells (Robertson and Lyttleton, 1982), mycorrhizal host cells (Barrosa and Pais, 1987), root hairs and cortical root cells (Emons and Traas, 1986), and cultured plant cells (Franke and Herth, 1974). Coated vesicles have been described in protoplasts prepared from a variety of plant sources (Tanchak *et al.,* 1984; Joachim and Robinson, 1984; Fowke *et al.,* 1983, 1989; Van der Valk and Fowke, 1981; Doohan and Palevitz, 1980).

In addition to demonstrating the occurrence of free coated vesicles, the early ultrastructural studies reported on the occurrence polyhedral, alveolate structures associated with the plasma membrane-coated pits and dictyosomes (Fig. 1) (Bonnet and Newcomb, 1980; Robertson and Lyttleton, 1982; Ryser, 1979; Nakamura and Miki-Hirosige, 1982; Emons and Traas, 1986; Van der Valk and Fowke, 1981). The dictyosome-associated vesicles were smaller (62–78 nm), with less conspicuous coats and vesicle membranes than the plasma membrane-associated vesicles, which had a diameter of 100 nm (Van der Valk and Fowke, 1981). A similar diversity of coated vesicles was reported in microspore mother cells (Nakamura and Miki-Hirosige, 1982) and root cells (Emons and Traas, 1986).

II. Function

A. Endocytosis

The observations of the association of coated vesicles with dictyosomes and plasma membrane resulted in early speculations that the coated vesicles

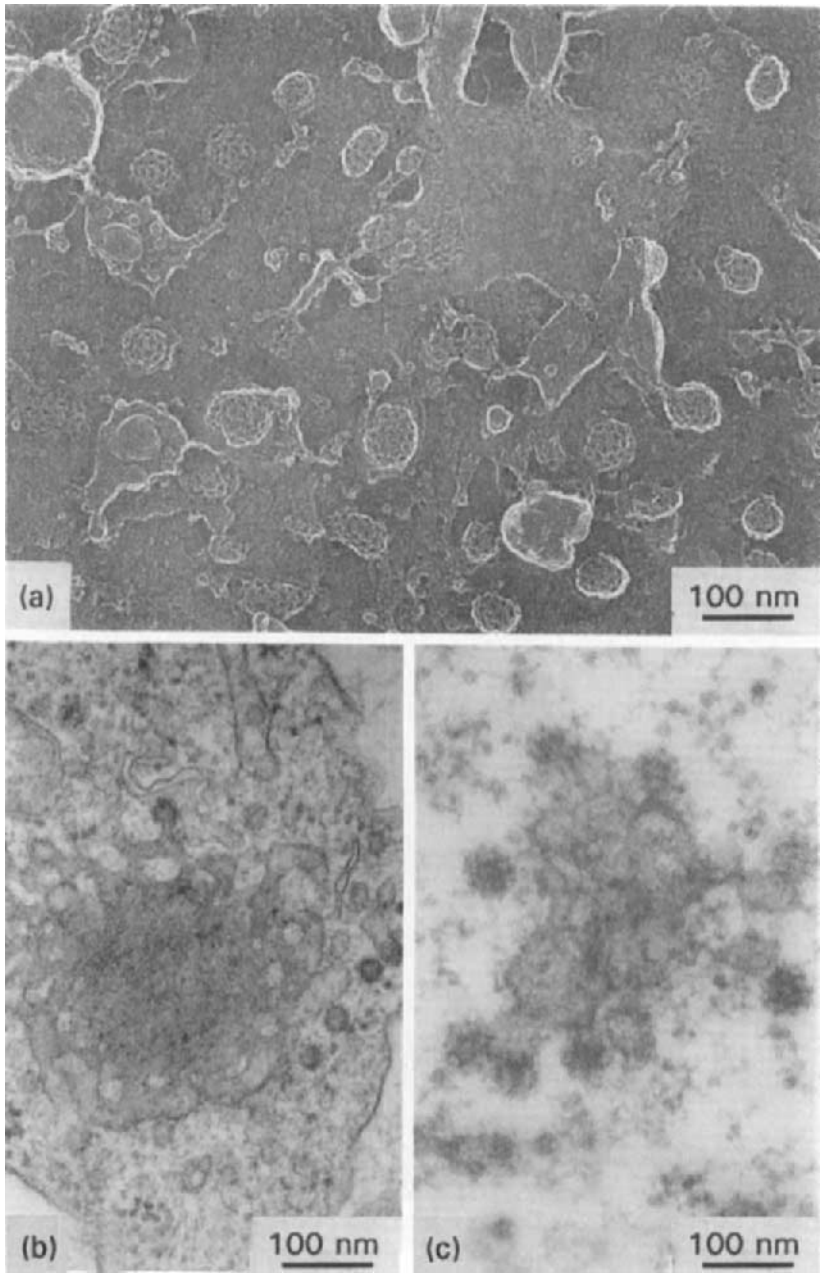


FIG. 1 Transmission electron micrographs of carrot suspension-cultured cells. (a) Coated pits on the plasma membrane of an osmotically burst protoplast. (b) Dictyosome-associated coated vesicles. (c) Clathrin-coated vesicles associated with partially coated reticulum (PCR). Bars = 100 nm. (From Coleman *et al.*, 1987, with permission of Blackwell Science Ltd.)

were the source of new plasma membrane and pectic wall components (Nakamura and Miki-Hirosige, 1982; Van der Valk and Fowke, 1981; Franke and Herth, 1974; Ryser, 1979; Robertson and Lyttleton, 1982; Doohan and Palevitz, 1980). Such a proposal of exocytotic function was consistent with the greater abundance of coated vesicles in root hair tips and in meristematic and expanding cells. The early suggestion of an exocytotic role for vesicles in plants contrasted with the research in animal systems which proposed an endocytotic function (see reviews in Ockleford and Whyte, 1980).

The first clearly defined role for coated vesicles in plant systems was provided in studies of endocytosis by isolated protoplasts (Tanchak *et al.*, 1984; Joachim and Robinson, 1984). The investigations demonstrated that in protoplasts surface labeled with cationized ferritin, the label became localized into ferritin-coated pits and became internalized in the cytoplasm in coated vesicles over time (Fig. 2). Serial sectioning confirmed that the

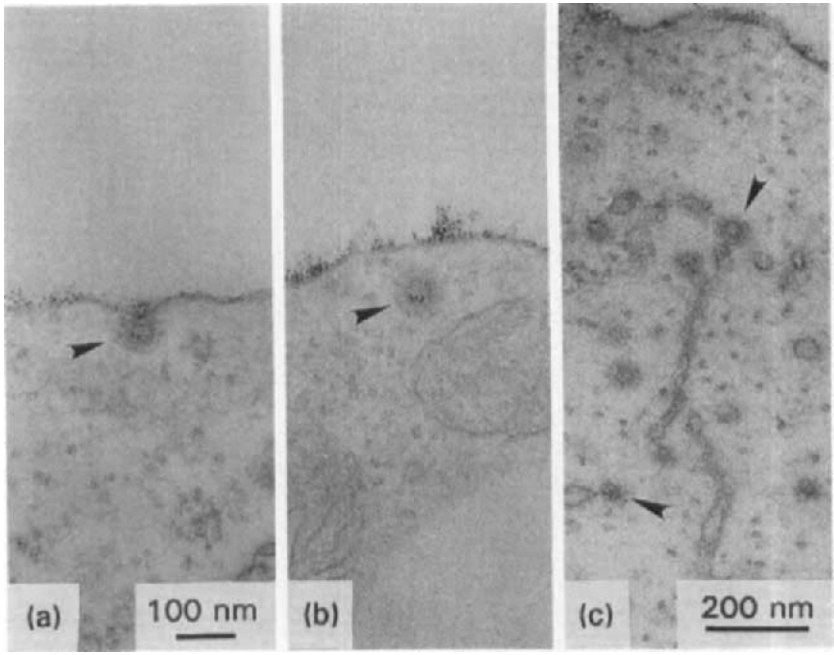


FIG. 2 Transmission electron micrographs of soybean protoplasts exposed to cationized ferritin (CF). (a) Invagination of coated pit. Note CF contents of pit. Bar = 100 nm. (b) Free coated vesicle containing CF at same magnification as (b). (Both a and b from Fowke *et al.*, 1991; reprinted with the permission of Cambridge University Press). (c) Coated vesicles with CF cargo associated with partially coated reticulum. Bar = 200 nm. (From Tanchak *et al.*, 1988, with permission).

ferritin was indeed localized in structurally distinct vesicles and not with peg-like invaginations of the plasma membrane (Fowke *et al.,* 1989). In addition to demonstrating an endocytotic role for coated vesicles, these studies identified the involvement of an additional coated membrane component in plant cells—the partially coated reticulum (PCR). This organelle consists of tubular membranes bearing clathrin-like coats over part of the cytoplasmic surface (Pesacreta and Lucas, 1985) and is frequently seen in the vicinity of the dictyosome (Golgi) (Hilmer *et al.,* 1988; Tanchak *et al.,* 1988) (Fig. 1c). However, whether there is a direct connection between PCR and dictyosomes is unresolved. It has been suggested that PCR is the plant equivalent of the trans-Golgi network (TGN) of animal cells (Hilmer *et al.,* 1988). In the trans-Golgi, there is a sorting of materials for transfer to the plasma membrane and cell exterior or the lysosome. Others (Fowke *et al.,* 1991) suggest that the PCR is equivalent to the compartment of uncoupling of receptor and ligand (Curl), which is equivalent to the early endosomal compartment of mammalian cells (Geuze *et al.,* 1983, 1984). Unfortunately, at present there are no reliable markers for the TGN or endosomal compartment in plant cells, and thus the controversy is unresolved. Certainly the PCR is a region involved in vesicle trafficking.

B. Membrane Recycling

The studies of endocytosis by plant protoplasts clearly demonstrate the internalization of plasma membrane through the involvement of coated pits and coated vesicles (Tanchak *et al.,* 1984; Joachim and Robinson, 1984; Fowke *et al.,* 1991; Galway *et al.,* 1993). It has been suggested that the plasma membrane in plant cells must be constantly recycled to compensate for the secretory activity of the Golgi. The elegant work of Staehelin's group has demonstrated that the growth and development of plant cell walls requires the deposition of matrix polysaccharides. These polysaccharides are synthesized in the Golgi apparatus and are transported to the cell wall in smooth secretory vesicles. These vesicles are generated from the medial and trans compartment of the Golgi stack (Staehelin *et al.,* 1991; Zhang and Staehelin, 1992). Since there is no proliferation of the plasma membrane during matrix deposition, it is evident that the membranes of the smooth-coated transport vesicles must be recycled. The plasma membrane of tobacco protoplasts initiating cell wall regeneration showed an eightfold increase in abundance of coated pits in comparison with membranes of protoplasts unable to form walls (Fig. 3) (Fowke *et al.,* 1983). It is now generally considered that coated pits and coated vesicles are involved in internalization (Fowke *et al.,* 1991; Steer and O'Driscoll, 1991). More recently, Samuels *et al.* (1995) have concluded that the coated membranes

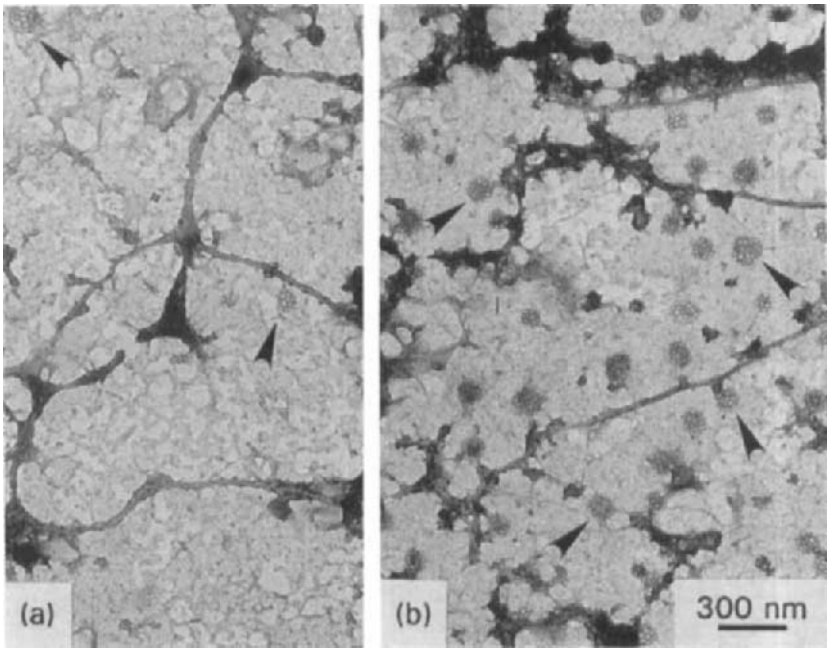


FIG. 3 (a) Low-frequency coated pits on inner surface of plasma membrane from tobacco leaf protoplasts that exhibit a lag period before deposition of new cell wall. (b) High-frequency coated pits on inner surface of plasma membrane from rapidly growing cultured tobacco cells capable of immediate cell wall formation. Bars = 300 nm. (From Fowke *et al.*, 1991; reprinted with permission of Cambridge University Press.)

and clathrin-coated vesicles which are abundant during cell plate formation are involved in membrane retrieval rather than exocytosis.

Thus the early demonstrations of coated pits and vesicles in meristematic and elongating cells, which were originally interpreted as being involved in exocytosis, can now be viewed as consistent with the retrieval of the plasma membrane by endocytosis. Emons and Traas (1986) have calculated that growing cells could recycle their complete plasms membrane in 20–40 min and suggested that coated vesicles were involved in the process.

C. Protein Sorting and Transport

Early ultrastructural studies of legume seeds showed that spherical bodies about 2 μm in diameter were prominent features of the cells. Various analytical procedures established that these bodies contained the reserve globulins of the legume seeds (Altschul *et al.*, 1961; Varner and Schidlovsky,

1963), and the term "protein body" has subsequently generally been applied to identify these entities. A considerable body of evidence has accumulated (Higgins, 1984) which demonstrates that the synthesis of storage proteins in maturing seeds takes place on the rough endoplasmic reticulum. In many seeds the protein bodies are considered homologous to the vacuolar system of the cell (Matile, 1975; Millerd, 1975; Pernollet, 1978). The most recent evidence, however, indicates that in legume seeds the protein bodies (storage vacuoles) arise *de novo* rather than by fragmentation or partitioning of the existing cell vacuole, as had been originally suggested (Hoh *et al.*, 1995; Robinson *et al.*, 1995).

Ultrastructural studies suggested that the endoplasmic reticulum (ER)-synthesized proteins are passed through the Golgi apparatus, where they are processed and concentrated. Golgi-derived vesicles were proposed to provide the vehicle for transport into existing vacuoles (Bergfeld *et al.*, 1980) as originally proposed by Dieckert and Dieckert (1976). The development of this concept was based on the increase in osmiophilic content of the vesicles surrounding the Golgi apparatus and the accumulation of osmiophilic material in storage bodies. Significantly, the vesicles were most prominent during the period of protein body formation. Confirmation that the Golgi apparatus was involved in protein storage and processing has been provided by the immunological localization of reserve proteins (Nieden *et al.*, 1984; Krishnan *et al.*, 1986), lectins (Herman and Shannon, 1984), and vacuolar hydrolases (Herman and Shannon, 1985) in Golgi cisternae and surrounding secretory vesicles. Pulse chase experiments demonstrate a time-dependent migration of labeled phytohemagglutinin from a Golgi-enriched fraction into dense vesicles (Chrispeels, 1983).

Clathrin-coated vesicles isolated from developing pea cotyledons have been demonstrated to contain precursors of lectin, hydrolytic enzymes (Harley and Beevers, 1989b), and the precursors of the storage proteins legumin and vicilin (Robinson *et al.*, 1989, 1991; Hoh *et al.*, 1991). In this situation the coated vesicles appear to be functioning in selectively recruiting material from the Golgi apparatus for deposition into the storage vacuoles, in a manner analogous to the transport of lysosomal enzymes in mammalian cells (von Figura and Hasilik, 1986). Such a sequence of events would be consistent with the proposal by Craig and co-workers (1979, 1980; Craig, 1986, 1988), who suggested that preexisting vacuoles in the cells of developing cotyledons are the depository of the storage proteins. The large vacuole(s) of the young cotyledonary cells are considered to be transformed into protein bodies by fragmentation or subdivision. Others have suggested that the protein storage vacuoles of developing legume cotyledons have dual origins, one set arising by fragmentation of the vegetative vacuole and an additional set arising *de novo* (Bain and Mercer, 1966; Neuman and Weber, 1978; Adler and Müntz, 1983). Recently Robinson *et al.* (1995)

have demonstrated that in cells of developing pea cotyledons, the bulk of the storage protein does not pass through the Golgi apparatus but instead never leaves the endoplasmic reticulum. The protein storage vacuoles are derived from the ER and replace the degenerating vegetative vacuoles. According to Robinson *et al.* (1995), the legumin and vicilin present in clathrin-coated vesicles represent a mechanism for retrieving storage proteins which have escaped retention in the ER. Since the "escaped" storage proteins are not secreted, there must be selectivity at the trans-Golgi to ensure that legumin and vicilin are packaged into clathrin-coated vesicles, with ultimate deposition of the cargo into the develoing protein bodies.

III. Isolation

The first isolation of a coated vesicle-enriched fraction from plant cells used homogenates from tobacco cell cultures and centrifugation through two sequential sucrose density gradients. Electron microscopy demonstrated that the preparations still contained smooth vesicles in addition to vesicles with characteristic alveolate coats. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) indicated that the most prominent polypeptide had a molecular weight of 190 kDa. The yield of vesicles was 1 mg protein/375 g fresh weight of cells (Mersey *et al.*, 1982). Subsequently, protoplasts from soybeans were used as a source of coated vesicles. Separation of vesicles from the homogenate was achieved through a series of isopycnic and rate zonal centrifugations through sucrose density gradients. SDS–PAGE demonstrated the presence of a prominent polypeptide of 190 kDa identified as plant clathrin, and also peptides of 105, 100, 96, 64, 50, 38 and 32 kDa (Mersey *et al.*, 1985). Following these original isolations, a series of manuscripts have described the successful isolation of coated vesicles from suspension-cultured cells (Depta and Robinson, 1986; Coleman *et al.*, 1987).

The initial isolations of coated vesicles from tissue cultures contained particulate material in addition to smooth vesicles. Smooth vesicle contamination was reduced by substitution of an isopycnic centrifugation step for the original rate zonal sucrose density gradient centrifugation (Mersey *et al.*, 1985). Depta and Robinson (1986) suggested that the particulate material in the original isolations was due to ribosomes. These investigators successfully applied the ribonuclease treatment pioneered by Pearse (1982) to remove contaminating ribosomes, in addition to the Ficoll/D_2O gradient method of Pearse (1982). The substitution of Ficoll/D_2O for sucrose avoids the dissociation of the clathrin coat that occurs in high sucrose concentrations (Nandi *et al.*, 1982). The yield of clathrin from carrot cells was estimated

at 1.7 mg/410 g fresh weight of cells, but it was indicated that the preparation was still contaminated (10–30%) with various small vesicles (Depta and Robinson, 1986). Subsequent work using sucrose density gradient analysis of the "purified" coated vesicle fraction from *Cucurbita* hypocotyls has revealed that at least some of the smooth vesicles are attributable to contamination by plasma membrane (Depta *et al.*, 1991). The ribonuclease digestion and Ficoll/D_2O gradient technique has been used successfully to isolate coated vesicles from bean leaves (Depta *et al.*, 1987); however, the yield of vesicles was extremely low—1 mg of coated vesicles from 2.4 kg of leaves. In order to achieve this yield, it was necessary to include thiol reagents to reduce phenol oxidase activity and the protease inhibitor phenylmethylsulfonylfluoride (PMSF).

In continuing efforts to improve the yield and purity of clathrin-coated vesicle preparations, Robinson's group has substituted the inclusion of ethylenediamine tetraacetic acid (EDTA) in the homogenization and isolation medium for the ribonuclease treatment (with the inherent danger of proteolysis) of the postmicrosomal pellet. The inclusion of the chelating agent EDTA lowers the magnesium concentration to the extent that contaminating ribosomes are dissociated and, in the case of zucchini hypocotyls, are no longer recovered in the clathrin-coated vesicle fraction. However, clathrin-coated vesicles recovered from developing pea cotyledons remain contaminated with ribosomes unless a ribonuclease treatment is included (Demmer *et al.*, 1993).

To avoid proteolysis of the protein components during isolation of vesicles, a "cocktail" of protease inhibitors is included in the homogenization and isolation media. The ribonuclease treatment, if needed, is conducted at 4°C. By using vertical rotors to perform isopycnic centrifugation, the vesicle isolation can be conducted in one day. Despite these precautions, it appears that some proteolysis of vesicle constituents may still occur, as indicated by the disappearance of β-adaptin components from bovine brain coated vesicles incubated in plant extracts containing a protease inhibitor cocktail (Holstein *et al.*, 1994).

The highest yields of coated vesicles have been recovered from developing cotyledons of peas (Harley and Beevers, 1989a; Robinson *et al.*, 1991). However, even the most purified preparations recovered by centrifugation through either Ficoll/D_2O or sucrose gradients are contaminated with ferritin, thus making assessment of clathrin yield unreliable (Hoh and Robinson, 1993). It was suggested that gel filtration on Sephacryl S-1000, which has been used to isolate clathrin-coated vesicles from yeast by Mueller and Branton (1984), may be a satisfactory alternative procedure. However, we have found this method unsatisfactory for the preparation of vesicles from homogenates of developing pea cotyledons.

IV. Composition of Coated Vesicles

A. Heavy and Light Chains

SDS–PAGE of the early preparations of coated vesicles from plant sources consistently demonstrated the presence of a prominent 190-kDa peptide identified as plant clathrin by Mersey *et al.* (1985). Although it was demonstrated that antibodies to bovine brain clathrin recognized proteins from coated vesicles from plants (Cole *et al.*, 1987), there has been little further confirmation of the homology between the 190-kDa protein from plants and the 180-kDa clathrin from animal sources. Antibodies to the 190-kDa clathrin have been difficult to raise and thus cross reactivity is difficult to establish. Interestingly, the 180-kDa clathrin from bovine brain is also poorly antigenic.

As investigations of mammalian clathrin-coated vesicles proceeded, Pearse (1978) identified a doublet of peptides of 30 and 36 kDa as constituents in association with the more prominent 180-kDa peptide. Subsequently Ungewickell and Branton (1981) demonstrated that treatment of clathrin-coated vesicles from bovine brain with 2 *M* urea solubilized proteins that were co-eluted from Sepharose 4B gel filtration chromatography columns. Analysis of the co-eluting components demonstrated the presence of 30-, 36-, and 180-kDa peptides. The low molecular weight components were identified as light chains and were associated with the 180-kDa component to form the characteristic three-legged structure termed a "triskelion."

The presence of triskelions in extracts of coated vesicles from plants was demonstrated by Coleman *et al.* (1987). Tris extracts of coated vesicles from protoplasts of carrot suspension-cultured cells were concentrated with ammonium sulfate. The recovered precipitated portion was redissolved in Tris and analyzed by SDS–PAGE and electron microscopy. The triskelions released from the coated vesicles with Tris were morphologically identical with those recovered from mammalian systems. Each arm radiated from a central vertex, was kinked midway, and had a globular terminal domain.

The orientation of the arms on the triskelions on grids used in electron microscopy depends on the method of preparation. Triskelions prepared in Tris show a clockwise orientation (Coleman *et al.*, 1987) whereas those prepared in 2 *M* urea show an anticlockwise orientation (Lin *et al.*, 1992). In mammalian triskelions, the arms are 45.5 nm in length with a kink occurring 16–20 nm from the vertex separating the proximal from the distal regions of the arm. The amino-terminal of the arm is in the globular terminal domain while the carboxy-terminal is at the vertex. The arms of triskelions from plant coated vesicles have an average length of 61 nm, which is consistent with the higher molecular mass of plant clathrin (Coleman *et al.*, 1991).

The triskelion forms the basis of the polyhedric coat characteristic of clathrin-coated vesicles. The center of each triskelion lies at the vertex of a polyhedron; each vertex is attached to three surrounding vertices by three struts (Fig. 4). Each strut corresponds to the edge of the polyhedron and consists of two proximal and two distal arms (Pearse and Crowther, 1981). Despite a difference in arm size of the triskelions, the polygon center-to-center distance of 21 nm is the same in plant and animal vesicles. This information suggests that the extra length in the triskelion arms must be in the distal and not the proximal components (Coleman *et al.*, 1987, 1991).

SDS–PAGE of the Tris extract of clathrin-coated vesicles from carrot cells demonstrated the presence of three prominent peptides of 190-, 60-, and 57-kDa (Coleman *et al.*, 1987). It was originally suggested that the 60- and 57-kDa peptides may be clathrin light chains; however this opinion has now been revised and the peptides 57 and 60 kDa are considered proteolytic artifacts. In studies of coated vesicles from soybean, it was demonstratred that the clathrin heavy chain was dissociated from vesicles by 4 *M* urea (Weidenhoeft, *et al.*, 1988). In general, clathrin-coated vesicles from plants are more resistant to dissociation than the well-characterized bovine brain vesicles. SDS–PAGE of the urea extract from soybean vesicles demonstrated the presence of a 185-kDa heavy chain and a number of polypeptides in the 35–50 kDa range which are possible light chain candidates. Electron microscopic evaluation of Sepharose 4B-fractionated 2 *M* urea extracts of clathrin-coated vesicles from developing pea cotyledons indicated the presence of triskelions which, on SDS–PAGE, demonstrated the 190-kDa heavy chain and four potential light chain candiates of 50, 46,

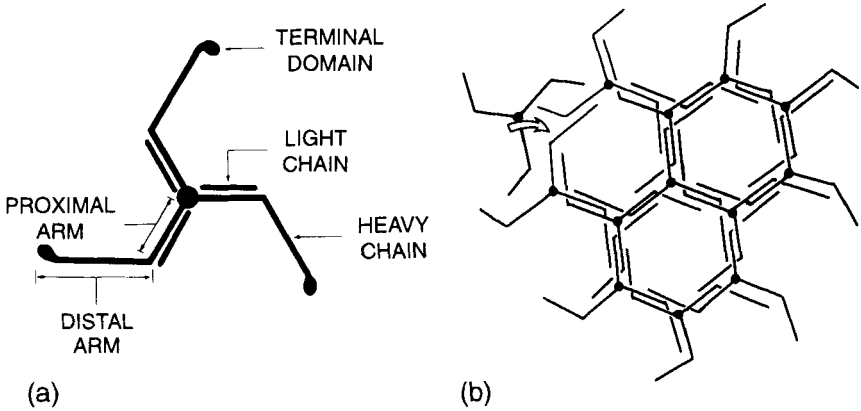


FIG. 4 (a) Clathrin triskelion model (based on current information on mammalian clathrin). (b) Illustration of the packing of triskelions to form cages as suggested by Pearse and Crowther (1981). (From Coleman *et al.*, 1988, with permission of Blackwell Science Ltd.)

40, and 31 kDa (Fig. 5). These polypeptides were elastase sensitive, heat stable, readily solubilized following trichloracetic acid precipitation, and could bind to calmodulin. The light chain candidates could be phosphorylated under appropriate conditions (Lin *et al.*, 1992). All of these properties are demonstrated by the well-characterized light chains from mammalian systems (Brodsky *et al.*, 1991).

On the basis of heat stability and calcium-binding properties, two polypeptides of 30 and 38 kDa, respectively, isolated from clathrin-coated vesicles prepared from zucchini hypocotyls were identified as potential light chains (Balusek *et al.*, 1988). However, more recent studies suggest that polypeptides of 45 and 52 kDa may be light chain candidates in zucchini with four or five polypeptides between 40 and 50 kDa being associated with the heavy chain present in triskelions prepared from pea cotyledons (Demmer *et al.*, 1993).

Most of the evidence points to a greater size and possible greater complexity of light chains in plants, in contrast to the two light chains normally encountered in animals (Brodsky *et al.*, 1991) or the one light chain of 38 kDa found in yeast (Mueller and Branton, 1984; Payne and Schekman, 1985). We have prepared antibodies to the 50- and 46-kDa light chain-like

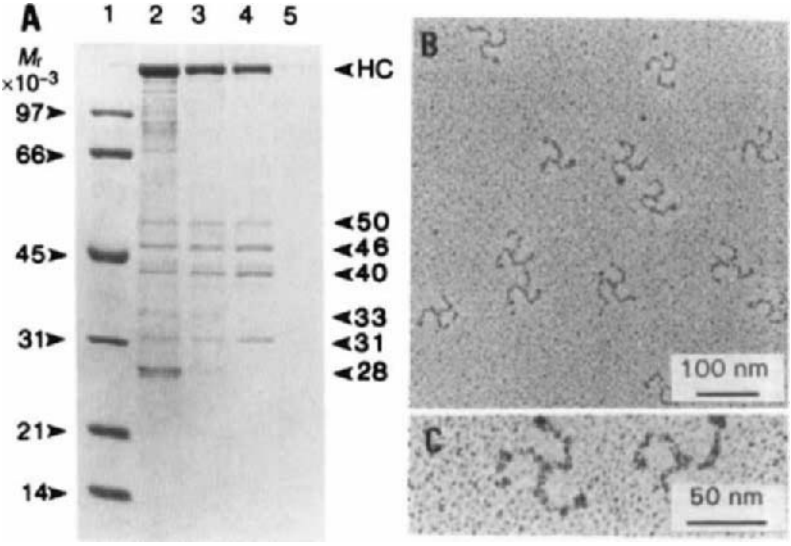


FIG. 5 (A) SDS–PAGE of pea clathrin coated vesicles. Lane 1, MW markers; lane 2, total coated vesicle proteins; lane 3; coat proteins isolated with buffer containing 2 *M* urea; lane 4, triskelion fraction from Sepharose CL-4B column; and lane 5, proteins in second peak from above column. (From Lin *et al.*, 1992, with permission from Company of Biologists, Ltd.) (B) and (C) Triskelions isolated from pea clathrin-coated vesicles with buffer containing 2 *M* urea. (B) bar = 100 nm (C) bar = 50 nm.

peptides from developing pea cotyledon vesicles and have demonstrated cross reactivity with all four light chain candidates (Lin, 1992). This cross immunoreactivity indicates some degree of structural similarity between the light chain candidates. This is in agreement with the situation encountered in mammalian light chains. Sequencing of cDNA from a variety of mammalian sources has demonstrated a 60% identity between Light Chain a (LCa) and Light Chain b (LCb). A comparison of sequences of individual light chains indicates a greater than 95% homology among mammalian sources (Jackson and Parham, 1988). If a similar degree of conservation occurs in plants, it should be possible to utilize the light chain antibody from peas to identify clathrin light chains from the plants.

The four light chain candidates coimmunoprecipitate with the heavy chain when homogenates of pea cotyledons are treated with antibodies to the 46-kDa light chain candidate. Furthermore, all of the light chain candidates are dissociated when clathrin-coated vesicles are treated with uncoating ATPase (Kirsch and Beevers, 1993). Thus there is a consistent association of the identified light chain candidates with the heavy chain. Further studies are needed to determine the sequences of the light chain candidates in order to establish their relationships and determine the extent of polymorphism. Sequencing information from plant sources will help identify motifs that have been determined to be important in the association of light chains with heavy chains and which have been proposed to regulate vesicle dynamics in mammalian systems (Brodsky *et al.*, 1991).

B. Assembly Proteins and Adaptors

Clathrin triskelions purified by Sepharose chromatography of Tris extracts of clathrin-coated vesicles from bovine brain reassemble into cages when dialyzed or diluted into mildly acidic and low ionic strength buffers. However, these cages formed *in vitro* from clathrin alone demonstrate a broad distribution on density gradients, with a proportion of unpolymerized protein remaining at the top of the gradients. The cages formed from the clathrin triskelions are composed of heavy chains and light chains, and demonstrate a wide range of diameters on electron microscopy (Zaremba and Keen, 1983). In contrast, when unfractionated Tris extracts of clathrin-coated vesicles were dialyzed or when the fractions from the two peaks from Sepharose chromatography were combined and dialyzed, the reassembled structures formed a narrow, discrete peak on sucrose density gradients. SDS–PAGE demonstrated that the structures formed contained heavy chains, light chains, and additional polypeptides of 100–110 and 50 kDa. The reassembled structures, which are termed "coats" since they contain proteins in addition to clathrin heavy and light chains (Pearse and Robinson,

1984), were smaller than the cages formed from triskelions and had a uniform diameter of 78 nm. The protein components from Sepharose chromatography that enable the clathrin triskelions to reassemble into uniformly sized coat structures *in vitro* were termed assembly polypeptides (APs) (Zaremba and Keen, 1983; Pearse and Robinson, 1984). Earlier studies had also implicated polypeptides of 100 kDa in the binding of clathrin to clathrin-depleted coated vesicles (Ungewickell *et al.*, 1981; Unanue *et al.*, 1981).

Pearse and Bretscher (1981) proposed that a family of molecular units would sort out components destined to travel in clathrin-coated vesicles from those that remained behind. Such molecular units, termed "adaptors," would (1) interact with clathrin on the cytoplasmic side of the membrane, (2) recognize specifically a motif carried by a receptor, and (3) perhaps have some signal indicating to which organelle they should go. Subsequently, two distinct adaptor complexes were separated by hydroxyapatite chromatography and were referred to as HA_1 and HA_2, respectively, based on their order of elution (Pearse and Robinson, 1984; Manfredi and Bazari 1987). The HA_1 group of assembly proteins is confined to the Golgi region whereas the HA_2 group is associated with coated vesicles and pits derived from the plasma membrane (Ahle *et al.*, 1988), reflecting the multifunctional nature of these protein complexes. The current preference is to use the term "adaptor complex AP" (Ungewickell *et al.*, 1994). The AP_2 complex at the plasma membrane and in coated vesicles derived from the plasma membrane is a heterotetramer composed of two distinct $\simeq$ 100 kDa polypeptides referred to as α and $\beta 2$ respectively, a $\mu 2$, and $\sigma 2$ subunit (formally 50- and 17-kDa subunits). AP_1 is also a heterotetramer consisting of $\beta 1$ and γ subunits of $\simeq$ 100 kDa and $\mu 1$ and $\sigma 1$ (formally 47 and 19 kDa subunits). In freeze etching by electron microscopy, the AP_2 from bovine brain looks like a brick with ears (Heuser and Keen, 1988; Virshup and Bennet, 1988). The proposed arrangement of the constituent peptides is depicted in Fig 6.

Reassembly of clathrin cages form Tris extracts of clathrin-coated vesicles from carrot suspension-cultured cells has been achieved by suspending concentrated extracts in buffered dilute $CaCl_2$ and $MgCl_2$. Electron microscopy indicated the presence of polyhedric structures with baskets of 79 nm diameter (Coleman, *et al.*, 1987). Since no fractionation of the Tris extract or reassembled material was performed, there is no evidence of the involvement of AP components in the reassembly. Dialysis of a urea extract of soybean clathrin-coated vesicles resulted in the production of pelletable material which on electron microscopic examination was shown to be composed of spherical baskets of 65 nm with a polygonal alveolate lattice. Many peptides were present in the baskets (Weidenhoeft *et al.*, 1988).

Our laboratory has recently investigated the assembly and disassembly of coated vesicles from developing pea cotyledons (Butler and Beevers,

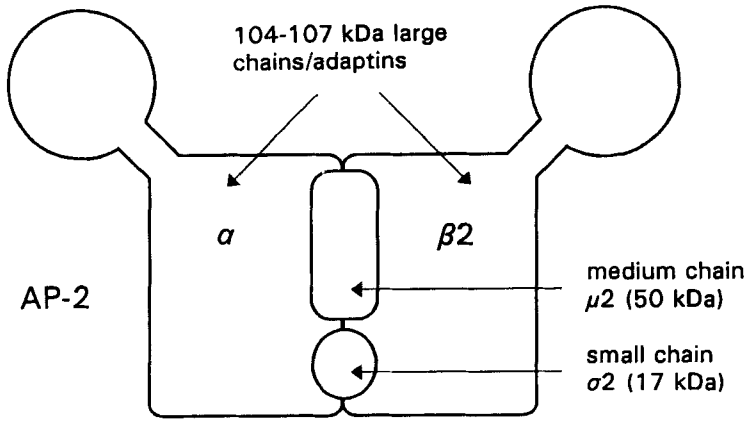
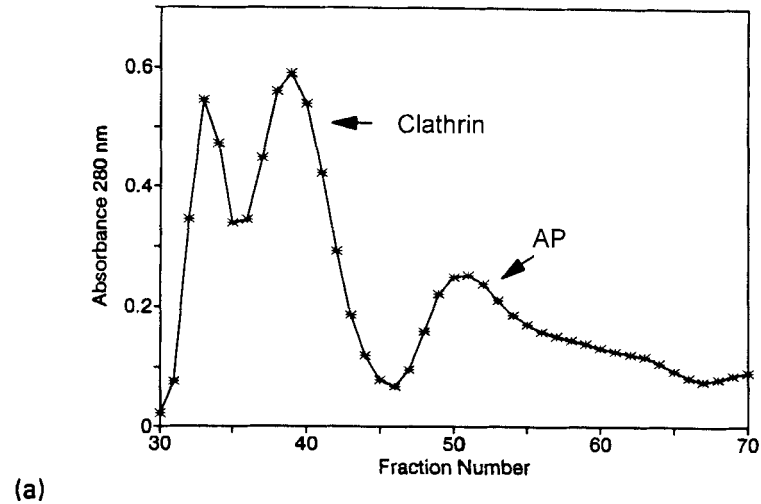


FIG. 6 Model AP-2 based on current knowledge of mammalian brain clathrin-associated proteins.

1993). The efficient removal of the vesicle coats requires a higher Tris concentration than bovine brain vesicles and has been demonstrated to be enhanced by inclusion of 0.75 *M* $MgCl_2$, as recommended by Holstein *et al.* (1994). Inclusion of $MgCl_2$ in the dissociation reduces the level of ferritin in subsequent extracts. Fractionation of the Tris extract by Superose chromatography results in the separation of three protein peaks, which is similar to the results obtained with bovine brain (Fig. 7a). SDS–PAGE indicates that peak 1 contains a range of proteins, including 190-kDa heavy chains and light chain candidates, and probably consists of undissociated vesicles. Peak 2 contains a 190-kDa heavy chain and polypeptides of 50, 46, 40, and 31 kDa which we identified previously as light chain candidates (Lin *et al.*, 1992). The third minor peak contains a range of polypeptides, including prominent bands at ≃100, 80, and 50 kDa. Electron microscopy of rotary-shadowed unfractionated Tris extract of coated vesicles demonstrated the presence of both triskelions and globular structures (Fig. 7b). In contrast, peak 2 from the Superose column contained only triskelions while peak 3 was enriched in the globoidal structures (Fig. 7c). These may be analogous to the AP complexes from bovine brain described by Heuser and Keen (1988).

When the Tris extract was dialyzed and the dialysate subjected to sucrose density gradient centrifugation, three protein peaks were produced (Butler and Beevers, 1993) (Fig. 8a). Electron microscopy indicated that peak 2 was composed of distinct reassembled coats with a mean diameter of 75 nm and vertex distance in the polyhedrons of 14.1 nm (Fig. 8b). When the reassembled coats were dissociated with Tris and refractionated by



(a)

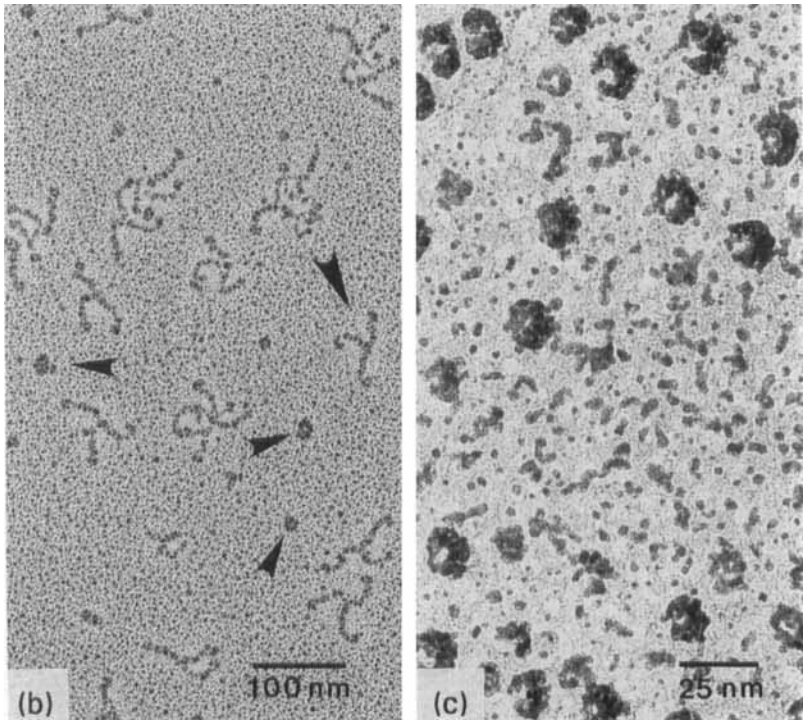
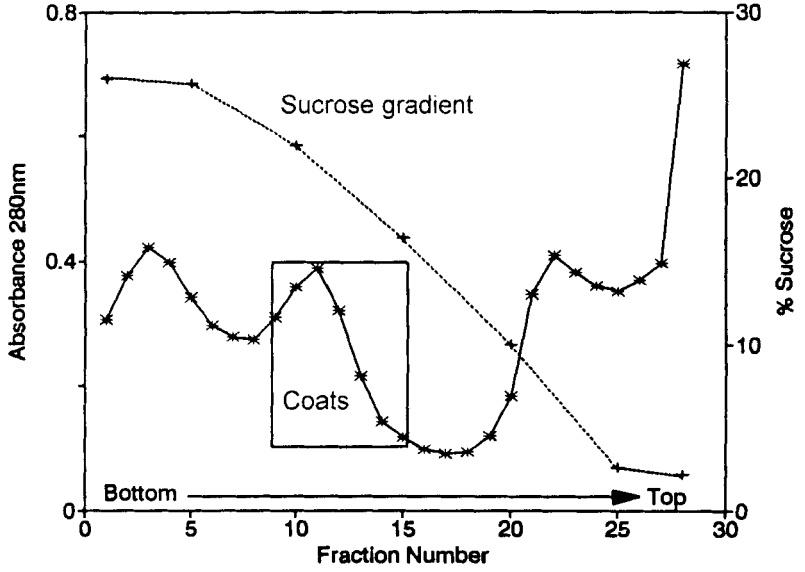


FIG. 7 (a) Superose 6 gel filtration chromatography of pea clathrin coated vesicle coat proteins isolated with 0.5 *M* Tris–HCl buffer containing 0.75 *M* $MgCl_2$. (b) Electron micrograph of pea clathrin coated vesicle coat proteins, sprayed, freeze-etched, and rotary shadowed with platinium and carbon. Note both triskelions (large arrow) and globular proteins (small arrows). Bar = 100 nm. (c) Electron micrograph of isolated globular proteins from Superose 6 AP peak treated as in (b). Bar = 25 nm.



(a)

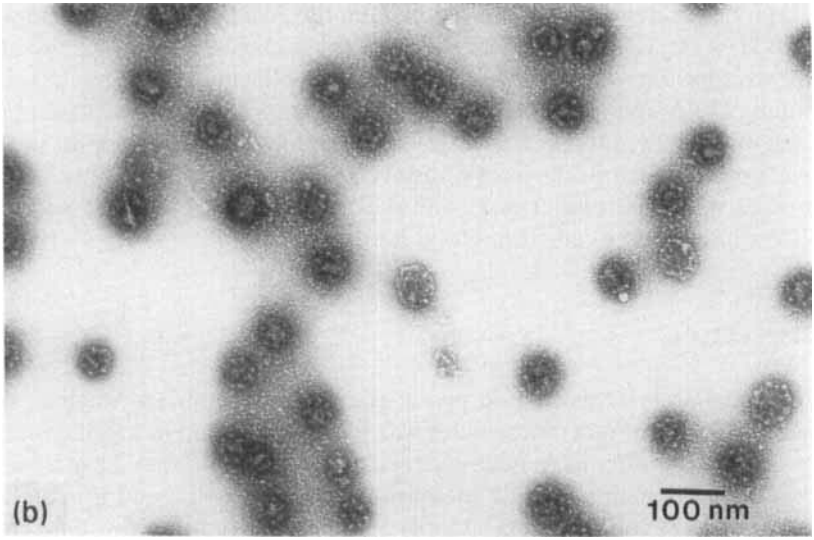


FIG. 8 (a) Sucrose gradient centrifugation of reassembled coats formed on dialysis of total pea clathrin coated vesicle coat proteins. (b) Electron micrograph of coats from peak 2 of (a) showing distinct polygonal lattice structure. Negatively stained with 2% uranyl acetate. Bar = 100 nm.

Superose chromatography, two protein peaks were obtained. The first peak contained the 190-kDa heavy chain and light chain components. The second peak demonstrated enrichment in ≃100-, 80-, and ≃50-kDa peptides. Thus in similarity to the mammalian system, the coats reassembled from Tris extracts of plant clathrin-coated vesicles incorporate peptides in addition to the clathrin constituents. Although it is tempting to speculate that these proteins would show the assembly-stimulating properties of mammalian APs, this characteristic has yet to be demonstrated. In further purification of the peak 3 component from the Superose column fractionation of the Tris extract, we can demonstrate the presence of polypeptides of an apparent M_r of 107, 100, 80, 54, 51, 18, 16, and 14 kDa, which is similar to the peptide of APs from mammalian systems.

Holstein *et al.* (1994) have recently used immunological probes to identify AP-like proteins in Tris extracts of coated vesicles prepared from zucchini hypocotyls. Fractionation of the coat proteins on Superose columns indicated that the AP complex, identified by the monoclonal antibody to the β_1, β_2 adaptin Mab-100/1, was eluted after the triskelions, indicating that the plant APs have a mass similar to those occurring in mammals. The immunoreactive polypeptide had a mass of 108 kDa and was cleaved to a 70-kDa product following hydrolysis with trypsin. Hydroxyapatite chromatography indicated that the β-adaptin from the zucchini-coated vesicle was of the HA_1 or AP_1 type. Confirmation of the presence of a β-type adaptin gene in zucchini was provided by Southern blotting experiments using genomic DNA and a β-adaptin cDNA clone from human fibroblasts. In a subsequent study, Drucker *et al.* (1995) located the β-type adaptin in the plasma membrane of cells from a variety of plant sources. This observation contrasts with their earlier report on the association of β-adaptin with the AP_1 component derived from Golgi bodies.

C. Receptors

In mammalian systems, coated pits at the plasma membrane mediate the selective uptake of macromolecules which are first bound to specific receptors. Many receptors have been characterized. Typically they have a large extracellular domain, a single membrane-spanning helix, and a cytoplasmic portion. Deletion of the cytoplasmic portion reduces the efficiency with which the receptors and ligands are internalized by endocytosis into clathrin-coated vesicles (Pearse and Robinson, 1990; Keen, 1990). In several cases the cytoplasmic domain of the receptor has been shown to contain sequences, usually containing tyrosine residues, that mediate interaction with clathrin coats and are necessary for efficient uptake (Ktistakis *et al.*, 1990; Chen *et al.*, 1990). In yeast also, clathrin acts at the plasma membrane

to selectively internalize the seven transmembrane segment receptors for mating pheromones (Tan *et al.*, 1993). The mechanism by which the receptors are concentrated into coated pits and become incorporated into clathrin-coated vesicles is not well understood but is thought to involve adaptors.

The involvement of adaptor–receptor interactions has been established in the case of sorting by the mannose-6-phosphate determinant. Enzymes bearing the mannose-6-phosphate label for lysosomal deposition interact with a 275-kDa mannose-phosphate/insulin-like growth factor II receptor in the trans-Golgi. The mannose-6-phosphate receptor complexed with lysosomal cargo protein interacts with adaptors and in time becomes surrounded by clathrin cages to form clathrin-coated vesicles. The clathrin-coated vesicles migrate to the endosome compartment. The receptor, clathrin, and adaptors are apparently recycled back to the trans-Golgi, and the mannose-6-phosphate-labeled cargo protein for lysosomal deposition is deposited in the acidic prelysosomal endosomal compartment (Kornfeld and Mellman, 1989; von Figura and Hasilik, 1986). As a consequence of this cycling, most of the mannose-6-phosphate receptor is localized in the trans-Golgi and endosome. Mannose-6-phosphate receptor is also located in the plasma membrane. Extracellular enzymes that bind to the mannose-6-phosphate receptor at the plasma membrane are concentrated into coated pits and are accumulated into clathrin-coated vesicles and eventually reach the lysosome after traversing the endosomal compartment. Thus there is a convergence of the endocytotic pathway and lysosomal delivery pathway at an endosomal site. A similar convergence of these pathways has been demonstrated in soybean protoplasts (Record and Griffing, 1988).

Evidence for receptor-mediated endocytosis in plants is sparse. A proteinaceous elictor from the fungus *Verticillium* and a polygalacturonic acid elictor are taken up by suspension-cultured soybean cells in a temperature-dependent manner. The uptake was saturable and the incorporation of radioactive elictor was completely inhibited by unlabeled elicitors. It has been suggested that biotin uptake also occurs by receptor-mediated endocytosis. Despite this evidence, to date the appropriate receptors have not been identified and their accumulation into coated pits and clathrin-coated vesicles at the plasma membrane has not been demonstrated (Low and Chandra, 1994; Hawes *et al.*, 1995).

In similarity to the lysosomal enzymes, the proteins destined for the vacuole or protein bodies in plants are synthesized with N-terminal signal sequences on membrane-bound ribosomes. After synthesis and translocation through the ER membrane, the signal sequence is removed and the proteins may undergo folding and in some cases glycosylation before transport to the Golgi apparatus. Although no direct evidence is available, it is assumed that similar to the lysosomal system, proteins are sorted at the

trans-Golgi for transport to the vacuole. Both sorting to the vacuole and retention within organelles of the secretory system (ER, Golgi) require specific targeting information. Soluble proteins lacking targeting information are by default secreted into the intracellular space (Bednarek and Raikhel, 1992). No mannose-6-phosphate containing proteins or receptors have been identified in plant systems (Gaudreault and Beevers, 1984). Instead, three types of targeting signal have been found to be involved in the sorting of proteins to the vacuole or protein body. Some proteins contain a targeting determinant in the N-terminal region; others contain targeting information in the C-terminal region; and some contain targeting determinants internally within the mature proteins (Chrispeels and Raikhel, 1992; Gal and Raikhel, 1993; Nakamura and Matsuoka, 1993). Plant vacuolar targeting signals are not recognized in yeast (Gal and Raikhel, 1993).

Since storage protein precursors, lectin precursors, and vacuolar hydrolases are found in clathrin-coated vesicles from plants (Hoh *et al.*, 1991; Harley and Beevers, 1989b; Robinson, *et al.*, 1989, 1991), it is thought that the vesicles may contain receptors capable of interacting with targeting determinants identified as necessary for sorting proteins for deposition in the vacuole. Kirsch *et al.* (1994a) prepared affinity columns bearing a peptide containing the amino acid sequence of the vacuolar targeting information of proaleurain, a proteolytic enzyme accumulated in the vacuole of barley aleurone cells. The N-terminal sequence of proendopeptidase, a secreted proteolytic enzyme, was used as control. When detergent (CHAPS) extracts of clathrin-coated vesicles from developing peas were applied to the affinity columns under appropriate ionic conditions, an 80-kDa protein was retained on the proaleurain columns. The retained protein was eluted by lowering the pH. In contrast, the 80-kDa protein was not retained on the proendopeptidase column. Thus it appears that the 80-kDa protein has affinity for vacuolar targeting sequences. This affinity has been confirmed by demonstrating that the 80-kDa protein is retained on affinity columns prepared with the sporamin-targeting ligand but not on mutant targeting sequences that *in vivo* result in sporamin secretion.

The 80-kDa protein is oriented in the clathrin-coated vesicle with a short C-terminal exposed to the cytoplasm. The ligand-binding domain is located in the N-terminal luminal portion of the protein. The protein is a glycoprotein. A cDNA clone from an *Arabidopsis* library has been identified which demonstrates predicted amino acid sequence homology to the N-terminal peptide and tryptic peptides of the 80-kDa peptide prepared from peas. The cDNA sequence indicates a coding for a 623-amino acid polypeptide, with a signal sequence, three N-linked glycosylation sites, a transmembrane domain, and a 37-amino acid cytoplasmic region.

In addition to occurring in the clathrin-coated vesicles, the 80-kDa protein is also found in a less dense membrane fraction. Although we have not

been able to positively identify this membrane, it fails to localize with Golgi or ER markers. It has a density of 1.10–1.13 g/cm^3 (24–29% sucrose), which is similar to that reported for a trans-Golgi network or endosomes in animal systems. A distribution of the 80-kDa protein in the plant endosome, PCR, or trans-Golgi would be similar to the distribution of the mannose-6-phosphate receptor in animal systems (Griffiths *et al.,* 1988).

When the lower density membrane fraction from developing pea cotyledons is incubated with Tris extracts of coated vesicles after buffer exchange to remove the Tris, the 80-kDa peptide associates with the clathrin components and sediments in sucrose density gradients at a density of 1.18–1.21 g/cm^3, which is equivalent to that of clathrin-coated vesicles (Kirsch *et al.,* 1994b). At this stage we do not know the mechanism(s) of interaction of the 80-kDa protein with components of the Tris extract, but the observation is similar to the assembly of the mannose-6-phosphate receptor into reconstituted clathrin coats reported by Pearse (1985).

It has been demonstrated in mammalian systems that adaptors are able to interact in a cell-free system with the cytoplasmic tail of the mannose-6-phosphate receptor (Pearse, 1985, 1988; Glickman *et al.,* 1989) and in turn the adaptors interact with clathrin components (Pearse and Robinson, 1990). However, the recruitment of receptors from the trans-Golgi appears to involve more than interaction with adaptors. The association of AP with Golgi membranes (and presumably receptors in the Golgi membrane) involves cytosolic fractions, guanosine triphosphate (GTP) binding proteins, and GTP (Traub *et al.,* 1993; Stamnes and Rothman, 1993), while the binding of receptors to adaptors does not require GPT (Pearse and Robinson, 1990). It is suggested that adaptor recruitment within the cell must be specifically regulated. Such regulation prevents the interaction of cytoplasmic adaptors with suitably oriented cytoplasmic domains of receptors present in the endosome, for example. Indication of the interaction of adaptors with components other than receptors is illustrated by the fact that membrane proteins of 85, 53, and 52 kDa bind specifically to AP_1 affinity columns. This binding of the proteins was promoted by $GTP_\gamma S$ and aluminum fluoride (Mallet and Brodsky, 1994).

A debate exists concerning which specific components of the adaptor complexes interact with clathrin and the membrane proteins, respectively. Traub *et al.* (1994) report that trypsin-treated γ- and β-adaptins were efficiently recruited onto Golgi membranes in a GTP and ADP-ribosylation factor (ARF)-GTP-binding protein-dependent manner. However, tryptic hydrolysis of the β_1 appendage eliminated clathrin binding. In contrast, Wang and Anderson (1994) indicate that the clathrin- and membrane-binding domains of adaptins map to the trunk or brick portion of the molecule. The adaptor appendages interacted with peptides present in detergent extracts of bovine brain.

V. Uncoating

It has been established that coated vesicles exist only transiently in the cytoplasm. It appears that the vesicles are rapidly uncoated and the vesicles and contents fuse with other membranous systems (the endosome?). A 70-kDa uncoating ATPase has been identified in plant cytosol. The enzyme was isolated by affinity chromatography on ATP-agarose columns followed by hydrophobic chromatography on phenyl-Sepharose or chromatofocusing (Kirsch and Beevers, 1993). The purified 70-kDa protein interacted with antibodies against the constitutive 70-kDa heat shock protein, demonstrating that, in similarity to mammalian and yeast cells, the uncoating protein is a member of the HSP70 family (Chappel *et al.*, 1986; Gao *et al.*, 1991). The purified ATPase from pea cotyledons uncoated clathrin vesicles from bovine brain, and bovine brain ATPase uncoated coated vesicles from developing peas. Susceptiblility of the clathrin-coated vesicles from pea was dependent upon the method of preparation. In general, vesicles prepared in the presence of sucrose were less susceptible to uncoating. Nandi *et al.* (1982) have demonstrated that long-term exposure to sucrose destabilizes clathrin-coated vesicles. Uncoating was impeded by treatment of vesicles with elastase, which was shown to partially digest the light chains. Thus uncoating appears to be dependent upon the presence of intact light chains. It has been suggested that proteolysis may account for the multiplicity of the light chain candidates detected in preparations of plant coated vesicles (Holstein *et al.*, 1994).

On the basis of the uncoating assay, it is clear that the light chains of isolated vesicles from pea cotyledon contain the uncoating ATPase binding site and have maintained some functional integrity. The observations that the uncoating ATPase from developing pea cotyledons can uncoat clathrin-coated vesicles from bovine brain indicates that the vesicles from both sources must have similar enzyme binding sites. Furthermore, the uncoating of coated vesicles from developing peas by the uncoating ATPase from bovine brain indicates a conservation of catalytic sites in the uncoating enzyme.

Studies with coated vesicles from animal systems have demonstrated that three 70-kDa uncoating ATPase molecules bind at the vertices of clathrin triskelions. The ATPase is fully active against isolated vesicles but does not affect plasma lemmal coated pits (Heuser and Steer, 1989). It has been demonstrated that the uncoating ATPase binds to a conformationally labile domain of clathrin light chain LCa to stimulate ATP hydrolysis (De Luca-Flaherty *et al.*, 1990). Characteristically, there is a rapid uncoating when ATP and HSP70 are added to clathrin-coated vesicles followed by a slow steady-state uncoating (Greene and Eisenberg, 1990). The uncoating of

synthetic clathrin baskets prepared with AP_2 requires a 100-kDa cofactor in addition to HSP70. This 100-kDa protein has been identified as auxilin, which is proposed to bind to clathrin baskets and expose the site of HSP action (Barouch *et al.*, 1994; Prasad *et al.*, 1994). Although the mechanism of uncoating is not established, it appears that ATP activates the HSP70. In this activated form the clathrin attaches and detaches very rapidly, whereas with ADP and Pi at the active site, clathrin neither binds nor dissociates from HSP70. It appears that the clathrin uncoating activity can be uncoupled from the peptide-stimulated ATPase activity (Tsai and Wang, 1994). On the basis of this accumulated evidence, it appears that ATP has a regulatory function, controlling the conformation of the enzyme rather than supplying energy to the process.

Confirmation of the role of uncoating ATPase in clathrin-coated vesicle function is provided by studies which demonstrated that antibodies against the uncoating ATPase retard receptor-mediated endocytosis when injected into cells. In the injected cells, the endocytosed ligand remained associated with clathrin and was not delivered to the endosome compartment (Honing *et al.*, 1994). Such evidence indicates that, as speculated from *in vitro* studies, the uncoating ATPase is essential for normal receptor-mediated endocytosis and is presumably involved in uncoating the coated vesicles before their fusion with endosomes. By analogy, it would be expected that the uncoating ATPase would also be necessary for the uncoating of trans-Golgi-derived vesicles.

Although the uncoating ATPase removes clathrin, it does not remove adaptors. Preliminary experiments with clathrin-coated vesicles from bovine brain indicate that AP release was dependent upon prior release of clathrin by HSP70, followed by the addition of a brain cytosol component. Fractionation of the cytosol by ion exchange and hydroxyapatite chromatography has tentatively identified a protein that catalyzes AP release (Hinshaw and Schmid, 1994).

VI. Acidification

A common feature of many receptor-mediated systems is the process of receptor recycling. Such recycling provides a mechanism by which a given receptor molecule may be used for multiple rounds of internalization as would occur during endocytosis at the plasma membrane or in the selection of cargo enzymes at the trans-Golgi. To enable recycling of unoccupied receptors to the plasma membrane or trans-Golgi, the ligand receptor complexes must dissociate within some intracellular compartment. There is an accumulating body of evidence which indicates that the ligand–receptor

dissociation is modulated by exposure of the complex to acidic pH. It is generally considered that in mammalian systems acidification is achieved through activities of a vacuolar-type H^+-ATPase (Mellman and Helenius, 1986; Forgac, 1989; Mellman, 1992). A similar vaculolar H^+-ATPase has been well characterized from plants (Sze, 1985; Sze *et al.*, 1992). Activity is highly enriched in the vacuolar membrane (tonoplast) but immunological and activity assays have also demonstrated the occurrence of the enzyme in other endomembrane components (Oberbeck *et al.*, 1994; Herman *et al.*, 1994). In addition to containing the V-type H^+-ATPase, plants also contain a proton pumping pyrophosphatase (H^+-PPase). This enzyme is also capable of generating electrochemical gradients and could provide a mechanism for the acidification of the vacuole (Rea and Sanders, 1987; Rea and Poole, 1993). Although the enzyme is located primarily in the tonoplast, immunological studies demonstrate the occurrence of the pyrophosphatase in other endomembranes (Oberbeck *et al.*, 1994).

The V-H^+-ATPases isolated from plant sources, similar to those from mammalian sources and yeast, (Stevens, 1992) are multimeric enzymes containing up to 10 subunits. There is variability in subunit composition among plants, and multiple genes for specific subunits have been identified. It has been suggested that the multiple genes may encode isoforms of the enzyme which could be differentially localized or specifically regulated during development (Sze *et al.*, 1992). In general, the V-ATPases from plants are composed of extrinsic V_1 peptides of 70, 57, 44, 42, 36, and 29 kDa and intrinsic Vo polypeptides of 100, 36, 16, 13, and 12 kDa. It is anticipated that the intrinsic polypeptides would be cotranslationally inserted into the membranes at the ER, but until recently the cellular site of addition of the peripheral extrinsic polypeptides had not been resolved. On the basis of immunological studies, V-ATPase intrinsic and extrinsic polypeptides have been located in the ER prepared from plant roots (Oberbeck *et al.*, 1994; Herman *et al.*, 1994) as well as the Golgi apparatus and clathrin-coated vesicles (Oberbeck *et al.*, 1994). The endomembranes from maize seedling roots also contained the 66-kDa subunit of H^+-pyrophosphatase (Oberback *et al.*, 1994).

On the basis of these data, it appears that the V-ATPase extrinsic and intrinsic peptides and the H^+-pyrophosphatase are assembled in the ER and then migrate through the endomembrane system for final incorporation into the tonoplast. However, the V-ATPase and pyrophosphatase are incapable of proton pumping and show little enzymatic activity in ER and Golgi preparations (Oberbeck *et al.*, 1994). No proton pumping and only low ATPase and H^+ pyrophosphatase are present in clathrin-coated vesicles (Drucker *et al.*, 1993; Lin, 1992).

Analysis of D_2O/Ficoll gradients of microsomal preparations from developing pea cotyledons demonstrates that only low V-ATPase activity is

present in the clathrin-coated vesicle fractions, but appreciable activity is associated with the less dense membrane fractions. The region of the gradient with V-ATPase activity is the same as that which demonstrated the occurrence of the 80-kDa receptor and it is speculated that it corresponds to the endosome or TGN (Lin, 1992).

The immunological and activity assays suggest that the V-ATPase and H^+-pyrophosphatase are inactive in the early stages of the secretory pathway (ER, Golgi apparatus and clathrin-coated vesicles) but are activated by the time of deposition in the tonoplast. Foreseeably, if ligand–receptor interaction is modulated by pH, the activation of the V-ATPase and pyrophosphatase could occur in the endosome (PCR) prior to eventual deposition in the tonoplast. Such speculation assumes that the membrane proteins of the vacuole follow the same pathway as soluble vacuolar or lysosomal proteins. This may not be the case. Gomez and Chrispeels (1993) have demonstrated that monensin, a monovalent carboxylic ionophore that leads to dissipation of pH gradients and alkalization of membrane compartments, inhibits transport of soluble, but not membrane-bound proteins to the vacuole. Monensin application leads to secretion of vicilin from pea cotyledon cells (Craig and Goodchild, 1984) and blocks movement of phytohemagglutinin from the Golgi compartment in beans (Chrispeels, 1983). These findings, coupled with the reported dilation of Golgi vesicles in some cells (Zhang *et al.,* 1993; Boss *et al.,* 1984), are consistent with a monensin-induced pH increase disrupting receptor-mediated selection of storage protein precursors in the Golgi compartment. In the absence of receptor-mediated selection for transport to the vacuole or protein body, the storage proteins enter the default secretory pathway and accumulate in the cell wall (Craig and Goodchild, 1984). The question arises of whether the monensin-induced alkalization counteracts acid pH in the Golgi induced by an active V-ATPase or increases the pH from neutrality in a Golgi in which the V-ATPase is inactive. Given the necessity for a pH approaching neutrality for the establishment of receptor–ligand interaction (Kirsch *et al.,* 1994a; Mellman, 1992), the latter possibility is favored. The immunological and enzyme activity data of Oberbeck *et al.* (1994) support this contention.

Much of the characterization of V-ATPase in animal systems has involved activity associated with clathrin-coated vesicles (Forgac, 1989). However, the internal acidity of such vesicles is debated. Given the necessity to establish ligand–receptor interaction at or near neutrality, it appears that at the time of vesicle formation the V-ATPase must be inactive or if active there must be modulation of pH in some manner. It has been suggested that the acidity of coated vesicles is modulated by a regulated chloride channel (Mulberg *et al.,* 1991) which could counteract the H^+ accumulated by an active V-ATPase. Similarly, a Na^+K^+-ATPase has been implicated in modulating ATP-dependent acidification (Fuchs *et al.,* 1989). Alterna-

tively, the V-ATPase activity *per se* may be regulated. In this regard it has been suggested that the association of different isoforms of specific subunits could modulate activity (Puopolo *et al.*, 1992). Intramolecular disulfide bonding in specific subunits may regulate activity (Feng and Forgac, 1992, 1994). It has also been indicated that the sulfhydryl status of the H^+-ATPase may regulate the association of the enzyme with the 50-kDa peptide of the AP_2 complex (Liu *et al.*, 1994).

Obviously investigations of the mechanism(s) of modulation of V-ATPase activity in clathrin-coated vesicles of the ER and Golgi apparatus in plant systems need to be conducted. In passing, it should be apparent that immunological localization of an enzyme to a membrane does not necessarily correspond to enzymatic activity.

VII. Prospects and Unresolved Problems

Reviews of clathrin-coated vesicles in plants (Robinson and Depta, 1988; Coleman *et al.*, 1988) indicated the much greater volume of literature on vesicles from animal systems in comparison to that from plants. For the most part, our concepts of the operation of clathrin-coated vesicles in plant cells continue to be modeled after our understanding of the function of vesicles in endocytosis and lysosomal enzyme delivery in mammalian cells. In this regard, the polyhedric alveolate structure of the vesicles and their occurrence at the patches of clathrin-coated membranes at the plasma membrane of plant cells are consistent with a role for clathrin-coated vesicles in endocytosis and/or membrane retrieval. The triskelion structure and similarity of size in heavy chains among animals, plants, and yeasts are consistent with related functions, although the significance of the larger size of the clathrin heavy chain in yeasts and plants is not immediately apparent. The distribution of light chains in plants remains enigmatic but indications point to a greater size and possible greater complexity of polypeptides than those characterized from mammalian systems. There seems to be some similarity at the level of adaptors. The preliminary immunological data and isolation information demonstrate a degree of homology between plant and mammalian systems. Clearly what is needed now is sequence information about the adaptor polypeptides isolated from plants in order to establish homology and function. The greater resistance of plant-coated vesicles to dissociation by Tris in comparison to those from bovine brain remains unexplained. Of course, the differential resistance of clathrin-coated vesicles from different tissues in mammalian systems is unresolved. The role of plant adaptors in clathrin assembly remains to be established.

At present we have no information from plants on receptors of the plasma membrane that might be endocytosed. The 80-kDa vacuolar targeting receptor identified in pea cotyledons and in *Arabidopsis* cDNA libraries shows structural homology in terms of luminal, membrane spanning, and cytoplasmic domains to the better-characterized mannose-6-phosphate receptor involved in delivery of soluble lysosomal proteins. Although progress has been made in determining ligand specificity of the vacuolar receptor from peas, to date there has been no demonstration of interaction of the receptor with endogenous proproteins delivered to the storage vacuole of pea cotyledons. Of course in light of Robinson's information on storage vacuole formation by dilation of the ER (Hoh *et al.*, 1995; Robinson *et al.*, 1995), the involvement of the other endomembrane components in delivery of storage proteins becomes diminished. Nevertheless, glycosylated proteins and the storage proteins which are retrieved must be selectively recruited from the Golgi by coated vesicles for delivery to the vacuole or protein body.

Although both C-terminal and N-terminal targeting ligands direct proteins to the same vacuole, there appear to be distinct mechanisms of selectivity. One system responsible for targeting C-terminal propeptides of barley lectin and chitinase is sensitive to inhibition by wortmannin, an inhibitor of phosphatidylinositol kinase, whereas the targeting of N-terminal propeptide of sporamin is insensitive (Matsuoka *et al.*, 1995). Significantly, prosporamin targeting sequences show affinity for the 80-kDa receptor, whereas the barley prolectin targeting sequences do not bind. Are clathrin-coated vesicles involved in the wortmannin-sensitive pathway?

Questions remain unanswered as to how clathrin vesicles are assembled on the cytoplasmic face of the plasma membrane or trans-Golgi. What constraints are imposed to ensure vesicles forming in coated pits or the trans-Golgi are not immediately uncoated by the cytoplasmic HSP70 uncoating ATPase?

How critical is acidification and how is it regulated? Based on the scenario established by the progression of measured acidification from early endosome → late endosome → lysosome in mammalian systems, it is anticipated that clathrin-coated vesicles would not be acidified yet they contain peptides of the V-ATPase and H^+-PPase which are responsible for acidification of other endomembrane systems. Yeast mutants deficient in components of V-ATPase demonstrate unhindered delivery of vacuolar proteins (Raymond *et al.*, 1992), but the monensin inhibition studies from plants indicate a necessity to maintain appropriate pHs in the endomembrane compartments.

Ultrastructural studies indicate that in endocytosis clathrin-coated vesicle contents are delivered to the PCR and multivesiculate bodies prior to accumulation in the vacuole. Vacuolar proteins and endocytosed materials are found in the same endomembrane compartment. These compartments have been identified at the ultrastructural level but we have no biochemical

information. Ultrastructural studies aimed at demonstrating the origin of protein bodies or storage vacuoles seldom demonstrate the fusion of coated vesicles with membranous structures. When fusion is observed, the vesicles fusing with storage vacuoles are much larger than clathrin coated vesicles.

In the better-studied early vesicular traffic from ER to Golgi and between Golgi stacks in mammalian systems, the role of low molecular weight GTP-binding proteins is well established. There is increasing evidence for the requirement of specific GTP-binding proteins for fusion processes in other endomembrane components (Balch, 1990; Nuoffer and Balch, 1994; Pfeffer, 1992). Genes for small GTP-binding proteins are being reported in plants (Ma, 1994; Nagano *et al.,* 1993; Terryn *et al.,* 1993). The products of some of these genes may be useful markers for endomembranes in plants and perhaps help indentify the membranes bearing the 80-kDa receptor and compartments into which clathrin-coated vesicles deposit their cargoes.

Increasingly, gene libraries are being established from plant sources. It is possible that this information will identify plant proteins homologous to those of clathrin constituents of mammalian or yeast systems. By applying improved isolation protocols pioneered by Robinson and co-workers, we now have the capability to reproducibly recover highly purified vesicles from plant sources. It is now possible to conduct biochemical studies. We have answered some of the questions raised by Robinson and Depta in their 1988 review. Plants do possess uncoating ATPase. Clathrin-coated vesicles from plants possess assembly polypeptides, receptors, and V-H^+ ATPase. A combination of biochemical and molecular approaches which are now available will rapidly advance our understanding of the structure and function of clathrin-coated vesicles from plants and permit detailed comparisons with vesicles from yeast and animal sources.

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Peptides in the Nervous Systems of Cnidarians: Structure, Function, and Biosynthesis

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Cnidarians are the lowest animal group having a nervous system and it was probably within this phylum or in a related ancestor group that nervous systems first evolved. The primitive nervous systems of cnidarians are strongly peptidergic. From a single sea anemone species, *Anthopleura elegantissima,* 17 different neuropeptides have been isolated so far, and we expect that many more neuropeptides (more than 30) must be present. All peptides are localized in neurons of cnidarians and we have demonstrated the presence of some of the peptides in neurosecretory dense-cored vesicles. Most neuropeptides have an excitatory or inhibitory action on whole cnidarians, muscle preparations, and isolated muscle cells, suggesting that these peptides are neurotransmitters or neuromodulators. One neuropeptide induces metamorphosis in planula larvae to become a polyp. This shows that cnidarian neuropeptides also are involved in developmental processes, such as cell differentiation and pattern formation. We have cloned the preprohormones for most of the cnidarian neuropeptides. These preprohormones have a high copy number of the immature neuropeptide sequence, which can be up to 37 neuropeptide copies per precursor molecule. In addition to well-known, "classical" processing enzymes, novel prohormone processing enzymes must be present in cnidarian neurons. These include a processing enzyme hydrolyzing at the C-terminal sides of acidic (Asp and Glu) residues and a dipeptidyl aminopeptidase digesting at the C-terminal sides of N-terminally located X-Pro and X-Ala sequences. All this shows that the primitive nervous systems of cnidarians are already quite complex, and that neuropeptides play a central role in the physiology of these animals.
KEY WORDS: Neurotransmitter, Neuropeptide, Preprohormone, Post-translational modification, Nervous system, Metamorphosis, Development, Cnidaria.

I. Introduction

Cnidarians are very primitive animals, and include sea anemones, corals, jellyfishes, and the freshwater polyp *Hydra.* The phylum of Cnidaria can be subdivided into four classes: the Hydrozoa (e.g., *Hydra*), Cubozoa (box jellyfishes), Scyphozoa (true jellyfishes), and Anthozoa (e.g., sea anemones and corals). Members of the first three classes have frequently a life cycle that includes a polyp, a medusa, and a planula larva stage. The medusa is usually produced by budding from a polyp stock (e.g., in Hydrozoa). The sexually mature medusa produces sperm and oocytes from which, after fertilization, a planula larva originates that undergoes metamorphosis to become a polyp again. Members of the Anthozoa have only a polyp and a planula larval stage. The above description is, of course, a generalization and there exist numerous variations on this general scheme. The freshwater polyp *Hydra,* for example, normally reproduces by budding off small polyps from the middle of its body column, and it is possible to culture *Hydra* in the laboratory for many decades and harvest millions of (cloned) animals without the animal having any sexual reproduction. It could even be that *Hydra,* kept under these laboratory conditions, is immortal. *Hydra,* however, can also be made sexual and in this case the polyp itself produces sperm or oocytes. No medusa stage exists in *Hydra.*

Cnidarians can live as individuals or as colonies. A colony may consist of only polyp forms, or of mixed polyps and medusae. The colonies may be sessile, such as in corals, or free-living, as is the case with siphonophores (e.g., "Portuguese man-of-war"). Siphonophores are pelagic, swimming or floating, colonial hydrozoans consisting of a central stem or disk to which pneumatophores (float), swimming bells (medusae), and a variety of modified medusae and polyps are attached.

Cnidarians are not only beautiful and impressive organisms, but they are also interesting to biologists as experimental animals for the following reasons. First, cnidarians have an anatomically simple nervous system and only a few, simple, behavioral patterns, which, in the case of hydromedusae, include swimming, feeding, and a special type of defensive behavior ("crumpling"). In hydromedusae, it is also easy to do intracellular recordings of neurons or muscle cells, even in intact or semi-intact animals. This makes the hydromedusae a good model system to study the cellular basis of behavior (Anderson and Schwab, 1982; Spencer and Arkett, 1984; Anderson and Spencer, 1989). Second, *Hydra* and other hydrozoan polyps have an amazingly high regeneration capacity: from slices of *Hydra,* or even dissociated and subsequently reaggregated cells, new animals develop within a few days (Gierer *et al.,* 1972; Gierer, 1977). *Hydra* has only a few (about 5–10) cell types and the animal can be easily manipulated. For

example, it is possible to create *Hydra* that consist of only one cell type, the epithelial cells (Campbell, 1976; Sugiyama and Fujisawa, 1978; Lepault *et al.,* 1980). These "epithelial" animals can bud and, therefore, be cultured if fed by hand. It is possible to introduce stem cells into the "epithelial" *Hydra* by implantation, and these stem cells then develop into their normal product cells, among them nerve cells. In this way it is possible to study the differentiation and development of a completely new nervous system in an originally nerveless animal (Minobe *et al.,* 1995). By inbreeding, it is also possible to obtain mutants from *Hydra* that have developmental defects and that are, for example, multiheaded or deficient in one of the cell types (Sugiyama and Fujisawa, 1977, 1978). For all of these reasons, *Hydra* and other hydropolyps are often used as model organisms by developmental biologists. Finally, nervous systems probably evolved in cnidarians or in a closely related ancestor (Mackie, 1990). This makes cnidarians interesting from an evolutionary point of view.

II. Anatomy of the Cnidarian Nervous System

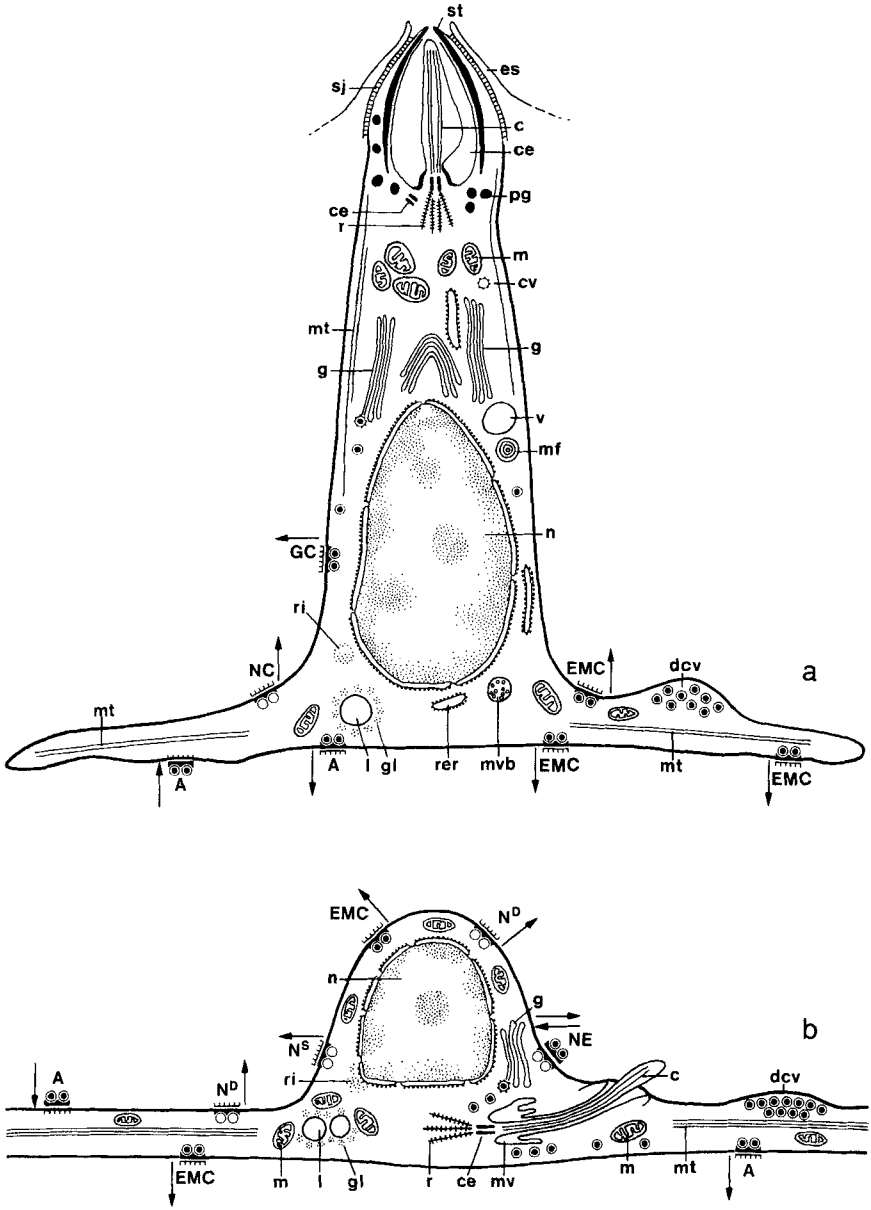
The basic organization of the cnidarian nervous system is that of a nerve net. At some locations, however, this nerve net has condensed to form a nerve plexus, or longitudinal or circular nerve tracts. An example of a longitudinal nerve tract is the "giant axon" in the stem of physonectid siphonophores. This nerve tract consists of fused neurons and enables, by virtue of its enlarged diameter, a fast signal transduction and, consequently, a fast escape reaction (Mackie, 1973, 1984; Grimmelikhuijzen, *et al.,* 1986). Examples of circular condensations are the inner and outer nerve rings at the bell margin of hydrozoan medusae. These rings, which consist of electrically coupled neurons, are capable of integrating a variety of sensory imputs and transmitting these signals rapidly throughout the margin (Spencer and Arkett, 1984).

In addition to a nerve net, nerve plexuses or nerve tracts, many hydro-, cubo- and scyphomedusae have light- and gravity-sensitive organs, which may have evolved in response to their free-living life style. The light-sensitive organs (ocelli) of hydromedusae are found at the bases of the marginal tentacles. These ocelli consist of a cluster of light-sensitive neurons that are often embedded in a cup of pigmented cells. The light-sensitive neurons of the ocelli are connected by two or more nerve tracts to the outer nerve ring of the bell margin, where their signals are integrated and transmitted to the motor neurons of the inner nerve ring (Spencer and Arkett, 1984; Grimmelikhuijzen and Spencer, 1984). In cubo- and scyphomedusae, the ocelli are often contained in larger organs, the rhopalia

that also harbor gravity-sensitive structures, the statocysts. These statocysts are pits or hollow structures filled with calcareous material and are surrounded by neurons that are connected to the nerve net of the bell. The statocyst neurons have a pacemaker function related to swimming (Horridge, 1954, 1956). In contrast to medusae, the sessile polyp forms do not have specialized sense organs.

The anatomy of the nervous system in cnidarians has been studied by both light and electron microscopy. It has always been difficult to see the nervous system using light microscopy because, until recently, no good histological method has been available that stains cnidarian neurons well. The best stain has been methylene blue, which was, in fact, already used about a century ago by Schneider (1890) and Hadži (1909). Using methylene blue staining, Schneider and Hadži found that two types of neurons exist in *Hydra* and other cnidarians: (1) The "sensory cells" are long, slender neurons oriented perpendicular to the mesoglea (an acellular layer of collagen located between the two cnidarian cell layers, ectoderm and endoderm), and project to the surface of one of the two cell layers. These sensory neurons are equipped with a cilium that extends into the outer medium, or into the lumen of the gastric cavity. (2) The "ganglion cells" are roundish neurons located in the more basal parts of either ectoderm or endoderm. Using electron microscopy, Westfall and co-workers have found that there is no essential, anatomical difference between sensory cells (Fig. 1a) and ganglion cells (Fig. 1b) in *Hydra* (Westfall, 1973a; Westfall and Kinnamon, 1978). Both types of neurons are apparently multifunctional, and contain a cilium (are "sensory"), store secretory dense-cored vesicles at nonsynaptic loci (are "neurosecretory"), make chemical synapses with epitheliomuscular cells (are "motorneurons"), and form synaptic contacts

FIG. 1. Schematic drawings of neurosecretory motor interneurons of *Hydra.* (a) Sensory cell from the tentacle showing neurosecretory dense-cored vesicles (dcv), a sensory cilium (c), and polarized synapses with epitheliomuscular cells (EMC), ganglion cells (GC), "en passant" axons (A) and nematocytes (NC). Other abbreviations are ce, paired centrioles; cv, coated vesicles; es, epitheliomuscular cell sheath; g, Golgi complex; gl, glycogen; l, lipid droplets; m, mitochondria; mf, myelin figure; mt, microtubules; mvb, multivesicular body; n, nucleus; pg, pigment granules; r, rootlets; rer, rough endoplasmic reticulum; ri, ribosomes; sj, septate junction; st, stereocilia; and v, vacuoles. (Modified from Westfall and Kinnamon, 1978.) (b) Ganglion cell from the tentacle showing, again, neurosecretory dense-cored vesicles (dcv), a sensory cilium (c), and polarized synapses with epitheliomuscular cells (EMC), axons (A), different types of nematocytes (N^S, N^D) and two-way, somatic, interneuronal synapses (NE). Other abbreviations are ce, paired centrioles; g, Golgi complex; gl, glycogen particles; l, lipid droplet; m, mitochondria; mt, microtubules; mv, microvilli; n, nucleus; r, rootlets; and ri, ribosomes. (Modified from Westfall, 1973a.)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with two or more other neurons (are "interneurons") (Figs. 1a and b). Westfall proposes that these primitive, multifunctional neurons are the ancestors of the more specialized neurons that we find in higher animals today. It is still uncertain, however, whether all cnidarian neurons have the same multifunctional properties as the neurons in *Hydra.* It is possible that many cnidarian neurons have these multifunctional features, but that, in addition, other neurons also exist that have only three, two, or one of the above-mentioned properties available, and are, for example, only sensory-motor, or interneurons. This is supported by recent scanning electron microscopy of dissociated neurons from the sea anemone *Calliactis parasitica,* showing that some ganglion cells lack the cilium observed in *Hydra* ganglion cells (J. A. Westfall, unpublished).

The existence of neurons in cnidarians having both sensory and motor functions suggests that these animals must have a reflex arc which is even simpler than the well-known monosynaptic reflex arc from mammals. Whereas in mammals at least two neurons are needed for this reflex (one sensory and one motor neuron), only one single sensory-motorneuron would be sufficient in *Hydra.* The function of this single, multifunctional neuron might be the control of local contractions, for example, those occurring during fishing and catching a prey (local tentacle shortening) and feeding (mouth opening).

III. Neurotransmission

Neuronal dense-cored vesicles (70–150 nm) associated with synaptic and nonsynaptic release sites have been found in many cnidarian species (Horridge and Mackay, 1962; Jha and Mackie, 1967; Westfall, 1973a,b, 1987; Westfall and Kinnamon, 1978, 1984; Quaglia and Grasso, 1986). This anatomical evidence for the presence of chemical synapses and nonsynaptic, chemical (paracrine) neurotransmission in the cnidarian nervous system has been confirmed by electrophysiological experiments. During intracellular recordings at both pre- and postsynaptic neurons of hydro- and scyphomedusae, excitatory postsynaptic potentials (EPSPs) were found with the expected, constant latency from the presynaptic spikes (Spencer, 1982; Spencer and Arkett, 1984; Anderson, 1985; Anderson and Spencer, 1989). Furthermore, neurotransmission was blocked after depletion of Ca^{2+} from the incubation medium, or after addition of excess of Mg^{2+}, indicating that classical, exocytotic release of neurotransmitter substances occurs (McFarlane, 1973; Satterlie, 1979; Spencer, 1982).

Several chemical synapses in the cnidarian nervous system are anatomically bidirectional, with synaptic vesicles found at both sides of the synapse

(Horridge and Mackay, 1962; Westfall, 1973a,b; Westfall and Kinnamon, 1984; Anderson and Grünert, 1988). Bidirectional or two-way synapses are especially frequent in the nerve nets of scyphomedusae, where they, indeed, transmit excitation in both directions: intracellular, transsynaptic recordings have shown that an action potential in one cell evokes an EPSP in the other cell with the same synaptic delay (1 msec), irrespective of which of the two cells is stimulated (Anderson, 1985; Anderson and Spencer, 1989). Bidirectionality could be responsible for the diffuse (nondirected) conduction that is often seen in cnidarian nerve nets, but bidirectional synapses are apparently not an absolute requirement and diffuse conduction can also be explained by other means (Josephson *et al.,* 1961; Anderson and Spencer, 1989).

In addition to chemical synapses, electrical synapses have also been found in the cnidarian nervous system, but so far this has only been observed in members of the Hydrozoa. Electrical synapses have been demonstrated by electrical and dye coupling between neurons and by the presence of structures that were similar to gap junctions (Anderson and Mackie, 1977; Spencer, 1978, 1979; Spencer and Satterlie, 1980; Westfall *et al.,* 1980; Spencer and Arkett, 1984). We have already mentioned that in some cases, such as in the nerve net and "giant axon" of the stem of physonectid siphonophores, the neurons have apparently fused with each other to form true syncytia (Mackie, 1973; Grimmelikhuijzen *et al.,* 1986). The presence of neuronal syncytia may be more common in the Cnidaria than has been realized so far and must be suspected wherever nerve tracts, nerve rings, or "giant axons" are observed. The first step in the formation of a neuronal syncytium may be the formation of gap junctions, followed by fusion and degradation of the adjacent neuronal cell membranes (cf. Mackie *et al.,* 1988; Mackie, 1989). Thus, the presence of electrical coupling, dye coupling, and gap junctions in, for example, the inner nerve ring of hydromedusae (Anderson and Mackie, 1977; Spencer, 1978, 1979; Spencer and Satterlie, 1980) could also mean that this nerve ring is in fact a syncytium.

IV. Neuropeptides

In general, it has been accepted that cnidarian neurons use neurotransmitters or locally acting (paracrine) hormones for signal transmission. For a long time, however, the nature of these transmitter substances has remained unknown. We ourselves have been unable to demonstrate catecholamines, serotonin, or acetylcholine in neurons of *Hydra* (Grimmelikhuijzen, 1986). These negative results have been confirmed by other researchers (O. Koizumi, personal communication). This means that the monoamines and ace-

tylcholine do not generally occur in the nervous systems of cnidarians and suggests that classical transmitters may not be the evolutionarily "oldest" neurotransmitters.

Several years ago, we showed that antisera against the molluscan neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide; Price and Greenberg, 1977) and especially against its C-terminal fragment Arg-Phe-NH_2 (RFamide) stain neurons in all classes of Cnidaria (Grimmelikhuijzen *et al.*, 1982, 1986, 1987, 1988a, 1989a,b, 1992a; Grimmelikhuijzen, 1983a, 1985; Grimmelikhuijzen and Spencer, 1984; Anderson *et al.*, 1992). These results have been confirmed by other research groups (Mackie *et al.*, 1985, 1988; Koizumi and Bode, 1986; Plickert, 1989; Weber, 1989; Koizumi *et al.*, 1992; Kroiher and Plickert, 1992; Martin, 1992; Minobe *et al.*, 1995). Staining with FMRFamide/RFamide antisera does, of course, not mean that FMRFamide is the transmitter substance in cnidarians; it only indicates that substances are present in cnidarian neurons that have structural similarities with the sequence FMRFamide or RFamide. Many cnidarians, such as hydrozoan polyps and medusae and their planula larvae, are transparent and can be stained as whole mounts by the RFamide antisera (Grimmelikhuijzen and Spencer, 1984; Grimmelikhuijzen, 1985; Grimmelikhuijzen *et al.*, 1986). This immunocytochemical staining method is superior to the methylene blue staining method of Schneider (1890) and Hadži (1909) and gives us a much clearer picture of the organization of the cnidarian nervous system than has previously been possible. Naturally, only a portion of all cnidarian neurons may be stained by the RFamide antisera (see also below), but this same drawback holds for any other staining method.

After the classic work by Hadži (1909), it was generally believed that *Hydra* and other hydroid polyps have a diffuse, loosely interconnected nerve net with no forms of centralization. This concept is still presented in most of the introductory zoology textbooks (Figs. 2a and b). In contrast to this picture, which held for over 70 years, however, staining with RFamide antisera shows that *Hydra vulgaris* (formerly called *Hydra attenuata*) has a strong agglomeration of sensory neurons and processes in the hypostome (around the mouth opening), and a densely packed collar of neurons in the peduncle (near the foot) (Fig. 2c, Fig. 3a). In a related species, *Hydra oligactis,* there is an obvious nerve ring lying at the border of hypostome and tentacles (Fig. 2d). These are clear examples of neuronal centralizations that have not been demonstrated in their full extent before using conventional histological techniques or electron microscopy (cf. Davis *et al.*, 1968; Kinnamon and Westfall, 1981; Matsuno and Kageyama, 1984). After staining with the RFamide antisera, polyps of the marine hydroid *Hydractinia echinata* turned out to have a very dense neuronal plexus in the body column and an aggregation of sensory neurons around the mouth opening (Fig. 3b). Thus, the density and complexity of the hydropolyp nervous

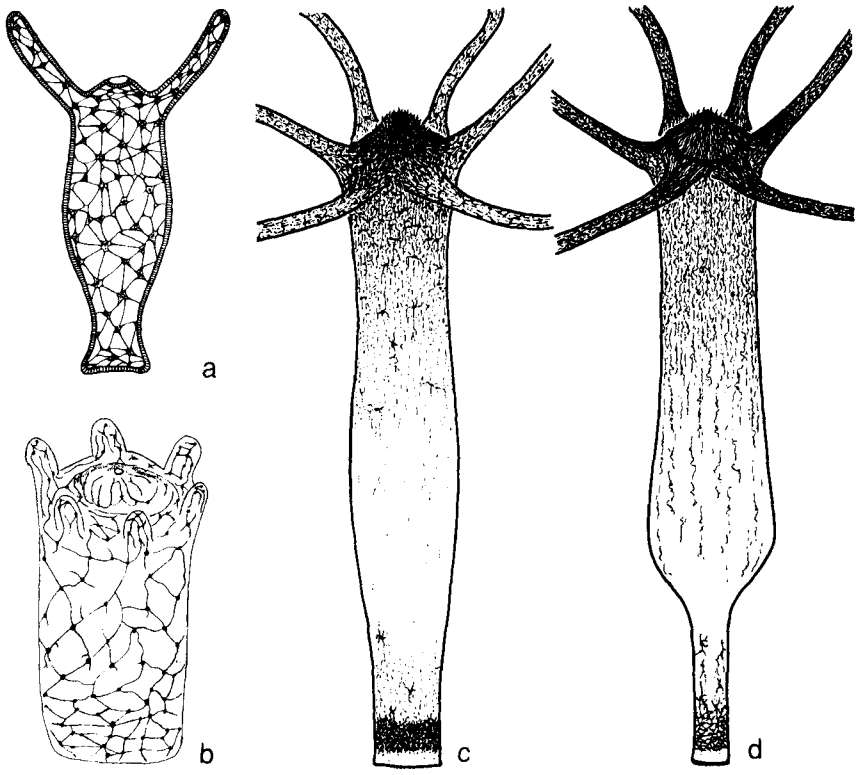

FIG. 2. Drawing showing the nervous system of *Hydra*. (a) From a zoology textbook by Marshall and Williams (1972); a similar picture is given in a textbook by Brusca and Brusca (1990). The nervous system is presented as a diffuse nerve net, with no regions of centralization. (b) From a zoology textbook by Barnes (1968). Again, only a diffuse, loosely interconnected nerve net is shown. (c) The nervous system of *Hydra vulgaris* stained with an antiserum against the sequence RFamide. Two centralizations occur: a strong agglomeration of sensory neurons and processes around the mouth opening and a densely packed collar of neurons in the peduncle. (From Grimmelikhuijzen, 1985.) (d) The nervous system of *Hydra oligactis* stained with an antiserum against RFamide. This species has a dense plexus of immunoreactive neurites in the hypostome, a cluster of sensory neurons around the mouth opening, and a nerve ring between hypostome and tentacle bases. The collar of neurons in the peduncle is less well developed. (From Grimmelikhuijzen, 1985.) Reproduced with permission from the publishers.

system has long been underestimated and this becomes even more true if one realizes that the RFamide-positive neurons form only a subpopulation of the total polyp nervous system and that additional, peptidergic neurons must exist (Grimmelikhuijzen, 1983b; Koizumi and Bode, 1991; Koizumi *et al.*, 1992).

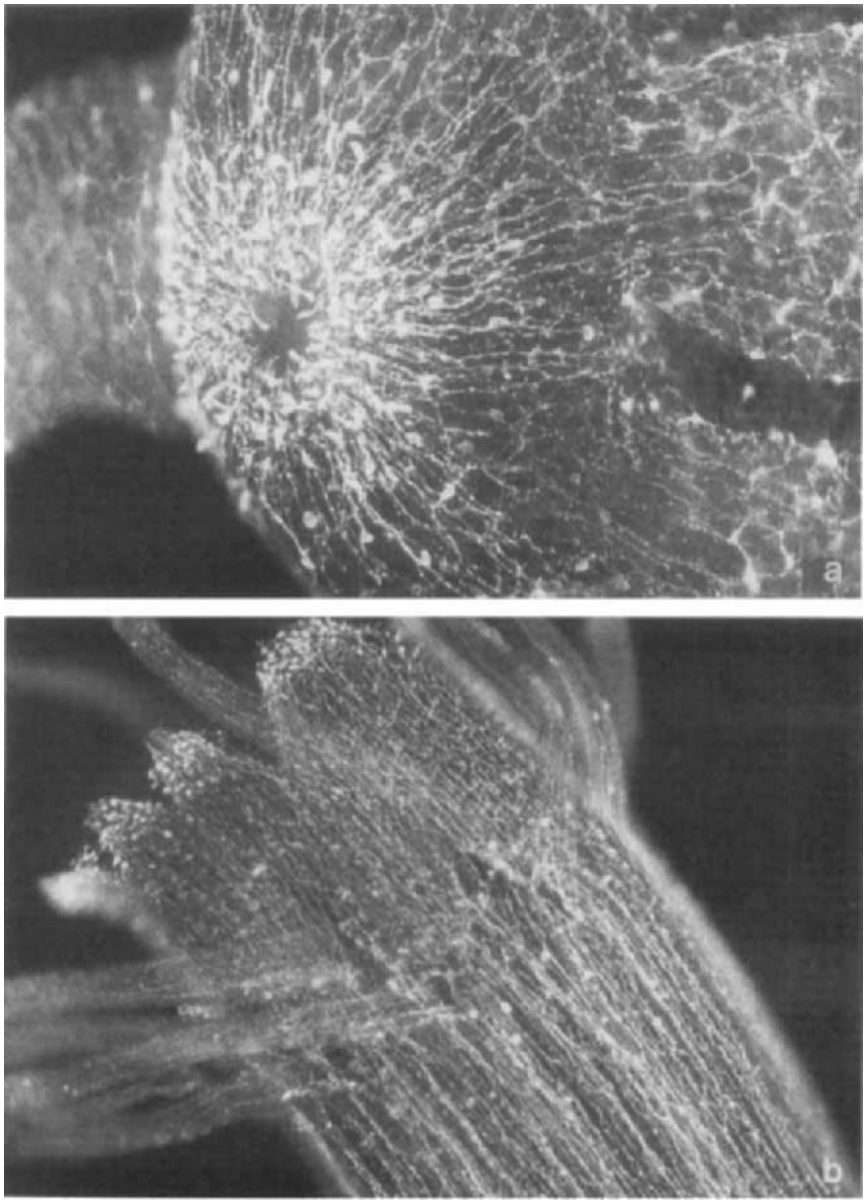

FIG. 3. Whole-mount staining of hydroid polyps with an RFamide antiserum. (a) Staining of the nervous system in the hypostome of *Hydra vulgaris*. Numerous sensory cells form a cluster around the mouth opening. ×180 (b) the nervous system in the head and body column of an adult gastrozooid (feeding polyp) of *Hydractinia echinata*. A very dense plexus of immunoreactive processes occurs throughout the polyp, and numerous sensory neurons are present around the mouth opening (the mouth area has been broken by pressure of the cover slip). ×130 (Both figures are adapted from Grimmelikhuijzen, 1985.)

A story similar to that of the hydroid polyps can be given for other members of the Hydrozoa (Grimmelikhuijzen and Spencer, 1984; Grimmelikhuijzen *et al.*, 1986), for Scyphozoa (Anderson *et al.*, 1992), Cubozoa and Anthozoa (C. J. P. Grimmelikhuijzen, unpublished). The nervous system of sea anemones, for example, has always been described as a diffuse, widely meshed nerve net, even in the most respectable zoology textbooks (Fig. 4a). The truth is, however, that such pictures are wrong, and that sea anemones have amazingly dense and highly complex nerve plexuses in the oral disk (Fig. 4b), tentacles and body column (C. J. P. Grimmelikhuijzen, unpublished).

Staining with RFamide antisera is not only an excellent technique to visualize a major portion of the cnidarian nervous system, but it also gives

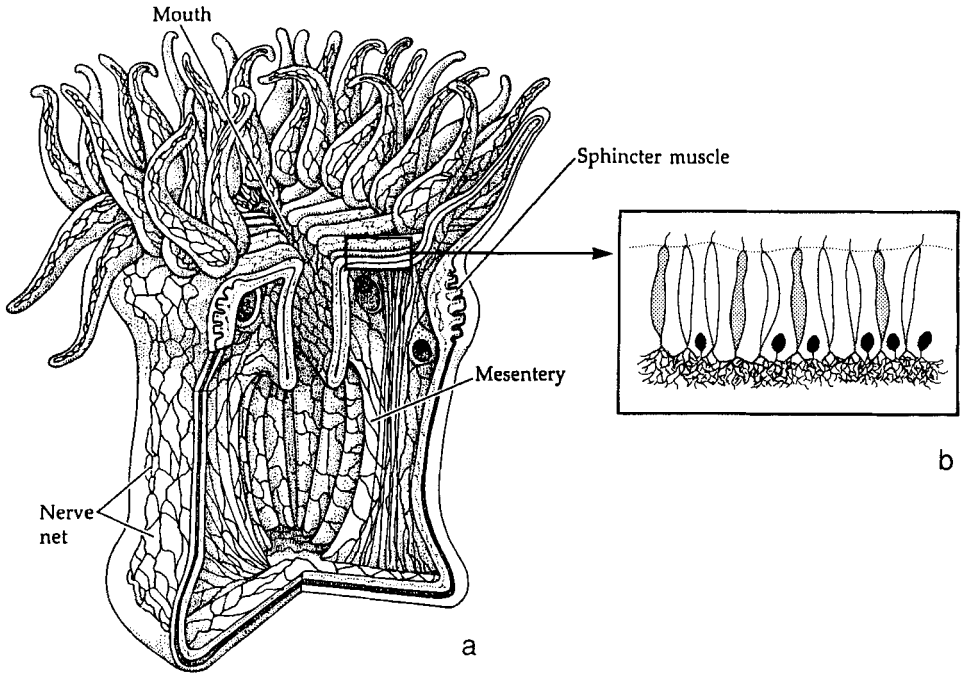

FIG. 4. Drawings showing the nervous system of sea anemones. (a) From a textbook of invertebrate zoology by Brusca and Brusca (1990), reprinted with permission. The nervous system is presented as widely meshed, uniformly distributed nerve net. (b) The nervous stystem in the ectoderm of the oral disk as it appears after staining with three antisera: against Antho-RFamide (black ganglionic neurones), Antho-RWamides I and II (white sensory neurons), and the Antho-RPamides (spotted sensory neurons) (see also Table 1). The nervous system in the oral disk is a complex organization of different types of neurons with a dense plexus of processes in the basal part of the ectoderm.

a clear indication of the neurotransmitter substances being used. In order to isolate these substances, we have developed a radioimmunoassay against the two-amino acid sequence RFamide. Using this assay as a monitoring system, we have purified a novel neuropeptide, < Glu-Gly-Arg-Phe-NH_2 (Antho-RFamide), from extracts of the sea anemone *Anthopleura elegantissima* (Grimmelikhuijzen and Graff, 1986). Antho-RFamide has also been isolated from the sea pansy *Renilla köllikeri* and appears to occur generally in the Anthozoa (Grimmelikhuijzen and Groeger, 1987). Antho-RFamide has, like many other neuropeptides from higher animals, an amidated C terminus and an N-terminal pyroglutamyl (< Glu) group. These groups, in addition to having other functions (e.g., in receptor binding), also protect the peptide against nonspecific carboxy- and aminopeptidases.

Using our RFamide radioimmunoassay, we have also isolated four RFamide neuropeptides from *Hydra* (A. Moosler and C. J. P. Grimmelikhuijzen, unpublished), two RFamide peptides from the hydromedusa *Polyorchis penicillatus* (Grimmelikhuijzen *et al.*, 1988b, 1992b), and three RFamide peptides from the scyphomedusa *Cyanea lamarckii* (A. Moosler and C. J. P. Grimmelikhuijzen, unpublished). The two *Polyorchis* peptides are given in Table I. These and all the other isolated cnidarian RFamide peptides

TABLE I
Neuropeptide Families in Cnidarians

Species	Structure	Name
Anthopleura elegantissima	L-3-phenyllactyl-Phe-Lys-Ala-NH_2	Antho-KAamide
Anthopleura elegantissima	L-3-phenyllactyl-Tyr-Arg-Ile-NH_2	Antho-RIamide I
Anthopleura elegantissima	Tyr-Arg-Ile-NH_2	Antho-RIamide II
Anthopleura elegantissima	L-3-phenyllactyl-Leu-Arg-Asn-NH_2	Antho-RNamide I
Anthopleura elegantissima	Leu-Arg-Asn-NH_2	Antho-RNamide II
Anthopleura elegantissima	<Glu-Ser-Leu-Arg-Trp-NH_2	Antho-RWamide I
Anthopleura elegantissima	<Glu-Gly-Leu-Arg-Trp-NH_2	Antho-RWamide II
Anthopleura elegantissima	<Glu-Asn-Phe-His-Leu-Arg-Pro-NH_2	Antho-RPamide II
Anthopleura elegantissima	Leu-Pro-Pro-Gly-Pro-Leu-Pro-Arg-Pro-NH_2	Antho-RPamide I
Anthopleura elegantissima	Gly-Pro-Hyp-Ser-Leu-Phe-Arg-Pro-NH_2	Antho-RPamide IV
Anthopleura elegantissima	<Glu-Val-Lys-Leu-Tyr-Arg-Pro-NH_2	Antho-RPamide III
Anthopleura elegantissima	Tyr-Arg-Pro-NH_2	Antho-RPamide V
Anthopleura elegantissima	<Glu-Gly-Arg-Phe-NH_2	Antho-RFamide
Renilla köllikeri	<Glu-Gly-Arg-Phe-NH_2	Antho-RFamide
Polyorchis penicillatus	<Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH_2	Pol-RFamide I
Polyorchis penicillatus	<Glu-Trp-Leu-Lys-Gly-Arg-Phe-NH_2	Pol-RFamide II

are structurally related and have the C-terminal sequence Gly-Arg-Phe-NH_2 in common (Table I). Thus, the Gly-Arg-Phe-NH_2 peptides appear to be ubiquitous in the Cnidaria.

During the purification of the sea anemone neuropeptide Antho-RFamide, we found other components that were weakly immunoreactive in our RFamide radioimmunoassay. Two of these components are the closely related peptides < Glu-Ser-Leu-Arg-Trp-NH_2 (Antho-RWamide I) and < Glu-Gly-Leu-Arg-Trp-NH_2 (Antho-RWamide II) (Graff and Grimmelikhuijzen, 1988a,b). A third component was only very weakly immunoreactive, and its structure turned out to be L-3-phenyllactyl-Leu-Arg-Asn-NH_2 (Antho-RNamide) (Grimmelikhuijzen *et al.*, 1990). The L-3-phenyllactyl group in Antho-RNamide is a novel N terminus that was not identified earlier in neuropeptides or peptide hormones from higher animals. There is no free amino group connected to the α-C atom (C2) of the L-3-phenyllactyl residue (Fig. 5) and therefore there is no possibility for a positive charge (NH_3^+) at this position at neutral pH. Thus, the L-3-phenyllactyl group renders the peptide resistant against nonspecific aminopeptidases, and it is a new way in which nature protects biologically active peptides against degradation. Of course, one could wonder why coelenterates (and perhaps also other animals) use two different protective residues for the N termini of their peptides. It is possible, however, that certain peptide receptors do not accept an aliphatic (< Glu), but require an aromatic (L-3-phenyllactyl) N terminus for peptide binding.

All peptides discussed so far have the C-terminal sequence Arg-X-NH_2 (where X is Phe, Trp, or Asn). In order to find out whether additional

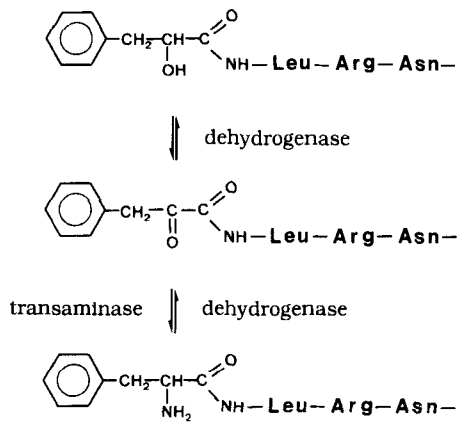

FIG. 5. A possible pathway for the biosynthesis of the L-3-phenyllactyl group (top) from an N-terminal phenylalanyl residue (bottom). (Adapted from Grimmelikhuijzen *et al.*, 1990.)

peptides exist in cnidarians that have a C-terminal Arg-X-NH_2 sequence, we raised antisera against a whole variety of Arg-X-NH_2 dipeptides and checked whether neurons could be stained in sections of sea anemones. With some of the antisera we obtained a very strong staining, and in these cases we developed a radioimmunoassay for the dipeptide in question and tested sea anemone extracts for the presence of immunoreactive material. In this way, we isolated altogether 16 different neuropeptides from extracts of the sea anemone *Anthopleura elegantissima* (Grimmelikhuijzen and Graff, 1986; Graff and Grimmelikhuijzen, 1988a,b; Grimmelikhuijzen *et al.*, 1990; Nothacker *et al.*, 1991a,b; Carstensen *et al.*, 1992, 1993). Thirteen *Anthopleura* neuropeptides are given in Table I. Not all the peptides in Table I contain the C-terminal sequence Arg-X-NH_2: L-3-phenyllactyl-Phe-Lys-Ala-NH_2 (Antho-KAamide) has the C terminus Lys-X-NH_2. Of course, there is not much difference between an Arg and a Lys residue, as both are positively charged.

Of all the peptides isolated, many have an N-terminal < Glu group (Table I). Three peptides have an N-terminal L-3-phenyllactyl residue, showing that this novel protecting group occurs generally in sea anemone peptides. Three C-terminal Arg-Pro-NH_2 peptides have an N-terminal X-Pro-Pro or X-Pro-Hyp sequence. Prolyl is not a normal amino acid, but an imino acid residue, and the X-Pro bond is not an amide but an imide bond. Therefore, peptides that start with an X-Pro sequence are normally resistant against degradation by nonspecific aminopeptidases. The enzyme dipeptidyl aminopeptidase (DPAP), however, especially requires such an N-terminal X-Pro (or X-Ala) sequence, but it cannot digest peptides with the N-terminal sequence X-Pro-Pro or X-Pro-Hyp (Carstensen *et al.*, 1992). The N-terminal X-Pro-Pro or X-Pro-Hyp sequence, therefore, is a third strategy of sea anemones to protect the N termini of their neuropeptides.

Why are so many cnidarian-peptides stabilized by protecting groups at both their N and C termini? This question is difficult to answer, but one answer could lie in the observation that there are not many well-developed synapses in the cnidarian nervous systems. Most transmitter substances in cnidarians are released at nonsynaptic ("neurosecretory") sites and must diffuse via the intercellular space to reach their target cells. This diffusion probably takes a relatively long time, and it must require rather stable transmitter molecules.

Antisera were raised against the C and N termini of most of the peptides of Table I. Immunocytochemistry showed that Antho-KAamide, the Antho-RIamides, -RNamides, -RWamides, -RPamides and -RFamide are each produced by a characteristic set of neurons (see e.g., Fig. 4b). This means that, neurochemically speaking, there are at least six different populations of neurons in sea anemones. Thus, although many neurons in cnidarians appear to be multifunctional, this does not mean that they are

all the same, because there is a clear differentiation with respect to the transmitter substance. Later we will see that these different transmitter substances also have different actions. This means that cnidarian neurons, although multifunctional, may nevertheless perform different tasks.

The different types of neurons can have different distributions. This is clearly shown in the area of the sphincter muscle in the upper body wall of the sea anemones (Fig. 4a and Fig. 6). Normally, there are only two cell layers in cnidarians: the ecto- and endoderm. In the area of the upper body wall, however, there is a third layer consisting of sphincter muscle cells embedded in the mesoglea between the ectoderm and the endoderm. The sphincter muscle cells have a circular orientation and, when danger is sensed, they contract and close the animal after the tentacles have been withdrawn. The sphincter muscle cells are innervated by a dense network of neuronal processes coming from Antho-RWamide-positive neurons in the endoderm (Fig. 6b). There are no other types of neurons containing one of the peptides from Table I, except for a few neurons containing Antho-KAamide-like material (Fig. 6a shows the absence of Antho-RFamide). Thus, the sphincter muscle is mainly innervated by Antho-RWamide neurons and to a lesser extent by neurons containing Antho-KAamide. This suggests a role for Antho-RWamide and Antho-KAamide at the neuromuscular junctions of the sphincter.

We have started to investigate the ultrastructural localization of the cnidarian neuropeptides. Using RFamide antisera and gold-labeled secondary antibodies, we have found RFamide-like material in the granular cores of neuronal dense-cored vesicles of *Hydra* (Koizumi *et al.,* 1989). These dense-cored vesicles were located in nerve processes paralleling the myonemes (muscle processes) of the epitheliomuscular cells and in nerve endings terminating on the myonemes, suggesting that the RFamide peptides are involved in neuromuscular transmission. In sea anemones, using antisera against the sea anemone neuropeptide Antho-RFamide, we found immunoreactive material in neuronal dense-cored vesicles that were associated with two-way neuroneuronal synapses (Westfall and Grimmelikhuijzen, 1993). In sea anemones, therefore, Antho-RFamide appears to be involved in neuroneuronal communication. A different, but very clear picture comes, again, from ultrasections of the sphincter muscle area of sea anemones. Here we found Antho-RWamide-like material located in neuronal dense-cored vesicles that were presynaptic to bundles of myofilaments making up the myonemes of the sphincter muscle cells (Westfall *et al.,* 1995). This strongly suggests that the Antho-RWamides are neurotransmitters at neuromuscular synapses of sea anemones.

We have investigated the actions of the sea anemone and sea pansy neuropeptides using various, simple muscle preparations, e.g., isolated tentacles (to measure the tentacle longitudinal muscles), circular rings of the

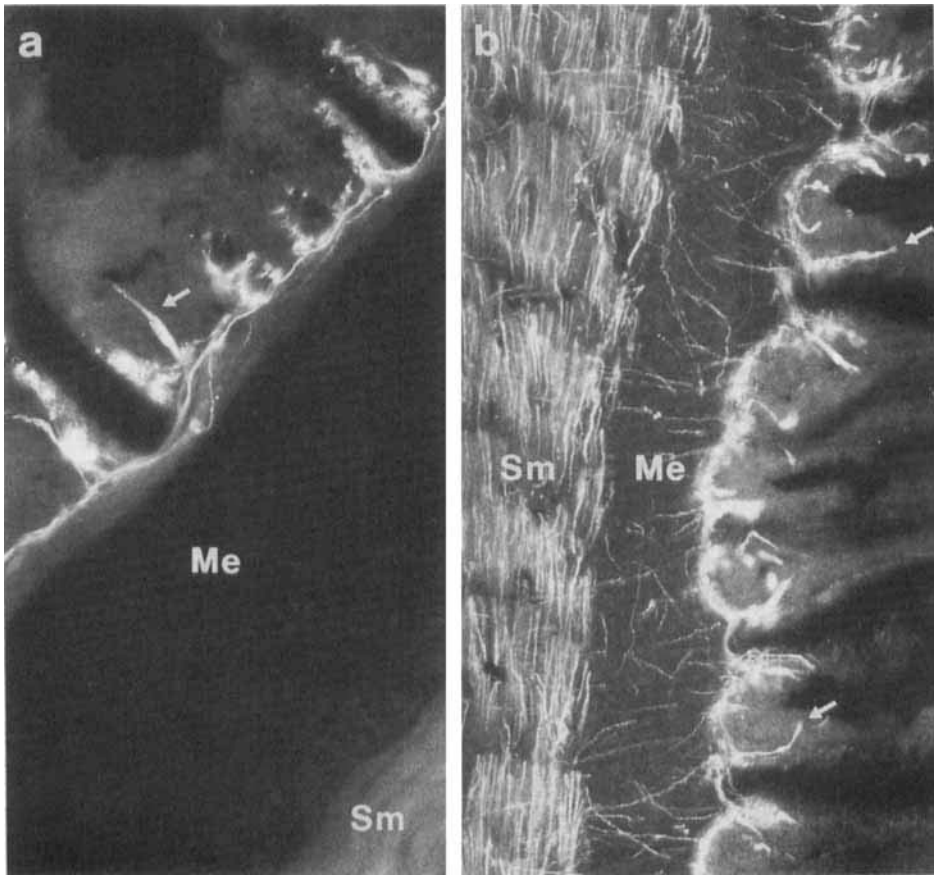

FIG. 6. Immunoreactive neurons in a cross section through the upper body wall of the sea anemone *Calliatis parasitica.* Me, mesoglea; Sm, sphincter muscle. (a) Staining with an antiserum direct against the N terminus (<Glu-Gly-Arg-Phe-) of Antho-RFamide. Immunoreactive "sensory cells" (arrow) are located in the endoderm. Their processes are associated with endodermal muscle fibers which are oriented either in a circular or longitudinal direction. Note that the muscle fibers of the sphincter muscle, which are embedded in the mesoglea, are not associated with immunoreactive neurites. A similar picture is obtained with antisera directed against the C terminus (-Gln-Gly-Arg-Phe-NH_2) of Antho-RFamide. ×290. (b) A lower power micrograph of the same region as in (a) Staining is now with an antiserum against the C terminus of both Antho-RWamide I and II (Arg-Trp-NH_2). A different population of neurons is stained: "sensory cells" (arrows), located in the endoderm, project through the mesoglea to join a dense plexus of neurites associated with fibers of the sphincter muscle. ×125 (Adapted from Grimmelikhuijzen *et al.,* 1992a.)

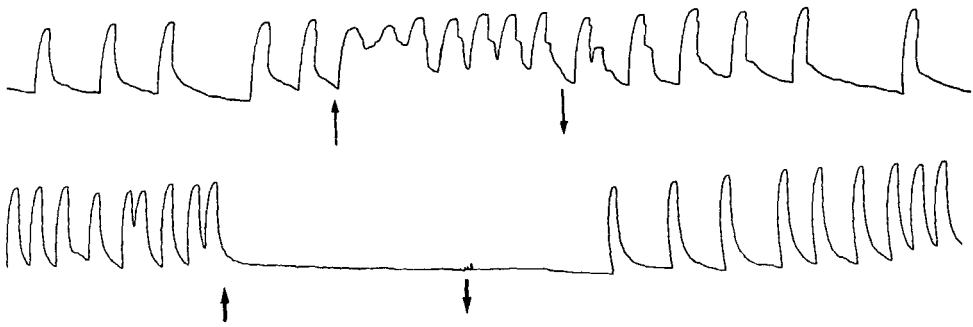

FIG. 7. Top: Biological action of Antho-RPamide I (Leu-Pro-Pro-Gly-Pro-Leu-Pro-Arg-Pro-NH_2). Application (↑) of the peptide to the bath (final concentration 10^{-5} *M*) induces an increase in the frequency and duration of spontaneous contractions in an isolated sea anemone tentacle. Normal activity restarts after washing (↓). The time scale is 5 min. (From Carstensen *et al.*, 1992.) Bottom: Biological action of Antho-RPamide II (<Glu-Asn-Phe-His-Leu-Arg-Pro-NH_2). Application (↑) of 5×10^{-6} *M* of the peptide fully inhibited the spontaneous contractions of a sea anemone tentacle. After washing (↓) activity restarts. The time scale is 5 min. (Modified from Carstensen *et al.*, 1993.)

upper body column (to measure the circular sphincter), and longitudinal body wall strips (to measure the longitudinal retractor muscles) (McFarlane *et al.*, 1987, 1991, 1992, 1993; Anctil and Grimmelikhuijzen, 1989; McFarlane and Grimmelikhuijzen, 1991; Nothacker *et al.*, 1991b; Carstensen *et al.*, 1992, 1993). Figure 7 gives an example of the excitatory action of Antho-RPamide I and the inhibitory action of Antho-RPamide II on isolated tentacles. Table II gives a summary of the actions of the neuropeptides (at 10^{-8} *M*) on the various muscle groups. It is clear that nearly all peptides

TABLE II

Excitatory (+) or Inhibitory (−) Actions of Neuropeptides on Different Muscle Groups of Sea Anemones

		Body column	
Peptide	Tentacle longitudinal	Longitudinal	Circular
Antho-RFamide	+	+	+
Antho-RWamide I, II	−	+	+
Antho-RNamide	+	+	−
Antho-RIamide I	−	−	−
Antho-KAamide	−	−	−
Antho-RPamide I	+		+
Antho-RPamide II, III	−		+

have different actions. For example, Antho-RFamide has a general excitatory action, whereas Antho-RIamide and Antho-KAamide are generally inhibitory. Antho-RNamide I has opposite actions on antagonistic muscle groups of the body column. We have injected some of the peptides into intact sea anemones and, by observing changes in volume or posture, we could confirm their actions on muscle groups in intact animals. Sea anemones have a hydroskeleton and their volume or posture is determined by muscle tension on one hand and, on the other hand, by the positive water pressure caused by ciliary beating of specialized ectodermal cells in the mouth opening. Injection of Antho-RIamide into the gastric cavity of intact animals led sea anemones to increase their volume by a factor of 4, which was obviously caused by inhibition of all their muscle groups (McFarlane *et al.*, 1993).

We have also checked the actions of some of the peptides on isolated cells. Addition of the Antho-RWamides (at 10^{-9}–10^{-7} *M*) to isolated sphincter muscle cells, for example, caused full contractions, showing that the Antho-RWamides act directly on these cells. This, together with the anatomical data at the light (Fig. 6b) and ultrastructural level (see earlier discussion), is strong evidence that the Antho-RWamides are transmitters at neuromuscular junctions. Antho-RFamide did not have an effect on isolated sphincter muscle cells. This was expected, as the Antho-RFamide neurons do not directly innervate the sphincter muscle (Fig. 6a). The excitatory effect of Antho-RFamide on the sphincter muscle in circular body column preparations (Table II) could be explained by an action of Antho-RFamide on the Antho-RWamide neurons innervating the sphincter. This, again, points to a role for Antho-RFamide in neuroneuronal communication. Resolving this kind of interaction among the different types of peptidergic neurons will be one of our research goals for the next few years. We might eventually be able to set up a neuronal or peptidergic hierarchy and, thus, explain the simple behavior of sea anemones and other cnidarians at the cellular and molecular level.

Cnidarian neuropeptides might have other functions than being a neurotransmitter. For example, they could play a role in reproduction or be important during regeneration, pattern formation, or metamorphosis. We ourselves have done very little in this respect so far, but another group associated with Thomas Leitz at the University of Heidelberg has found some exciting results. The marine hydroid *Hydractinia echinata* normally grows on molluscan shells inhabited by hermit crabs. The animal has a life cycle that includes various types of colonial polyps and a planula larva (there are no medusae). As mentioned earlier, a planula larva originates from a fertilized egg, and it is covered by cilia by which it swims around in search of a favorable location to settle (which is most often the outer surface of a molluscan shell inhabited by a hermit crab). After settlement,

the larva undergoes metamorphosis to become a primary polyp. Later, it produces tube-like connections (stolons) covering the shell and, subsequently, the secondary polyps and all the other colonial polyp types originate by budding from these stolons. Thomas Leitz cut the planula larva transversely one third of the way from its anterior part (which normally will contact the substratum). In this case the remaining two-thirds of the larva compromising the posterior part (the "tail" of the larva) was no longer able to perform metamorphosis. The addition of a *Hydractinia* extract to this posterior part of the planula larva, however, restored its ability to undergo metamorphosis. This implies that *Hydractinia* contains a substance that can induce metamorphosis, and that this substance is normally present in the anterior parts of *Hydractinia* planula larvae, but not (or to a too low degree) in the posterior parts. *Hydractinia* larval tissue is available in too small quantities to use it as a source for substance purification, so Leitz used extracts from the sea anemone *Anthopleura elegantissima,* and, using metamorphosis induction in posterior planula fragments as a bioassay, purified a neuropeptide from sea anemones that had the following structure: < Glu-Gln-Pro-Gly-Leu-Trp-NH_2 ("Metamorphosin A" or "MMA"; Leitz *et al.,* 1994; Leitz and Lay, 1995). It is, of course, not certain that the structure of the sea anemone peptide is the same as that of the authentic *Hydractinia* peptide, but it is likely to be related, the same way as the Pol-RFamides are related to Antho-RFamide (Table I). These recent findings from Leitz and co-workers show us two things: (1) in addition to the Arg-X-NH_2 and Lys-X-NH_2 neuropeptides, there are other types of peptides in cnidarians, and (2) neuropeptides are also involved in developmental processes.

Our own group has recently confirmed the structure of the metamorphosis-inducing peptide < Glu-Gln-Pro-Gly-Leu-Trp-NH_2 by cloning its preprohormone (Leviev and Grimmelikhuijzen, 1995). This preprohormone has an exciting structure (more than 40 peptide copies) and it is discussed in Section VI,E.

V. Peptide Receptors

If one is interested in the actions of peptides, one should also be interested in the peptide receptors. Until recently, no peptide receptors have been cloned from cnidarians. In higher animals, peptides bind to receptors belonging to the large family of G-protein-coupled (7-transmembrane) receptors. We have started our peptide receptor project in cnidarians by using oligonucleotide probes derived from consensus sequences of known G-protein-coupled receptors from higher animals and by applying polymerase chain reaction (PCR), using cDNA and genomic DNA from sea anemones

as templates. In this way, we have cloned a presumed G-protein-coupled receptor from the sea anemone *Anthopleura elegantissima* that shows a striking homology with members of the glycoprotein hormone [follicle-stimulating hormone (FSH), thyroid stimulating hormone (TSH), lutenizing hormone (LH), choriogonadatropin (CG)] receptor family from mammals (Nothacker and Grimmelikhuijzen, 1993). The mammalian glycoprotein hormone receptors differ from the other G-protein-coupled receptors by having a very large N terminus that is of the same size as the whole 7-transmembrane region plus the intercellular C-terminal loop. The N terminus of the glycoprotein hormone receptors constitutes the ligand binding site, which is in contrast with the other G-protein-coupled receptors, where the ligand binding site is situated at the outside loops of the 7-transmembrane area. The sea anemone receptor has a large N terminus similar to that of the mammalian glycoprotein hormone receptors, and this N terminus has 18–25% sequence identity with that of the mammalian counterparts. In the transmembrane region, there exists 44–48% sequence identity. The genes coding for the mammalian receptors have several introns, especially in the area coding for the N terminus. In mammals, there are several splicing variants coding for receptors containing shortened N termini and some variants code for a large extracellular N terminus that is soluble because it lacks the anchor of the 7-transmembrane region. Similar receptor variants have been found for the sea anemone receptor, caused by alternative splicing at exactly the same exon–intron transitions as in the mammalian receptor genes (Nothacker and Grimmelikhuijzen, 1993). All this is strong evidence that the sea anemone receptor is evolutionarily related to the glycoprotein hormone receptors from mammals.

The FSH, TSH, and LH/CG receptors have only been cloned from mammals and not from lower vertebrates. The fact that a similar type of receptor is present in cnidarians suggests that the glycoprotein hormone/receptor couple has been conserved throughout evolution, from the earliest nervous systems in cnidarians to the most advanced nervous systems in mammals. This was confirmed shortly after our own discovery in cnidarian by a report on the cloning of a related receptor in molluscs (Tensen *et al.*, 1994).

The ligand for the sea anemone receptor is probably not a peptide of the type given in Table I, but a larger molecule related to FSH, TSH, LH, and CG. This means that the endocrinology of sea anemones must be quite complex, involving at least 17 neuropeptides and, in addition, a new class of larger peptides, or protein hormones. As there are no endocrine cells in cnidarians, but only neurons, these presumed peptide or protein hormones are likely to be neurohormones.

VI. Biosynthesis of Neuropeptides

In higher animals, neuropeptides are made as large precursor proteins (preprohormones) which consist of a hydrophobic, N-terminal signal sequence (for translocation across the rough endoplasmic reticulum membrane; the "pre" part) and a prohormone part containing one or more copies of the immature peptide. The signal sequence of the preprohormone is cleaved off during translocation into the endoplasmic reticulum. The prohormone part is subsequently transported into the Golgi system, sorted, and finally packaged into the neurosecretory, dense-cored vesicles. Together with the prohormones, several processing enzymes are co-packaged into the dense-cored vesicles. These processing enzymes convert the prohormones into their biologically active peptides. Figure 8 gives a summary of these processing steps, as they are known from higher animals, mostly mammals.

First, there is an initial, endoproteolytic cleavage at the C-terminal side of paired or single basic residues. This first step liberates the immature neuropeptide sequence from its prohormone. Several neuropeptide precursor processing enzymes responsible for the endoproteolytic cleavage at paired basic residues have been cloned, among them the prohormone convertases PC1 (also named PC3) and PC2 from mammals (Smeekens and Steiner, 1990; Seidah *et al.*, 1990, 1991; Smeekens *et al.*, 1991). The endoprotease cleaving at single basic residues has not been cloned and characterized yet. It is clear, however, that the enzyme should not cleave at every basic

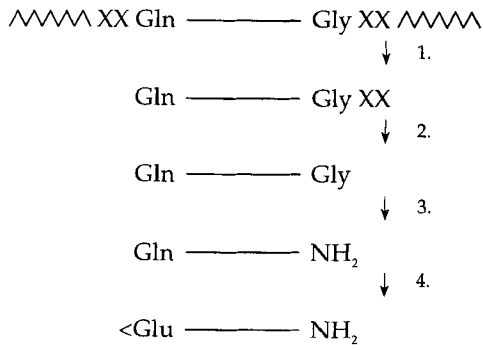

FIG. 8. Neuropeptide precursor processing in higher invertebrates and vertebrates. Top: The precursor protein contains one or several copies of an immature neuropeptide sequence, which are flanked by basic residues (marked by XX). The cleavage or processing steps are catalyzed by the following enzymes: 1. An endoproteinase cleaving at the C-terminal side of pairs or single basic residues. 2. A carboxypeptidase B-like enzyme. 3. Peptidyl-glycine hydroxylase and peptidyl-hydroxyglycine N-C lyase. 4. Glytaminyl cyclase.

residue, because some of the neuropeptide sequences themselves contain an interval Arg or Lys residue. Therefore, the prohormone must contain recognition sequences that direct cleavage at monobasic sites. The exact nature of these recognition sequences, however, has still to be determined (Schwartz, 1986; Devi, 1991; Leviev and Grimmelikhuijzen, 1995). As a second step, a carboxypeptidase B-like enzyme removes the remaining basic residues at the C terminus of the immature neuropeptide (Fricker *et al.,* 1989). Third, if the mature neuropeptide carries a C-terminal amide group (and, therefore, is a peptidyl-amide), its immature structure is a peptidyl-glycine. This peptidyl-glycine is converted into peptidyl-amide by the concerted action of two enzymes: peptidyl-glycine hydroxylase and peptidyl-hydroxyglycine N-C lyase (Bradbury and Smyth, 1991). Both enzymatic activities are located on a common proenzyme, the bifunctional peptidyl-glycine α-amidating mono-oxygenase (PAM), but the two activities can be separated by endoproteolysis (Kato *et al.,* 1990; Katopodis *et al.,* 1990, 1991; Perkins *et al.,* 1990; Eipper *et al.,* 1992). Fourth, if the mature neuropeptide carries an N-terminal < Glu group, the immature neuropeptide carries an N-terminal Gln, which is then converted into < Glu by the enzyme glutaminyl cyclase (Fischer and Spiess, 1987; Pohl *et al.,* 1991).

A. Biosynthesis of Antho-RFamide in Sea Anemones

Based on our knowledge from higher animals, we expected that the precursor of the anthozoan neuropeptide Antho-RFamide (< Glu-Gly-Arg-Phe-NH_2) was a protein that had an N-terminal signal sequence and one or more immature Antho-RFamide sequences (Gln-Gly-Arg-Phe-Gly) flanked by basic amino acid residues. Antho-RFamide was first isolated from the sea anemone *Anthopleura elegantissima* (Table I). The action of Antho-RFamide (Table II), however, was mostly investigated using another sea anemone species, *Calliactis parasitica* (living on the shell of a hermit crab), because this animal was more amenable to physiological experimentation. This was the reason that we first investigated the biosynthesis of Antho-RFamide in *Calliactis.* We have used two strategies to clone the Antho-FRamide precursor from *Calliactis.* First, we have raised antibodies against the internal, immature Antho-RFamide sequence that was coupled *via* two flanking Lys residues (Lys-Gln-Gly-Arg-Phe-Gly-Lys) to a carrier protein, thyroglobulin. The antibodies were subsequently affinity-purified and used to screen an expression cDNA (λgt11) library from *Calliactis.* Second, we have used a 384-fold degenerate pool of 15-mer oligonucleotide probes coding for the sequence Gln-Gly-Arg-Phe-Gly to screen the same

λgt11 cDNA library. Both strategies were successful and Fig. 9 shows the cDNA and deduced amino acid sequence of the Antho-RFamide precursor from *Calliactis* (Darmer *et al.*, 1991). The Antho-RFamide precursor protein contains 19 copies of immature Antho-RFamide (Gln-Gly-Arg-Phe-Gly) which are tandemly arranged in the C-terminal part of the protein. Each Antho-RFamide sequence is followed by one or more basic amino acid residues. Thus, so far, the biosynthesis of Antho-RFamide is "classical" and all the processing enzymes mentioned in Fig. 8 are probably present in sea anemone neurons.

Before the N terminus of each immature Antho-RFamide sequence, we expected the precursor to have one or two basic residues functioning as cleavage sites, but to our surprise, these residues are lacking and instead one or more acidic residues (Asp or Glu) occur (Fig. 9). These acidic residues, therefore, must be the cleavage sites for a new type of processing enzyme occurring in neurons (Darmer *et al.*, 1991). This processing enzyme could either be an endoproteinase cleaving at the C-terminal sides of Asp or Glu residues, or an aminopeptidase that starts to become active after an initial cleavage at basic residues has liberated the aminoterminus. This aminopeptidase would then sequentially eliminate the 2–3 acidic residues in the N-terminal extensions of the immature Antho-RFamide sequences (Fig. 9).

In addition to the 19 Antho-RFamide sequences, there are several other, putative Antho-RFamide-related sequences in the Antho-RFamide precursor. Two of these (at amino acid positions 101–114 of Fig. 9) could be N-terminally Phe-extended forms of Antho-RFamide. As a possible alternative, however, an additional processing enzyme could exist in sea anemone neurons that cleaves at the C-terminal side of Phe residues (see also below). This processing would yield two more authentic Antho-RFamide peptides. There is another putative neuropeptide which contains the C-terminal sequence Arg-Tyr-NH_2, instead of Arg-Phe-NH_2 (at amino acid positions 92–98 of Fig. 9). The sequence of the mature peptide could be Tyr-Val-Pro-Gly-Arg-Tyr-NH_2, or, if an aminopeptidase cleaving at the C-terminal side of aromatic amino acid residues exists, Val-Pro-Gly-Arg-Tyr-NH_2.

The N-terminal part of the precursor protein contains four other, repetitive sequences, which might also yield mature neuropeptides (at amino acid positions 43–84 of Fig. 9). These sequences are flanked by acidic or basic residues, but, at present, we have no concrete data on the final structures of the putative neuropeptides. Using immunocytochemistry we could not find staining with antibodies against the N-terminal sequences Pro-Gln-Phe-Trp-Lys-Gly-Arg-Phe, but antibodies against < Glu-Phe-Trp-Lys-Gly-Arg-Phe stained the same neurons, which were previously stained with antibodies against Antho-RFamide. This suggests a cleavage between the Pro and Gln residues (Fig. 9). As mentioned in Section IV, N-terminal X-Pro sequences are the substrates for dipeptidyl aminopeptidase (DPAP).

```
                                                                    TAAAGAAAAACGAAGAAGGTACACAGACAAGGTTTCAAGTGCGCAGACTCGT   52

ATG CTG GTC GCC ATG ACT ACA GCC TCC TAC GTC ACC ATC CTC GTG ACT CTA CTT TTC CAT  112
Met Leu Val Ala Met Thr Thr Ala Ser Tyr Val Thr Ile Leu Val Thr Leu Leu Phe His   20

ATA TTA ACA ATT AAT GCC AAG ACA GTG ACA AAG CGT GCG AAA GAA ACC AAT TTA GAA GAT  172
Ile Leu Thr Ile Asn Ala Lys Thr Val Thr Lys Arg Ala Lys Glu Thr Asn Leu Glu Asp   40

GAT GAA CCA CAG TAT TGG CGA GGT CGC TTT GCA AAA GAC GTA GTG CCA CAA TTC TGG AAG  232
Asp Glu Pro Gln Tyr Trp Arg Gly Arg Phe Ala Lys Asp Val Val Pro Gln Phe Trp Lys   60

GGA CGT TTT TCA GAT CCT CAA TTC TGG AAG GGA CGT TTT TCT GAT CCT CAG TTC TGG AAG  292
Gly Arg Phe Ser Asp Pro Gln Phe Trp Lys Gly Arg Phe Ser Asp Pro Gln Phe Trp Lys   80

GGA CGT TTT AGC TCT CAT GGA AAC AAG AGA CGC TAT GTC CCT GGA CGA TAT GGA CGT GAG  352
Gly Arg Phe Ser Ser His Gly Asn Lys Arg Arg Tyr Val Pro Gly Arg Tyr Gly Arg Glu  100

TTT CAG GGA CGC TTC GGA CGA GAA TTT CAA GGA CGT TTT GGC CGA GAA CAG GGA CGC TTT  412
Phe Gln Gly Arg Phe Gly Arg Glu Phe Gln Gly Arg Phe Gly Arg Glu Gln Gly Arg Phe  120

GGC AGA GAG GAG GAC CAA GGA CGC TTT GGC AGA GAG GAG GAC CAG GGA CGT TTT GGG AGA  472
Gly Arg Glu Glu Asp Gln Gly Arg Phe Gly Arg Glu Glu Asp Gln Gly Arg Phe Gly Arg  140

GAA GAA CAG GGA CGC TTT GGT CGA GAG GAG GAC CAG GGA CGC TTT GGT CGA GAA GAA GAC  532
Glu Glu Gln Gly Arg Phe Gly Arg Glu Glu Asp Gln Gly Arg Phe Gly Arg Glu Glu Asp  160

CAG GGA CGC TTT GGT CGA GAA GAG GAC CAG GGA CGC TTT GGT CGA GAG GAG GAA CAG GGA  592
Gln Gly Arg Phe Gly Arg Glu Glu Asp Gln Gly Arg Phe Gly Arg Glu Glu Glu Gln Gly  180

CGC TTT GGT CGA GAG GAG GAC CAA GGA CGC TTT GGT CGA GAG GAG GAA CAG GGA CGC TTT  652
Arg Phe Gly Arg Glu Glu Asp Gln Gly Arg Phe Gly Arg Glu Glu Glu Gln Gly Arg Phe  200

GGT CGA GAG GAG GAC CAA GGA CGC TTT GGC AGA GAA GAG GAC CAA GGA CGC TTT GGT CGA  712
Gly Arg Glu Glu Asp Gln Gly Arg Phe Gly Arg Glu Glu Asp Gln Gly Arg Phe Gly Arg  220

GAG GAG GAA CAG GGA CGC TTT GGA AAA CGA GAC GAG GAT CAA GGA CGC TTT GGA AAA CGA  772
Glu Glu Glu Gln Gly Arg Phe Gly Lys Arg Asp Glu Asp Gln Gly Arg Phe Gly Lys Arg  240

GAA GAT CAG GGA CGC TTT GGA AAA CGC GAC GAG GAC CAA GGA CGC TTT GGA AAA CGC GAC  832
Glu Asp Gln Gly Arg Phe Gly Lys Arg Asp Glu Asp Gln Gly Arg Phe Gly Lys Arg Asp  260

GAG GAT CAA GGA CGC TTT GGA AAA CGC GAA GAC CAG GGA CGC TTT GGA AAA CGA GAA GAT  892
Glu Asp Gln Gly Arg Phe Gly Lys Arg Glu Asp Gln Gly Arg Phe Gly Lys Arg Glu Asp  280

CAG GGA CGC TTT GGG AGA GAG TTA TTA GCA AAA CTG AAT AAA AGA ACC ACT TCA ATT CAA  952
Gln Gly Arg Phe Gly Arg Glu Leu Leu Ala Lys Leu Asn Lys Arg Thr Thr Ser Ile Gln  300

GAA GAT CCC CAA ACA CGA TTT AGA GAT GTA CAG ATG ACA AGA CGA AAT GTA GCA AAA AAG 1012
Glu Asp Pro Gln Thr Arg Phe Arg Asp Val Gln Met Thr Arg Arg Asn Val Ala Lys Lys  320

GAT AAG ATT GAA GAA TCC AAT GAC GAA GAG GCA AAT AAA TCT TAA CAATCGTAGAACTTTAATG 1076
Asp Lys Ile Glu Glu Ser Asn Asp Glu Glu Ala Asn Lys Ser  .                       334

CTGATATGGATTATGTCCAACCATAAATACTCTTCCAAGGGATCTACAAGAGCTGTTCACTTCACTAGTAATTCTGTAG 1155

TTCAGTGTATCAATAGTTAAAAGTACATAGGGTCTAGAAATAAACACTGGAGGAAATTATATCACATAGAGCATGTATA 1234

ATATTATGCAACAAAATATATGTCACTTCAAATTATCCAAAGGCAATTTCATTTCCAATTTCAAGGGATGGTACGAACA 1313

TCATGATGGTTCACGTTAATAGGAAGACCAAATCTCGATCACACTGTAATAAATTGTCAAATGTCTAGCAATTTGCTTC 1392

AATAAATGAGGCTGAGAATGAAAAAAAA                                                     1420
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FIG. 9. cDNA and deduced amino acid sequence of the *Calliactis* Antho-RFamide precursor. Nucleotide residues are numbered from the 5′ to 3′ end and amino acid residues are numbered starting with the first ATG codon in the open reading frame. The N terminus contains a hydrophobic signal sequence for rough endoplasmic reticulum membrane translocation which is probably cleaved off at Ala-26. Antho-RFamide copies are underlined and printed in boldfaced type, whereas putative neuropeptide sequences are underlined only. Polyadenylation signals in the untranslated 3′ region are marked by broken lines. (Modified from Darmer *et al.*, 1991.)

It is already known that DPAP plays a role in the final processing of N-terminal X-Pro or X-Ala elongated forms of yeast α-mating factor, honey bee melittin, frog skin xenopsin, and other, non-neuronal, bioactive peptides (Kreil, 1990). Since DPAP is an aminopeptidase, processing by this enzyme would imply an earlier cleavage at the C-terminal side of Asp, Val, and Ser residues (Fig. 9). We will see later in this chapter (Sections VI,D,E,F), that DPAP indeed plays a very important role in cnidarian prohormone processing.

The four N-terminally located, putative neuropeptides form a peptide family, as do the other Antho-RFamide-related neuropeptides (Fig. 10). The two neuropeptide families are related and have the sequence Gly-Arg-Phe in common.

Authentic Antho-RFamide has only been purified from *Anthopleura* and not from *Calliactis*. Therefore, it was still possible that *Calliactis* did not produce authentic Antho-RFamide, but variants which were N-terminally extended by Asp and Glu residues (Fig. 9). For these reasons, we also cloned the Antho-RFamide precursor from *Anthopleura* using the *Calliactis* Antho-RFamide precursor cDNA (Fig. 9) as a probe to screen an *Anthopleura* cDNA library (Schmutzler *et al.*, 1992). Figure 11 shows the primary structure of the Antho-RFamide precursor from *Anthopleura*. It contains 13 copies of immature Antho-RFamide, which are all preceded by acidic residues, exactly as in the Antho-RFamide precursor from *Calliactis*. In addition to these sequences, five copies of immature Antho-RFamide are preceded by a Leu, two by a Phe and one by an Ala residue (Fig. 10 and Fig. 11). These sequences could yield N-terminally elongated forms of Antho-RFamide. Again, if processing enzymes cleaving at the C-terminal sides of aliphatic (Leu, Val), aromatic (Phe, Tyr), or X-Ala sequences (DPAP) exist (see above), these sequences would yield authentic Antho-RFamide. It is interesting that, in the latter case, the total number of mature Antho-RFamide copies that would originate from one precursor molecule would be the same (21) in the *Anthopleura* and *Calliactis* precursor.

As in *Calliactis*, there is one copy of a putative neuropeptide with the C-terminal sequence Arg-Tyr-NH_2 (at amino acid positions 184–190 of Fig. 11; see also Fig. 10). The complete sequence of this putative neuropeptide could be Ser-Val-Pro-Gly-Arg-Tyr-NH_2. If a processing enzyme exists that cleaves at the C-terminal side of a Ser residue (see earlier discussion), this sequence would be Val-Pro-Gly-Arg-Tyr-NH_2, which would be the same sequence as that postulated in *Calliactis*. With DPAP, both the *Anthopleura* and the *Calliactis* peptide would be converted into Gly-Arg-Tyr-NH_2.

In contrast to the Antho-RFamide precursor from *Calliactis*, there is a much larger number of repetitive peptide sequences (11) in the N-terminal part of the *Anthopleura* Antho-RFamide precursor (Figs. 10 and 11). As with *Calliactis*, we assume that DPAP removes the X-Pro, or X-Ala se-

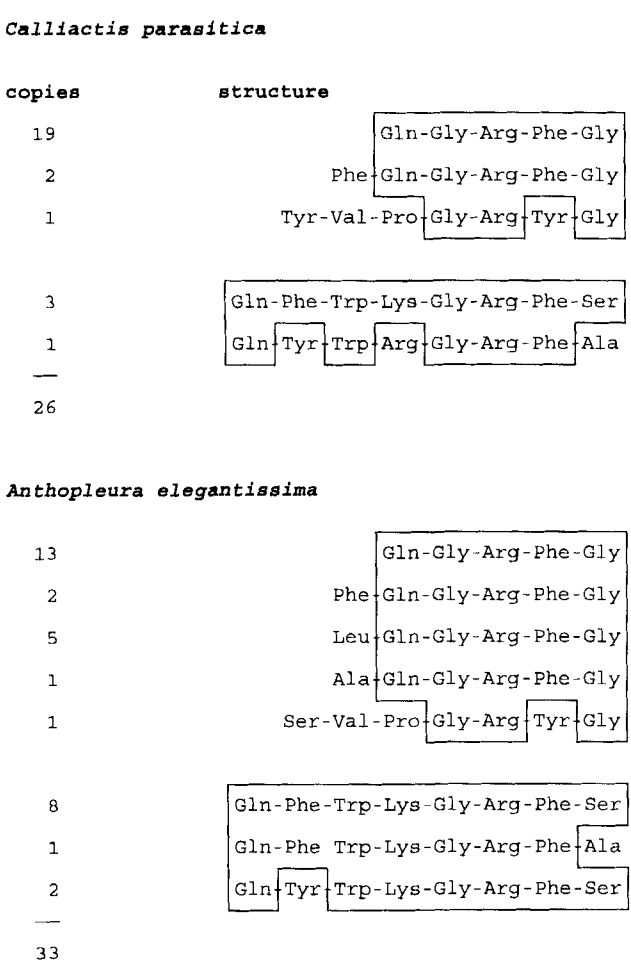

FIG. 10. Established and putative neuropeptide sequences found within the Antho-RFamide precursor from *Calliactis* (top) and *Anthopleura* (bottom). The neuropeptide sequences from *Calliactis* are deduced from Fig. 9, those from *Anthopleura* from Fig. 11.

quences at the N termini of each repetitive sequence, after an initial processing has taken place at the C-terminal side of Asp, Glu, Ser, and Lys-Arg residues.

In summary, the primary structures of both Antho-RFamide precursor proteins from sea anemones provide compelling evidence for the existence of a novel processing enzyme that cleaves at the C-terminal sides of acidic residues. In addition, there might be other processing sites and, therefore other, unknown processing enzymes.

```
                                                                AAGAAGCAACTGGCTTGTAGTGCGCAGACTCGTACCACTGTTACC   45

ATG ACG ACG GTT TCA TAC GTC ACA ATC TTG CTG ACC GTA CTC GTC CAA GTA CTT ACT TCA  105
Met Thr Thr Val Ser Tyr Val Thr Ile Leu Leu Thr Val Leu Val Gln Val Leu Thr Ser   20

GAT GCC AAA GCT ACA AAT AAC AAA AGA GAA CTA TCC AGT GGT TTG AAA GAA CGA AGC CTG  165
Asp Ala Lys Ala Thr Asn Asn Lys Arg Glu Leu Ser Ser Gly Leu Lys Glu Arg Ser Leu   40

AGC GAT GAT GCG CCA CAG TTT TGG AAA GGA CGC TTT TCC AGA AGT GAG GAA GAC CCC CAG  225
Ser Asp Asp Ala Pro Gln Phe Trp Lys Gly Arg Phe Ser Arg Ser Glu Glu Asp Pro Gln   60

TTC TGG AAG GGC CGT TTT TCT GAC CCC CAG TTC TGG AAG GGA CGT TTT TCT GAC CCC CAG  285
Phe Trp Lys Gly Arg Phe Ser Asp Pro Gln Phe Trp Lys Gly Arg Phe Ser Asp Pro Gln   80

TTC TGG AAG GGA CGT TTT TCT GAT CCT CAG TTT TGG AAG GGA CGT TTT TCT GAT CCT CAG  345
Phe Trp Lys Gly Arg Phe Ser Asp Pro Gln Phe Trp Lys Gly Arg Phe Ser Asp Pro Gln  100

TTT TGG AAG GGA CGT TTT TCT GAC CCA CAA TTC TGG AAG GGC CGT TTT TCT GAT CCT CAG  405
Phe Trp Lys Gly Arg Phe Ser Asp Pro Gln Phe Trp Lys Gly Arg Phe Ser Asp Pro Gln  120

TTC TGG AAG GGA CGT TTC TCT GAT GGC ACC AAG CGT GAA AAT GAC CCA CAA TAC TGG AAG  465
Phe Trp Lys Gly Arg Phe Ser Asp Gly Thr Lys Arg Glu Asn Asp Pro Gln Tyr Trp Lys  140

GGA CGT TTC TCA CGT TCC TTC GAG GAT CAG CCA GAC AGT GAG GCC CAG TTC TGG AAG GGA  525
Gly Arg Phe Ser Arg Ser Phe Glu Asp Gln Pro Asp Ser Glu Ala Gln Phe Trp Lys Gly  160

CGC TTT GCA AGG ACA AGC AGT GGA GAA AAA AGA GAA CCT CAG TAC TGG AAG GGA CGT TTT  585
Arg Phe Ala Arg Thr Ser Ser Gly Glu Lys Arg Glu Pro Gln Tyr Trp Lys Gly Arg Phe  180

TCG AGG GAT TCT GTC CCT GGG AGA TAT GGT CGC GAA CTT CAA GGA AGA TTT GGT AGG GAA  645
Ser Arg Asp Ser Val Pro Gly Arg Tyr Gly Arg Glu Leu Gln Gly Arg Phe Gly Arg Glu  200

CTC CAG GGA CGC TTT GGA CGT GAG GCC CAG GGA CGT TTT GGC CGA GAG CTA CAG GGA CGT  705
Leu Gln Gly Arg Phe Gly Arg Glu Ala Gln Gly Arg Phe Gly Arg Glu Leu Gln Gly Arg  220

TTT GGT CGG GAA TTT CAG GGA CGC TTT GGA CGC GAG GAC CAG GGA CGC TTT GGG CGC GAG  765
Phe Gly Arg Glu Phe Gln Gly Arg Phe Gly Arg Glu Asp Gln Gly Arg Phe Gly Arg Glu  240

GAC CAG GGA CGC TTT GGA CGC GAG GAC CAG GGA CGC TTT GGG CGC GAG GAC CAG GGA CGC  825
Asp Gln Gly Arg Phe Gly Arg Glu Asp Gln Gly Arg Phe Gly Arg Glu Asp Gln Gly Arg  260

TTT GGG CGC GAG GAC CAG GGA CGC TTT GGG CGC GAG GAC CAG GGA CGC TTT GGC CGA GAG  885
Phe Gly Arg Glu Asp Gln Gly Arg Phe Gly Arg Glu Asp Gln Gly Arg Phe Gly Arg Glu  280

CTA CAG GGA CGT TTT GGG CGA GAG TTT CAG GGA CGC TTT GGA CGC GAG GAC CAG GGA CGC  945
Leu Gln Gly Arg Phe Gly Arg Glu Phe Gln Gly Arg Phe Gly Arg Glu Asp Gln Gly Arg  300

TTT GGA CGT GAG GAC CAG GGA CGC TTT GGC CGA GAG CTC CAG GGA CGC TTT GGC CGA GAG 1005
Phe Gly Arg Glu Asp Gln Gly Arg Phe Gly Arg Glu Leu Gln Gly Arg Phe Gly Arg Glu  320

GAC CAG GGA CGC TTT GGA CGC GAG GAC CAG GGA CGT TTT GGA CGC GAA GAC CTC GCA AAA 1065
Asp Gln Gly Arg Phe Gly Arg Glu Asp Gln Gly Arg Phe Gly Arg Glu Asp Leu Ala Lys  340

GAG GAC CAG GGA CGC TTT GGT CGC GAA GAC CTC GCA AAA GAG GAC CAA GGA CGC TTT GGT 1125
Glu Asp Gln Gly Arg Phe Gly Arg Glu Asp Leu Ala Lys Glu Asp Gln Gly Arg Phe Gly  360

CGC GAA GAC ATC GCA GAA GCT GAC CAG GGA CGC TTT GGT CGA AAT GCA GCA GCA GCA GCA 1185
Arg Glu Asp Ile Ala Glu Ala Asp Gln Gly Arg Phe Gly Arg Asn Ala Ala Ala Ala Ala  380

GCA GCA GCA GCA GCA GCA AAG AAA AGA ACT ATT GAC GTG ATT GAT ATT GAA TCA GAT CCA 1245
Ala Ala Ala Ala Ala Ala Lys Lys Arg Thr Ile Asp Val Ile Asp Ile Glu Ser Asp Pro  400

AAA CCT CAA ACA AGA TTT AGA GAT GGA AAA GAT ATG CAA GAA AAA AGA AAA GTA GAG AAA 1305
Lys Pro Gln Thr Arg Phe Arg Asp Gly Lys Asp Met Gln Glu Lys Arg Lys Val Glu Lys  420

AAA GAT AAA ATC GAA AAA TCA GAT GAC GCA CTG GCA AAG ACT TCT TAA CGACGGTTCAAATAG 1368
Lys Asp Lys Ile Glu Lys Ser Asp Asp Ala Leu Ala Lys Thr Ser  .                    435

GGATCCTGATACTTTGATGAATGACAAGACAAGACAAAATAAATCCCTACCCTTCCAAGGGTAAATCTATGTTTTATTC 1447

ACAATTTGTATAATAGTTGTTTGTTGAAGCTCCATGAGTATGAGGTAGATAACCACACAGATAATATCATGGACACATG 1526

CATGTCAAATACAACAAAAAACAACAATAATAATAATGATAGTATATTCTTGTTTTATTCTATATTACCAATAACCTTT 1605

GAGGGAAAGGTTTAGGGGTCAGAGGATGAGACTCAGTGTTAGGAACTTCTAAAACTAGGGATACTGTAGATAGATTAGA 1684

ATCATGATACAAACTCAGAGTCATTAATCCAAATTGATAAGGTAAATTAAGTGCTGCAAATGAATGCTTCAATAAAATG 1763
AGGCAAGACTAAAA                                                                    1777
```

FIG. 11. cDNA and deduced amino acid sequence of the *Anthopleura* Antho-RFamide precursor. Neuropeptide sequences are underlined as in Fig. 9. (Modified from Schmutzler *et al.* 1992.)

The total number of all established and putative neuropeptides that may be cleaved from the Antho-RFamide precursor from *Anthopleura* is 33 (Fig. 10). Thus, this Antho-RFamide precursor is a very complex precursor protein.

B. Biosynthesis of Antho-RFamide in Sea Pansies

We have mentioned in Section IV that Antho-RFamide has also been isolated from the sea pansy *Renilla köllikeri* (Grimmelikhuijzen and Groeger, 1987). Sea anemones and sea pansies both belong to the class of Anthozoa, but they are members of a different subclass: Sea anemones are Hexacorallia (order: Actiniaria), whereas sea pansies are Octocorallia (order: Pennatulacea). The presence of Antho-RFamide in two remote groups of Anthozoa strongly suggests that Antho-RFamide occurs generally in anthozoans. To see whether the organization of the Antho-RFamide precursor in Octocorallia is the same as in Hexacorallia, we cloned the Antho-RFamide precursor protein from *Renilla.* For screening a *Renilla* λgt10 cDNA library, we used a cDNA probe encoding the Antho-RFamide precursor from *Anthopleura.* Figure 12 shows the cDNA and the deduced amino acid sequence of the Antho-RFamide precursor from *Renilla* (Reinscheid and Grimmelikhuijzen, 1994). This precursor contains 36 copies of immature Antho-RFamide and two additional, putative neuropeptide sequences which are regularly distributed over the whole precursor protein.

The putative, N-terminally located neuropeptide sequences that we found in the Antho-RFamide precursors from sea anemones (Figs. 9–11) are not present in the Antho-RFamide precursor from *Renilla.* Of the 36 Antho-RFamide sequences, 31 copies are preceded by one or more acidic (Glu) residues (Fig. 12, Table III). This illustrates the widespread use and importance of acidic residues for processing of cnidarian neuropeptide precursors. The processing enzyme that cleaves at acidic residues is most likely an endoproteinase, because the simplest way to generate Antho-RFamide from its precursor protein is by endoproteolytic cleavage at the C-terminal side of Glu (Fig. 12, Table III). The enzyme could possibly also be an aminopeptidase, but in this case other proteases are needed to remove the residual amino acid residues from the N-terminal extensions of Antho-

FIG. 12. cDNA and deduced amino acid sequence of the *Renilla* Antho-RFamide precursor. Neuropeptide sequences are underlined. (Modified from Reinscheid and Grimmelikhuijzen, 1994.)

```
                                                 TTTAGTATTGCGTCGTGTGTGTGTGTGTGTGTGTG    35
TTTGGCTCATTTTCTACGATCAACGTTGCTCTACACATTTGTATGGAGAAAACCTGTCAGTCTATAACGTATATACTTC   114
AAAATAATGGACTTAGTAATATAAGTATCAACTGCATTATTGTTGTCAATGTCCA ATG GTT AGT CTG GGT TTT   187
                                                        Met Val Ser Leu Gly Phe     6

TTC GTT CGT GAT GTT ACT CCA ACA TTC ATT GTA GAT CAT ATG TTC TAC ATG CTA TTC CCG   247
Phe Val Arg Asp Val Thr Pro Thr Phe Ile Val Asp His Met Phe Tyr Met Leu Phe Pro    26

ATG GAT TTT ACG TGT TAC GTT GCC GGG CTG TTG TTG ATA TTA AAT ACT TAC AGT TTG GCC   307
Met Asp Phe Thr Cys Tyr Val Ala Gly Leu Leu Leu Ile Leu Asn Thr Tyr Ser Leu Ala    46

GGG CCC TCA ACT AGC GAA GGA CTA AAC GAA CGG AAC TTG CTG GAT AAA ACA GAG TTG TCG   367
Gly Pro Ser Thr Ser Glu Gly Leu Asn Glu Arg Asn Leu Leu Asp Lys Thr Asp Leu Ser    66

ATA AAT GAC GAG ATA TTT AGC GAA GAT GAT GAT ATG CTG GCA AGG GAC GCT GAA GAC AAA   427
Ile Asn Asp Glu Ile Phe Ser Glu Asp Asp Asp Met Leu Ala Arg Asp Ala Glu Asp Lys    86

CAA GGA CGA TTT AAT CGT AAA TTA AAT AAC AAG TTG AAT GAA GCG GTA CAA GGA CGC TTC   487
Gln Gly Arg Phe Asn Arg Lys Leu Asn Asn Lys Leu Asn Glu Ala Val Gln Gly Arg Phe   106

GGG AGA AAT GAG AGA AAA GAA TCG GAA GAA GAA CAA GGA AGG TTC GGG CGA GAA AAT GAA   547
Gly Arg Asn Glu Arg Lys Glu Ser Glu Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   126

AAA CAA GGA AGA TTT GGA AGG GAA AGC GAG GAG CAA GGG AGA TTT GGA CGA GAA AAC AAA   607
Lys Gln Gly Arg Phe Gly Arg Glu Ser Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Lys   146

GAA CAA GGA AGA TTT GGA AGG GAA AAC AAA GAA CAA GGA AGG TTT GGA CGA GAA AAC GAG   667
Glu Gln Gly Arg Phe Gly Arg Glu Asn Lys Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   166

GAA CAA GGA AGA TTT GGA AGG GAA AGC GAG GAG CAA GGG AGA TTT GGA AGA GAA AAC GAA   727
Glu Gln Gly Arg Phe Gly Arg Glu Ser Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   186

GAA CAA GGA AGA TTT GGA CGA GAA AAC GAA GAA CAA GGA AGA TTT GGA CGA GAA AAC GAA   787
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   206

GAA CAA GGA AGG TTT GGA CGA GAA AAC GAG GAG CAA GGG AGA TTT GGA AGG GAG AAC GAA   847
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   226

GTT CAA GGA AGA TTT GGA CGA GAG AAC GAG GAG CAA GGA AGA TTT GGA CGA GAA AAC GAA   907
Val Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   246

GAA CAA GGA AGG TTT GGA AGA GAA AAC GAA GAA CAA GGA CGA TTT GGA CGA GAA AAC GAG   967
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   266

GAG CAA GGA AGA TTT GGA CGA GAA AAT GAA GAA CAA GGA AGA TTT GGA CGA GAA AAC GAG  1027
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   286

GAG CAA GGA AGA TTT GGA CGA GAA AAC GAA AAA CAA GGA CGA TTT GGA CGA GAA AAC GAA  1087
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Lys Gln Gly Arg Phe Gly Arg Glu Asn Glu   306

GAA CAA GGA AGA TTT GGA CGA GGA AAC GAG GAG CAA GGG AGA TTT GGA AGG GAA AAC GAA  1147
Glu Gln Gly Arg Phe Gly Arg Gly Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   326

GAA CAA GGA AGA TTT GGA CGA GAA AAC GAA GAA CAA GGA AGA TTT GGA CGA GAA AAC GAA  1207
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   346

GAA CAA GGA CGA TTT GGG CGA GAA AAC GAA GAA CAA GGA AGG TTT GGG CGA GAA AAC GAA  1267
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu   366

GAA CAA GGA AGG TTT GGA AGG GAG AAC GAG AAG CAA GGG AGA TTT GGA AGA GGG GAC GAA  1327
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Lys Gln Gly Arg Phe Gly Arg Gly Asp Glu   386

GAA CAA GGA AGG TTT GGA AGG GAG AAC GAG GAG CAA GGG AGA TTT GGA AGA GGG GAC GAA  1387
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Gly Asp Glu   406

GAA CAA GGA AGA TTT GGA CGA GAA AAC GAA GAG CAA GGG AGA TTT GGA AGA GAA AAC AAA  1447
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Glu Asn Lys   426

GAA CAA GGA AGA TTT GGA AGA GAA AAC GAA GAA CAA GGA AGA TTT GGA AGG GGG AAC AAA  1507
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Glu Gln Gly Arg Phe Gly Arg Gly Asn Lys   446

GAA CAA GGA AGA TTT GGA CGA GAA AAC GAA CAA GGA AGA TTT GGA AGA GAA AAC GAA GTT  1567
Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Gln Gly Arg Phe Gly Arg Glu Asn Glu Val   466

CAA GGA AGG TTT GGA AGA TTC AGT CGG GAG TTG GCG AAA GGT TTA AAG ATT GAC GAT GTT  1627
Gln Gly Arg Phe Gly Arg Phe Ser Arg Glu Leu Ala Lys Gly Leu Lys Ile Asp Asp Val   486

CTC TGA  CAATGAACTAATTACGTGAATTACTAGAAACAAACTTAGAGAAAGTTGTTTATGAATCTATCAATAGTAT  1703
Leu  *                                                                             487

TTAAAAAGCGTTTCCAAATATTTAGTGTGAAATGATATTTTAAAAAAATATACACCGAAAAAAAAAAAAAGGCCGATCG  1782
GGCCG(A)32                                                                        1819
```

TABLE III

N- and C-Terminal Extensions of Antho-RFamide and Related Neuropeptide Sequences in the Antho-RFamide Precursor Protein from *R. köllikeri*[a]

N- and C-terminal extensions and neuropeptide sequence	Copy number
Arg↓Glu-Asn-Glu-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	20
Arg↓Glu-Asn-Lys-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	3
Arg↓Glu-Asn-Glu-Lys-Gln-Gly-Arg-Phe-Gly-Arg ↓	3
Arg↓Glu-Ser-Glu-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	2
Arg↓Gly-Asp-Glu-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	2
Arg↓Gly-Asn-Glu-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	1
Arg↓Gly-Asn-Lys-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	1
Arg↓Glu-Asn-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	1
Arg-Lys↓Glu-Ser-Glu-Glu-Glu-Gln-Gly-Arg-Phe-Gly-Arg ↓	1
Arg-Lys ↓ Leu-Asn-Asn-Lys-Leu-Asn-Asp-Ala-Val-Gln-Gly-Arg-Phe-Gly-Arg ↓	1
Arg↓Glu-Asn-Glu-Val-Gln-Gly-Arg-Phe-Gly-Arg ↓	1
Arg↓Glu-Asn-Glu-Val-Gln-Gly-Arg-Phe-Gly-Arg-Phe-Ser-Arg ↓	1
Lys↓Gln-Gly-Arg-Phe-Asn-Arg-Lys ↓	1
	38

[a] The sites of initial cleavage at basic residues are indicated by arrows. Neuropeptide sequences are underlines.

RFamide after initial cleavage has taken place at monobasic sites. Such proteases should be able to cleave at Gly, Asn, Ser, or Lys residues (Table III).

Three Antho-RFamide sequences are not preceded by a Glu, but by a single Lys residue (Table III). We assume that these sequences also yield normal, authentic Antho-RFamide. Two Antho-RFamide sequences are preceded by an Asp-Ala-Val, or Glu-Val sequence (Table III). After initial processing at acidic residues, these sequences could produce N-terminally elongated forms of Antho-RFamide, but again, if a processing enzyme cleaving at the C-terminal side of Val residues exists, these sequences would yield normal Antho-RFamide.

One neuropeptide sequence, which is related to Antho-RFamide (Gln-Gly-Arg-Phe-Asn at amino acid positions 87–91 of Fig. 12), is flanked by basic amino acid residues, and will probably be released from its precursor and converted into <Glu-Gly-Arg-Phe-Asn. Its C-terminal will not be amidated because a C-terminal Gly residue necessary for amidation is lacking. Another amino acid sequence located at the very C terminus of the precursor (at amino acid positions 467–474 of Fig. 12) includes the complete Antho-RFamide sequence. However, the single Arg residue at position 472 (Fig. 12) is probably not used as a monobasic cleavage site because it is followed by a Phe residue (in position +1). Aromatic residues are rare at the +1 position of a monobasic cleavage site (Devi, 1991). Thus, cleavage probably does not occur at Arg-472, but more likely at the Arg-475 residue (Fig. 12, Table III). Furthermore, Arg-475 is followed by a Glu residue, as are most (32×) of the other monobasic cleavage sites in the precursor which, again, argues for cleavage at this site. The neuropeptide sequence is preceded by the sequence Glu-Val (Fig. 12, Table III). Therefore, the structure of the putative, mature neuropeptide is either Val-Gln-Gly-Arg-Phe-Gly-Arg-Phe-Ser, or, if a processing enzyme accepting Val residues exists, <Glu-Gly-Arg-Phe-Gly-Arg-Phe-Ser. Also, this peptide will not be amidated.

Of course we do not know whether every Antho-RFamide sequence in the *Renilla* precursor is processed into mature Antho-RFamide. However, since the spacer regions in-between the Antho-RFamide sequences are so similar (Fig. 12, Table III), we expect that if one Antho-RFamide sequence is processed, all of them will be. Therefore, the processing of one precursor molecule probably generates 36 copies of mature Antho-RFamide (of which a few might be N-terminally extended) and two additional, putative Antho-RFamide-related peptides (Table III). This makes the Antho-RFamide precursor from *Renilla* one of the most productive precursor proteins known so far.

C. Biosynthesis of Antho-RPamide I in Sea Anemones

The peptide Antho-RPamide I (Leu-Pro-Pro-Gly-Pro-Leu-Pro-Arg-Pro-NH_2; Table I) was isolated from the sea anemone *Anthopleura elegantissima* using a radioimmunoassay against the sequence Arg-Pro-NH_2. The peptide is located in sensory neurons (Carstensen *et al.,* 1992) and it increases the frequency of spontaneous tentacle contractions in various sea anemones (Fig. 7). The peptide is remarkable because five out of nine amino acid residues are Pro residues, which makes it one of the most proline-rich neuropeptides that has ever been isolated. The high proline content and especially the N-terminal sequence Leu-Pro-Pro make the peptide resistant against most degrading enzymes, including DPAP (Carstensen *et al.,* 1992).

We have cloned the *Anthopleura* Antho-RPamide I precursor protein by screening an *Anthopleura* cDNA library with an oligonucleotide pool derived from the immature Antho-RPamide I sequence (Leu-Pro-Pro-Gly-Pro-Leu-Pro-Arg-Pro-Gly). Figure 13 shows the cDNA and the deduced primary structure of the Antho-RPamide I precursor (K. Carstensen and C. J. P. Grimmelikhuijzen, unpublished). In contrast to the Antho-RFamide precursor proteins, which contain numerous neuropeptide copies, this precursor only contains one copy of the neuropeptide (at amino acid positions 53–62 of Fig. 13). The immature Antho-RPamide I sequence is followed by three basic residues and preceded by two acidic residues. This Antho-RPamide I precursor provides the proof that there must indeed be processing as at acidic residues: there is only one copy of Antho-RPamide I and the only way to generate the mature neuropeptide is by cleavage at the C-terminal side of an Asp residue. From the structure of the Antho-RPamide I precursor, the processing enzyme cleaving at the C-terminal side of acidic residues is most likely to be an endoproteinase, because the simplest way to liberate Antho-RPamide I is by endoproteolytic cleavage.

```
                                                    CAACCAATTAAGATCAGTTCAAATTGAACCGAACAAAAAAAAA  43

ATG GCA AGT AAG TTA CTG CTA TGT ATG GCT CTG CTG GTG GTG GTG TTT GTG CTG TCT GTA 103
Met Ala Ser Lys Leu Leu Leu Cys Met Ala Leu Leu Val Val Val Phe Val Leu Ser Val  20

GAA AGT CGA CAA AAA GCT GGA GTA GTA CAA GCC GAT GAG TAT CGC CCA AGA CCA GGA AAG 163
Glu Ser Arg Gln Lys Ala Gly Val Val Gln Ala Asp Glu Tyr Arg Pro Arg Pro Gly Lys  40

AAA CAA TAC GAT GGG CCA GAA GGT GAC TAT GAA GAT TTA CCA CCC GGC CCT CTT CCC CGA 223
Lys Gln Tyr Asp Gly Pro Glu Gly Asp Tyr Glu Asp Leu Pro Pro Gly Pro Leu Pro Arg  60

CCC GGC AGA AAA CGC TTT TTT GAA GAG AAT TAC TGA ATAATGGACGCACAGAGAAGACGACATGTTA 290
Pro Gly Arg Lys Arg Phe Phe Glu Glu Asn Tyr  *                                    71
```

FIG. 13. cDNA and duced amino acid sequence of the *Anthopleura* Antho-RPamide I precursor. Neuropeptide sequences are underlined as in Fig. 9. (K. Carstensen and C. J. P. Grimmelikhuijzen, unpublished.)

In addition to Antho-RPamide I, there might be a second, putative neuropeptide sequence, Tyr-Arg-Pro-Arg-Pro-Gly, contained within the precursor (at amino acid positions 34–39 of Fig. 13). This sequence is followed by two basic residues (Lys-Lys) and preceded by acidic amino acid residues. Its mature sequence might be Tyr-Arg-Pro-Arg-Pro-NH_2. However, if the neurons producing the Antho-RPamide I precursor also produce a processing enzyme specific for aromatic residues (Tyr) and DPAP (Arg-Pro), this putative neuropeptide sequence will be degraded.

D. Biosynthesis of Antho-RPamides II–IV in Sea Anemones

In contrast to Antho-RPamide I, Antho-RPamide II inhibits spontaneous tentacle contractions (Fig. 7; Carstensen *et al.*, 1993). Using an oligonucleotide pool coding for immature Antho-RPamide II (Gln-Asn-Phe-His-Leu-Arg-Pro-Gly), we have cloned the Antho-RPamide II precursor from *Anthopleura* (K. Carstensen and C. J. P. Grimmelikhuijzen, unpublished). Table IV gives and overview of the neuropeptides that are contained in this precursor protein: There are two copies of Antho-RPamide II, one copy of Antho-RPamide III, two copies of Antho-RPamide IV, and five other, putative neuropeptide sequences. Each of the established neuropeptide sequences is followed by a single basic residue (Arg).

For our understanding of the N-terminal processing of immature neuropeptide sequences in the Cnidaria, the Antho-RPamide II precursor from *Anthopleura* is a true "Rosetta stone," as it permits the code for several cleavage steps to be deciphered. First, the single and only copy of Antho-RPamide III is preceded by a Tyr residue (Table IV). This means that there must be cleavage at the C-terminal side of Tyr residues. Such cleavage at aromatic residues was assumed earlier (Section VI,A). The processing enzyme cleaving at the C-terminal side of Tyr residues could either be an endoproteinase, or an aminopeptidase. However, it is more likely to be an aminopeptidase, as an internal Tyr residue in the Antho-RPamide III sequence itself should not be cleaved. A Tyr-specific aminopeptidase would imply an additional cleavage at Asn residues for which, however, we have furhter evidence (see Section VI,F). Second, both Antho-RPamide II sequences are preceded by an X-Pro sequence. This means that there must be cleavage at the C-terminal side of Pro residues, a situation that, again, we suspected earlier. The processing enzyme cleaving at the C-terminal side of Pro residues is most likely DPAP, as the X-Pro sequences are preceded by acidic residues, where we know that cleavage could occur (Table IV). Third, one Antho-RPamide IV is preceded by an X-Ala sequence. Again, this suggests processing by DPAP (it requires, however, cleavage at an N-terminal Gly residue). The other Antho-RPamide IV

TABLE IV

N- and C-Terminal Extensions of Authentic and Putative Antho-RPamide Sequences in the Antho-RPamide II Precursor from *A. elegantissima*[a]

N- and C-terminal extension and neuropeptide sequence	Name	Copy number
Lys-Arg↓Asn-Tyr-Gln-Val-Lys-Leu-Tyr-Arg-Pro-Gly-Arg ↓	Antho-RPamide III	1
Arg ↓ Glu - Asp-Val-Pro-Glu-Gly-Pro-Gln-Asn-Phe-His-Leu-Arg-Pro-Gly-Arg ↓	Antho-RPamide II	1
Arg↓Glu-Asp-Val-Pro-Gln-Asn-Phe-His-Leu-Arg-Pro-Gly-Arg ↓	Antho-RPamide II	1
Arg↓Gly-Met-Ala-Gly-Pro-Pro-Ser-Leu-Phe-Arg-Pro-Gly-Arg ↓	Antho-RPamide IV	1
Arg ↓ Glu-Asp-Val-Pro-Asn-Gly-Pro-Pro-Ser-Leu-Phe-Arg-Pro-Gly-Arg ↓	Antho-RPamide IV	1
Lys↓Gly-Pro-Pro-Lys-Leu-Tyr-Arg-Pro-Gly-Arg ↓		1
Lys↓ Ile-Glu-Lys-Pro-Pro-Trp-Pro-Pro-Arg-Pro-Gly-Arg-Arg ↓		1
Arg↓Asp-Ala-Met-Pro-Gln-Thr-Leu-Leu-Arg-Pro-Gly-Arg ↓		1
Arg↓Glu-Asp-Val-Pro-Gln-Lys-Leu-Leu-Arg-Pro-Gly-Arg ↓		1
Arg↓Asp-Glu- Ile-Pro-Glu-Gln-Phe-Asn-Asn-Val-Arg-Ala-Gly-Arg-Arg ↓		1
		10

[a] The sites of initial cleavage at basic residues are given by arrows. Established and putative peptide sequences are underlined.

sequence is preceded by an Asn residue, which strongly suggests cleavage at the C-terminal side of Asn. The Asn residue is preceded by the sequence Glu-Asp-Val-Pro, which could be removed by the acidic residue-specific protease followed by DPAP (Table IV). The putative, Asn residue-specific protease, therefore, could be an aminopeptidase.

The other, putative neuropeptide sequences of the Antho-RPamide II precursor (Table IV) could be processed in the same way as described above (using DPAP and an acidic residue-specific proteinase), but we do not want to stress this too much because these sequences have not been isolated yet. It is interesting to see, however, that the Antho-RPamide II precursor probably contains eight different Arg-Pro-NH_2 peptides.

E. Biosynthesis of Metamorphosis-Inducing Peptides in Sea Anemones

We discussed in Section IV that cnidarian peptides are also involved in developmental processes such as metamorphosis, and that a peptide inducing metamorphosis in planula larvae of *Hydractinia echinata* has been isolated from extracts of the sea anemone *Anthopleura elegantissima* (Leitz *et al.*, 1994; Leitz and Lay, 1995). This peptide, <Glu-Gln-Pro-Gly-Leu-Trp-NH_2 (metamorphosin A, or MMA), has an interesting structure because it does not belong to the large family of Arg-X-NH_2/Lys-X-NH_2 neuropeptides present in sea anemones (Tables I and IV; Fig. 10).

We have cloned the preprohormone for the metamorphosis-inducing neuropeptide MMA by using an oligonucleotide probe derived from the immature neuropeptide sequence (Gln-Gln-Pro-Gly-Leu-Trp-Gly) and screening a λgt11 cDNA library from *Anthopleura elegantissima.* Figure 14 shows the cDNA and the deduced amino acid sequence of the peptide precursor (Leviev and Grimmelikhuijzen, 1995). The precursor contains 10 copies of immature MMA. These immature peptide copies have mostly dibasic processing sites at their C termini (Fig. 14, Table V). At their N termini, however, 9 copies are preceded by the sequence Ser-Ala-Asp-Pro. One copy of the peptide is preceded by the sequence Ser-Ala-Ala-Pro. Since authentic <Glu-Gln-Pro-Gly-Leu-Trp-NH_2 has been isolated from sea anemone extracts, this, again, clearly proves that there must be processing at X-Pro and X-Ala sequences, most likely catalyzed by a DPAP. This precursor structure, therefore, stresses the general importance of DPAP in cnidarian neuropeptide precursor processing.

In addition to the 10 copies of immature MMA, there is a large number of other, putative peptides that are closely related to authentic MMA (Table V). For reasons of simplicity, we have named the most frequent, putative peptide Antho-LWamide I (14 copies), the authentic metamorphosis-

```
CAATGAACTGAGTGGAACACAAGTAATACATATTCTTCACTTCGGTTGATA ATG GCC CTC AAG TGT CAT CTA GTT CTA CTG      81
                                                    Met Ala Leu Lys Cys His Leu Val Leu Leu      10

GCC ATT ACT TTA CTA TTA GCA CAG TGT TCA GGG TCA GTA GAC AAG AAG GAT AGT ACG ACG AAT CAC TTA     150
Ala Ile Thr Leu Leu Leu Ala Gln Cys Ser Gly Ser Val Asp Lys Lys Asp Ser Thr Thr Asn His Leu      33

GAT GAG AAG AAA ACA GAT TCC ACA GAA GCA CAT ATT GTA CAA GAA ACA GAC GCG TTA AAA GAA AAT TCT     219
Asp Glu Lys Lys Thr Asp Ser Thr Glu Ala His Ile Val Gln Glu Thr Asp Ala Leu Lys Glu Asn Ser      56

TAT CTT GGC GCC GAG GAG GAA TCT AAA GAA CAA GAC AAG AAG AGA TCC GCC GCT CCT CAG CAG CCT GGC     288
Tyr Leu Gly Ala Glu Glu Glu Ser Lys Glu Glu Asp Lys Lys Arg Ser Ala Ala Pro Gln Gln Pro Gly      79

CTC TGG GGG AAA CGC CAG AAA ATA GGA CTA TGG GGA AGA TCC GCT GAC GCA GGA CAG CCA GGC CTC TGG     357
Leu Trp Gly Lys Arg Gln Lys Ile Gly Leu Trp Gly Arg Ser Ala Asp Ala Gly Gln Pro Gly Leu Trp     102

GGC AAA CGA CAA AGT CCC GGA TTA TGG GGA AGA TCC GCT GAC GCA GGA CAG CCA GGC CTC TGG GGC AAA     426
Gly Lys Arg Gln Ser Pro Gly Leu Trp Gly Arg Ser Ala Asp Ala Gly Gln Pro Gly Leu Trp Gly Lys     135

CGT CAA AAT CCC GGA TTA TGG GGA AGA TCC GCT GAC GCA GGA CAG CCA GGC CTC TGG GGC AAA CGT CAA     495
Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala Asp Ala Gly Gln Pro Gly Leu Trp Gly Lys Arg Gln     148

AAT CCC GGA TTA TGG GGA AGA TCG GCT GAC GCA GGA CAG CCA GGC CTC TGG GGC AAA CGT CAA AAT CCC     564
Asn Pro Gly Leu Trp Gly Arg Ser Ala Asp Ala Gly Gln Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro     171

GGA TTA TGG GGA AGG TCC GCT GAC GCA AGA CAA CCC GGA CTC TGG GGC AAA CGT GAA ATC TAC GCA TTA     633
Gly Leu Trp Gly Arg Ser Ala Asp Ala Arg Gln Pro Gly Leu Trp Gly Lys Arg Glu Ile Tyr Ala Leu     194

TGG GGA GGA AAA CGT CAA AAT CCC GGA CTT TGG GGA AGA TCC GCT GAT CCA GGA CAG CCC GGC CTC TGG     702
Trp Gly Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala Asp Pro Gly Gln Pro Gly Leu Trp     217

GGC AAA CGT GAA CTC GTC GGA TTA TGG GGG GGA AAA CGT CAA AAC CCC GGA TTG TGG GGA AGA TCG GCT     771
Gly Lys Arg Glu Leu Val Gly Leu Trp Gly Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala     240

GAA GCA GGA CAG CCA GGA CTT TGG GGA AAA CGC CAA AAA ATA GGA TTG TGG GGA CGT TCG GCT GAC CCA     840
Glu Ala Gly Gln Pro Gly Leu Trp Gly Lys Arg Gln Lys Ile Gly Leu Trp Gly Arg Ser Ala Asp Pro     263

CTT CAG CCT GGC CTC TGG GGC AAA CGT CAA AAT CCC GGA TTA TGG GGA AGA TCT GCT GAC CCG CAG CAG     909
Leu Gln Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala Asp Pro Gln Gln     286

CCT GGC CTC TGG GGC AAA CGT CAA AAT CCC GGA TTA TGG GGA AGA TCT GCT GAC CCG CAG CAG CCT GGC     978
Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala Asp Pro Gln Gln Pro Gly     309

CTC TGG GGC AAA CGT CAA AAT CCC GGA TTA TGG GGA AGA TCT GCT GAC CCG CAG CAG CCT GGC CTC TGG    1047
Leu Trp Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala Asp Pro Gln Gln Pro Gly Leu Trp     332

GGC AAA CGT CAA AAT CCC GGA TTA TGG GGA AGA TCT GCT GAC CCG CAG CAA CCT GGC CTC TGG GGC AAA    1116
Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala Asp Pro Gln Gln Pro Gly Leu Trp Gly Lys     355

AGC CCC GGT TTA TGG GGA CGA TCC GCT GAC CCA CAA CAG CCT GGA CTT TGG GGG AAA CGC CAA AAT CCC    1185
Ser Pro Gly Leu Trp Gly Arg Ser Ala Asp Pro Gln Gln Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro     378

GGA TTT TGG GGA AGA TCT GCT GAC CCG CAG CAG CCT GGC CTC TGG GGC AAA CGT CAA AAT CCC GGA TTA    1254
Gly Phe Trp Gly Arg Ser Ala Asp Pro Gln Gln Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro Gly Leu     401

TGG GGA AGA TCT GCT GAC CCG CAG CAA CCT GGC CTC TGG GGC AAA CGT CAA AAT CCC GGA TTA TGG GGA    1323
Trp Gly Arg Ser Ala Asp Pro Gln Gln Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly     424

AGA TCT GCT GAC CCG CAG CAA CCT GGC CTC TGG GGC AAA CGT CAA AAC CCC GGT TTA TGG GGA CGA TCC    1392
Arg Ser Ala Asp Pro Gln Gln Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser     447

GCT GAC CCA CAA CAG CCT GGA CTT TGG GGG AAA CGC CAA AAT CCA GGA CTA TGG GGA AGA AGT GCT GGC    1461
Ala Asp Pro Gln Gln Pro Gly Leu Trp Gly Lys Arg Gln Asn Pro Gly Leu Trp Gly Arg Ser Ala Gly     470

TCC GGT CAA CTC GGA CTT TGG GGT AAA AGG CAA TCA CGC ATT GGA TTA TGG GGA AGA TCT GCC GAG CCT    1530
Ser Gly Gln Leu Gly Leu Trp Gly Lys Arg Gln Ser Arg Ile Gly Leu Trp Gly Arg Ser Ala Glu Pro     493

CCA CAA TTT GAA GAT TTA CAA GAT TTA AAG AAA AAA TCA GCA ATT CCC CAA CCA AAA GGA CAA TGA TAA    1599
Pro Gln Phe Glu Asp Leu Glu Asp Leu Lys Lys Lys Ser Ala Ile Pro Gln Pro Lys Gly Gln Stop Stop    514

TATCCTAGGATCTTCAAAAGTTATCCCGATCATCAATCCCCGGACAAGAGATATTTTAATTTCTGCCGCACGATTGACAGTTCCATTCCAT    1690
TACGAAGAACAAAAAGCTACGTTTCTTTAAGATAATAAATCAAATTCAATATTGTTTCAAGCAATGCACTTCAGGTTTTCACACAAAACTA    1781
ATACAAAAGTTATAAACATAAATAATAAACAAAAAAGGGCTAACAAACCTGGTTTTTCGTTTTAGACTTTTCAAATTGGTCCTGCATGTCA    1872
TTGTAAGAGGTCGGCAGTAGAAGCTCTTTGAAACACTGTAAGTAGTCATTGAATGCACATTTATGGCAATAAAGATCACCCAGTGGTCTGC    1963
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FIG. 14. cDNA and deduced amino acid sequence of the precursor for the metamorphosis-inducing peptide from *Anthopleura.* Copies of the authentic metamorphosis-inducing peptide are underlined and printed in boldfaced type, whereas the numerous related but putative neuropeptide sequences are underlined only. (Modified from Leviev and Grimmelikhuijzen, 1995.)

TABLE V

N- and C-terminal Extensions of MMA and Related, Putative Neuropeptide Sequences in the MMA Precursor from *A. elegantissima*[a]

N- and C-terminal extensions and neuropeptide sequence	Copy number	Name
Arg ↓ Ser-Ala-Asp-Pro-**Gln-Gln-Pro-Gly-Leu-Trp-Gly**-Lys-Arg↓[a]	8	MMA (Antho-LWamide II)
Arg ↓ Ser-Ala-Asp-Pro-**Gln-Gln-Pro-Gly-Leu-Trp-Gly**-Lys↓[b]	1	MMA (Antho-LWamide II)
Arg ↓ Ser-Ala-Ala-Pro-**Gln-Gln-Pro-Gly-Leu-Trp-Gly**-Lys-Arg↓[c]	1	MMA (Antho-LWamide II)
Lys-Arg↓**Gln-Asn-Pro-Gly-Leu-Trp-Gly**-Arg↓[d]	14	Antho-LWamide I
Lys-Arg↓**Gln-Ser-Pro-Gly-Leu-Trp-Gly**-Arg↓[e]	1	Antho-LWamide VII
Lys-Arg↓**Gln-Lys-Ile-Gly-Leu-Trp-Gly**-Arg↓[f]	2	Antho-LWamide IV
Lys-Arg↓**Gln-Ser-Arg-Ile-Gly-Leu-Trp-Gly**-Arg↓[g]	1	Antho-LWamide VIII
Arg ↓ Ser-Ala-**Gly-Ser-Gly-Gln-Leu-Gly-Leu-Trp-Gly**-Lys-Arg↓[h]	1	Antho-LWamide IX
Arg ↓ Ser-Ala-Asp-Ala-**Gly-Gln-Pro-Gly-Leu-Trp-Gly**-Lys-Arg↓[i]	4	Antho-LWamide III
Arg ↓ Ser-Ala-Glu-Ala-**Gly-Gln-Pro-Gly-Leu-Trp-Gly**-Lys-Arg↓[j]	1	Antho-LWamide III
Arg ↓ Ser-Ala-Asp-Pro-**Gly-Gln-Pro-Gly-Leu-Trp-Gly**-Lys-Arg↓[k]	1	Antho-LWamide III
Arg ↓ Ser-Ala-Asp-Pro-**Leu-Gln-Pro-Gly-Leu-Trp-Gly**-Lys-Arg↓[l]	1	Antho-LWamide V
Arg ↓ Ser-Ala-Asp-Ala-**Arg-Gln-Pro-Gly-Leu-Trp-Gly**-Lys-Arg↓[m]	1	Antho-LWamide VI
Lys↓Ser-Pro-**Gly-Leu-Trp-Gly**-Arg↓[n]	1	
Lys-Arg↓**Glu-Leu-Val-Gly-Leu-Trp-Gly-Gly**-Lys-Arg↓[o]	1	
Lys-Arg↓**Glu-Ile-Tyr-Ala-Leu-Trp-Gly-Gly**-Lys-Arg↓[p]	1	
Arg ↓ Ser-Ala-**Glu-Pro-Pro-Gln-Phe-Glu-Asp-Leu-Glu-Asp-Leu**-Lys-Lys-Lys ↓[q]	1	

[a] The sites of initial cleavage at basic residues are indicated by arrows. MMA copies are underlined and printed boldfaced type. Highly likely, but putative peptide sequences are underlined only. Uncertain mature sequences or residues are underlined by a dotted line. The neuropeptide sequences given in this table can be found in Fig. 14 at the following amino acid positions: *a*, 285, 306, 327, 367, 388, 409, 430, 451; *b*, 348; *c*, 76; *d*, 127, 148, 169, 200, 231, 273, 294, 315, 336, 376, 397, 418, 439, 460; *e*, 106; *f*, 85, 252; *g*, 481; *h*, 470; *i*, 97, 118, 139, 160; *j*, 243; *k*, 212; *l*, 264; *m*, 181; *n*, 358; *o*, 221; *p*, 190; *q*, 492.

inducing peptide isolated by Leitz *et al.* (1994) Antho-LWamide II (10 copies), a third peptide occurring in high-frequency Antho-LWamide III (7 copies) and other, closely related peptides Antho-LWamides IV-IX (Table V). The 14 immature Antho-LWamide I sequences are followed by single basic amino acid residues (Arg) and preceded by Lys-Arg. Thus, although Antho-LWamide I is still a putative peptide that has not been isolated yet, it is quite certain that it will exist and be released in a very high copy number from its precursor protein. The Antho-LWamides IV, VII, and VIII have exactly the same processing sites as Antho-LWamide I, and it is likely that these peptides will also be released from the Antho-LWamide precursor. All mature Antho-LWamides I, II, IV, VII, and VIII will have an N-terminal <Glu group and the C-terminal structure Gly-Leu-Trp-NH_2 (Table V).

There are other, putative peptides (the Antho-LWamides III, V, VI and IX) that are very similar or nearly identical to the authentic peptide MMA (Antho-LWamide II). Their immature sequences are followed by Lys-Arg residues and they are preceded by X-Ala or X-Pro sequences (Table V), so they will certainly be released and processed. Their final structures, however, are still uncertain. They might start with a <Glu group, or be N-terminally elongated by a single Gly, Leu, or Arg residue, or by the sequence Gly-Ser-Gly (see Leviev and Grimmelikhuijzen, 1995, for details). Finally, there are other, putative peptide sequences flanked by basic residues, but it is quite uncertain whether they will be processed into intact peptides (Table V).

F. Biosynthesis of Neuropeptides in Hydrozoans

We mentioned in Section IV that we have isolated two peptides from the hydrozoan medusa *Polyorchis penicillatus,* using an RFamide radioimmunoassay. These peptides, Pol-RFamide I and II, are closely related and form a peptide family with Antho-RFamide from anthozoans (Table I). The Pol-RFamides are located in neurons (Grimmelikhuijzen *et al.,* 1988b, 1992b).

We have cloned the Pol-RFamide precursor by screening a *Polyorchis* cDNA library with an oligonucleotide probe coding for immature Pol-RFamide II (Schmutzler *et al.,* 1994). The Pol-RFamide precursor contains 11 copies of Pol-RFamide II, 1 copy of Pol-RFamide I and 1 novel, putative neuropeptide sequence (Fig. 15; Table VI). Eight copies of Pol-RFamide II are preceded by an Asp residue, 3 copies by an Asn residue. Pol-RFamide I, however, is flanked by pairs of basic residues (Table VI). Thus, the unusual processing sites that we found in neuropeptide precursors of anthozoans (Asp or Glu in the Antho-RFamide and Antho-RPamide I precur-

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                                                    CAAAGAGAGACACAGTGGAAGCAGTCGAGAATATTAAA   38

ATG AAT CTA ATA ACA CTA CTT GTA CTT GGC GTG TCT ACT TGT TTA ATA TAT GGT ATT GAA   98
Met Asn Leu Ile Thr Leu Leu Val Leu Gly Val Ser Thr Cys Leu Ile Tyr Gly Ile Glu   20

GCT GAC GAA AAA ACA TCA AGT GCT CTT GAA AAT GAA ATT GTG GAA ATC TTA AAT GGA AAT  158
Ala Asp Glu Lys Thr Ser Ser Ala Leu Glu Asn Glu Ile Val Glu Ile Leu Asn Gly Asn   40

TTT AAA AAT GAA AAA AAA TCT ATA GAA ACA TCA GAT CAG TGG TTA AAA GGG CGA TTT GGT  218
Phe Lys Asn Glu Lys Lys Ser Ile Glu Thr Ser Asp Gln Trp Leu Lys Gly Arg Phe Gly   60

CGT GAA GTG AAC CAA TGG TTA AAA GGG AGA TTT GGT CGC GAG TTG TCA GAT CAG TGG TTA  278
Arg Glu Val Asn Gln Trp Leu Lys Gly Arg Phe Gly Arg Glu Leu Ser Asp Gln Trp Leu   80

AAA GGG AGG TTT GGT CGC GAG TTG TCA GAT CAG TGG TTA AAA GGA AGA TTT GGA CGA GAG  338
Lys Gly Arg Phe Gly Arg Glu Leu Ser Asp Gln Trp Leu Lys Gly Arg Phe Gly Arg Glu  100

GTA CTC GAT CAG TGG TTG AAA GGA AGA TTT GGT CGT GAT GCA TCA AAC CAA TGG TTG AAA  398
Val Leu Asp Gln Trp Leu Lys Gly Arg Phe Gly Arg Asp Ala Ser Asn Gln Trp Leu Lys  120

GGG AGA TTT GGT CGC GAG TTG TCA GAT CAA TGG TTA AAA GGA AGA TTT GGT CGT GAA GGA  458
Gly Arg Phe Gly Arg Glu Leu Ser Asp Gln Trp Leu Lys Gly Arg Phe Gly Arg Glu Gly  140

TCA AAC CAA TGG TTA AAA GGA AGA TTT GGT CGT GAA GCA TCA AAG AAC GAT CTG GAA AAA  518
Ser Asn Gln Trp Leu Lys Gly Arg Phe Gly Arg Glu Ala Ser Lys Asn Asp Leu Glu Lys  160

CAA AAT GGT AGG GGT GAT TCG GAC CAG TGG CTT AAA GGA AGA TTT GGC CGT GAA GCA AGG  578
Gln Asn Gly Arg Gly Asp Ser Asp Gln Trp Leu Lys Gly Arg Phe Gly Arg Glu Ala Arg  180

AAG CAG TTA TTG GGA GGA AGA TTT GGG CGT AAA GAT ATG AAT CAG TTA TTA GCA GAA CGA  638
Lys Gln Leu Leu Gly Gly Arg Phe Gly Arg Lys Asp Met Asn Gln Leu Leu Ala Glu Arg  200

CAT GGG CGT GAA ACA TCC GAT CAA TGG TTA AAG GGG AGG TTT GGA CGA CAA CTT TCA GAT  698
His Gly Arg Glu Thr Ser Asp Gln Trp Leu Lys Gly Arg Phe Gly Arg Gln Leu Ser Asp  220

CAA TGG TTG AAA GGA AGA TTC GGC CGT GAA GTA AAA AAT GAT AAA AAT AAT CCA TTT CGC  758
Gln Trp Leu Lys Gly Arg Phe Gly Arg Glu Val Lys Asn Asp Lys Asn Asn Pro Phe Arg  240

AGT CGT TAC ACA GGA GAT TCA ACA CAA CTG CAG CGA GAG AAC AAC CAA CCT ATT GAA GAA  818
Ser Arg Tyr Thr Gly Asp Ser Thr Gln Leu Gln Arg Glu Asn Asn Gln Pro Ile Glu Glu  260

TTA AGA GAC AAT ACA GAA AAA GTG TCG ATA GAA AAT AAA CCA ATC ATG AAA AAA ACT AGC  878
Leu Arg Asp Asn Thr Glu Lys Val Ser Ile Glu Asn Lys Pro Ile Met Lys Lys Thr Ser  280

GTC AAA ATT AGC AAA ACT GTT TAA GTAGAAAATAAAGTTTTATAAAGCTTGTTCTTTACTATGAAACACAG  949
Val Lys Ile Ser Lys Thr Val  *                                                    287

TGTTGAAAAGATTTTAGATCGAGTAGTAATTAATACTTAATACTATGTTTAACAATTTACCCAAATAATTT        1020
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FIG. 15. cDNA and deduced amino acid sequence of the precursor for Pol-RFamide I and II from *Polyorchis.* Copies of the authentic peptides are underlined and printed in boldfaced type, whereas the single putative neuropeptide sequence is underlined only. (Modified from Schmutzler *et al.,* 1994).

sors; Asn in the Antho-RPamide II precursor) are fully confirmed in the hydrozoans.

Using the same RFamide radioimmunoassay, we have also isolated four RFamide neuropeptides from the hydrozoan polyp *Hydra magnipapillata.* These peptides (Hydra RFamides I–IV) are very similar in structure to the Pol-RFamides. They are not given in Table I because we have not published them yet. The Hydra-RFamides I–IV are localized as single copies on one common precursor protein (D. Darmer and C. J. P. Grimmelikhuijzen, unpublished). The immature Hydra-RFamide I sequence is pre-

TABLE VI

N- and C-terminal Extensions of the Pol-RFamides and a Putative Neuropeptide Sequence in the Pol-RFamide Precursor Protein from *P. penicillatus*[a]

N- and C-terminal extensions and neuropeptide sequence	Name	Copy number
Arg-Lys↓Gln-Leu-Leu-Gly-Gly-Arg-Phe-Gly-Arg-Lys↓	Pol-RFamide I	1
Arg↓Glu-Leu-Ser-Asp-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	3
Arg↓Gln-Leu-Ser-Asp-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Arg↓Glu-Thr-Ser-Asp-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Lys-Lys↓Ser-Ile-Glu-Thr-Ser-Asp-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Lys↓Gln-Asn-Gly-Arg-Gly-Asp-Ser-Asp-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Arg↓Glu-Val-Leu-Asp-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Arg↓Glu-Gly-Ser-Asn-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Arg↓Asp-Ala-Ser-Asn-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Arg↓Glu-Val-Asn-Gln-Trp-Leu-Lys-Gly-Arg-Phe-Gly-Arg↓	Pol-RFamide II	1
Arg-Lys↓Asp-Met-Asn-Gln-Leu-Leu-Ala-Glu-Arg-His-Gly-Arg↓		1

[a] Initial cleavage sites are indicated by arrows. Neuropeptide sequences are underlined.

ceded by an Asp residue, Hydra-RFamide II by Thr, and Hydra-RFamide IV by an Asn residue. Hydra-RFamide III originates from Hydra-RFamide IV by elimination of an N-terminal X-Pro sequence (DPAP). Thus, the Hydra-RFamide precursor fully confirms processing at acidic residues (Asp), at Asn residues, and at N-terminal X-Pro sequences. Furthermore, there is now clear evidence for processing at Thr residues, which, because the two amino acids are so similar, is related to processing at Ser residues that we suspected earlier.

VII. Discussion

From our present review, it is clear that neuropeptides play an extremely important role in the physiology of cnidarians. Many neuropeptides may be neuromuscular or neuroneuronal transmitters released at synaptic or nonsynaptic sites, but others may be hormones controlling developmental processes such as metamorphosis. Nervous systems probably first evolved in cnidarians or in a closely related ancestor group. This suggests that the first nervous systems in evolution used peptides as transmitters. Of all the neuropeptides studied so far, the Gly-Arg-Phe-NH_2 peptides appear to be the most ubiquitous, as they occur in species belonging to all classes of Cnidaria. These types of peptides, therefore, are good candidates for being the first transmitters.

It is amazing that the primitive nervous systems of cnidarians are already so complex. From a single sea anemone species, *Anthopleura elegantissima,* for example, we have isolated 16 different neuropeptides (Table I; C. J. P. Grimmelikhuijzen, unpublished) and Leitz and coworkers (1994) have isolated one further peptide. If we look at the biosynthesis of these 17 peptides, we often find preprohormones containing many additional, putative neuropeptide sequences. Most of these putative neuropeptide sequences are likely to be liberated from their prohormones and converted into biologically active peptides (Fig. 10; Tables III–VI). If we add these likely neuropeptide structures to the number of peptides that we have actually isolated, we reach a number of about 30. This number will increase because we have probably not isolated all the neuropeptides that have a function in sea anemones. Do all these different peptides have a different action? At present, this is difficult to answer, but for the RPamide peptides we have found that Antho-RPamide I stimulates tentacle contractions, whereas the Antho-RPamides II–IV inhibit spontaneous contractions of the tentacle (Fig. 7; K. Carstensen, unpublished). Antho-RPamide I is produced by a precursor protein (Fig. 13) that is different from the one producing the Antho-RPamides II–IV (Table IV). So this could mean that

for different actions one would need a different precursor (and perhaps a different type of neuron), and that all the neuropeptide sequences located on the same precursor might give the same or similar actions.

We find it very impressive that some of the cnidarian preprohormones have such a high copy number of the immature neuropeptide sequences. The Antho-RFamide precursor from *Renilla köllikeri* (Fig. 12), for example, contains 36 copies of Antho-RFamide and 2 additional neuropeptide sequences. We have formatted Fig. 12 in such a way to show that the Antho-RFamide copies in the *Renilla* precursor are arranged in a very regular and condensed manner. Also, the Antho-LWamide precursor from *Anthopleura elegantissima* (Fig. 14) contains 37 neuropeptide copies. Such a high number of neuropeptide copies has never been found in vertebrate preprohormones. Although there are vertebrate precursors that have a similar organization, for example, prepro-enkephalin A or prepro-TRH (Comb *et al.*, 1982; Noda *et al.*, 1982; Lechan *et al.*, 1986), the number of repetitive neuropeptide copies never exceeds seven. Also among the invertebrate preprohormones, the cnidarian precursors are unmatched. The repetitive structure of neuropeptide precursors is often explained by "unequal crossing over," which would have occurred repeatedly during evolution (Schaefer *et al.*, 1985). These recombinations have apparently occurred more frequently in invertebrates than in higher animal groups, with cnidarians, especially the anthozoans, being the most extreme examples.

In higher animals, neuropeptide preprohormones are converted into their mature neuropeptides with the help of about four to five processing enzymes (Fig. 8). The same, or very similar processing enzymes must act on the cnidarian preprohormones because each immature neuropeptide sequence in the preprohormone is followed by pairs of basic or single basic residues, indicating that the enzymes catalyzing steps 1 and 2 of Fig. 7 are present. Furthermore, a C-terminal amide in the mature neuropeptide originates from a C-terminal Gly residue in the immature peptide sequence (step 3 of Fig. 8) and an N-terminal < Glu originates from an N-terminal Gln residue (step 4 of Fig. 8). In addition to these four to five processing enzymes known from mammals, however, many more enzymes must be involved in the maturation of cnidarian preprohormones. It is interesting that all these additional, novel processing enzymes act on processing sites located at the N termini of the immature neuropeptide sequences. In Fig. 16 we have made a list of all the established ("certain") and putative ("uncertain") processing sites and the corresponding novel processing enzymes. We have seen that in nearly all cnidarian preprohormones, there must be processing at the C-terminal sites of acidic (Glu or Asp) residues. Therefore, acidic residues are established processing sites in cnidarians. It is not certain whether the processing enzyme acting at these acidic residues is an endopro-

	Novel or unconventional processing enzymes		Status
*	Glu↓	endoproteinase or aminopeptidase	certain
	Asp↓		certain
*	Asn↓	endoproteinase or aminopeptidase	certain
*	Tyr↓	endoproteinase or aminopeptidase	certain
	Phe↓		possible
	Leu↓		possible
	Val↓		possible
*	Thr↓	endoproteinase or aminopeptidase	certain
	Ser↓		possible
*	X-Pro↓	dipeptidylaminopeptidase (DPAP)	certain
	X-Ala↓		certain
*	Gly↓	endoproteinase or aminopeptidase	possible

FIG. 16. Novel processing sites and corresponding processing enzymes discovered by cloning of a large number of neuronal preprohormones from cnidarians. The arrows indicate the sites of proteolysis.

teinase or an aminopeptidase, but the amino acid sequence preceding the single immature Antho-RPamide I in its precursor protein (Fig. 13) suggests that the enzyme is and endoproteinase.

Processing might also occur at the C-terminal side of single Asn residues (Antho-RPamide IV sequence in Table IV; Pol-RFamide II sequence in Table VI) and the Hydra-RFamide precursor shows that, indeed, there must be processing at Asn residues in order to liberate the authentic peptide Hydra-RFamide IV. Thus, Asn residues are also established processing sites in cnidarian preprohormones. The same is true for Tyr residues (Antho-RPamide III sequence in Table IV), Thr residues (Hydra-RFamide II sequence), and X-Ala or X-Pro sequences (Antho-RPamide II sequences in Table IV; Antho-LWamide II sequences in Table V). The processing enzyme cleaving at the C-terminal side of Tyr residues is probably an aminopeptidase because an internal Tyr-Arg sequence contained within

the immature Antho-RPamide III sequence (Table IV) should not be cleaved. Also, the presumed DPAP, cleaving at X-Ala or X-Pro sequences is, of course, an aminopeptidase. For the other processing sites (Asn and Thr residues), it is uncertain whether the corresponding processing enzyme is an aminopeptidase or an endoproteinase.

In addition to the above-mentioned seven "certain" processing sites (Fig. 16), there are other residues that might perhaps be used for cleavage. These are Phe, Leu, and Val, which have a bulky, neutral, aromatic, or aliphatic side chain, and which are similar to the Tyr residue, Ser (similar to Thr), and Gly (Fig. 16). The existence of the Phe, Leu, Val, and Gly processing sites is suggested by the presence of putative, immature neuropeptide sequences on the preprohormone that differ from the other, authentic sequences by having a single, additional, N-terminal Phe, Leu, Val, or Gly residue. Thus, processing at N-terminal Phe, Leu, and Val residues would yield many more copies of authentic Antho-RFamide from the Antho-RFamide precursors from sea anemones (Fig. 10), and *Renilla* (Table III). Similarly, processing at N-terminal Leu and Gly residues would yield many more copies of the putative peptide Antho-LWamide III from the sea anemone Antho-LWamide precursor (Table V). The existence of Ser as a putative processing site (Fig. 16) is mainly suggested by the ocurrence of multiple (up to 11) putative neuropeptide sequences located in the N terminus of the Antho-RFamide precursor from sea anemones (Figs. 9–11). Cleavage at the C-terminal side of Ser residues would enable DPAP to remove an X-Ala or X-Pro sequence and to yield a large group of related peptides starting with a <Glu group (Figs. 9–11). The processing enzyme cleaving at the Ser residue needs to be an endoproteinase. Again, it should be stressed that only (1) Glu and Asp, (2) Asn, (3) Tyr, (4) Thr, and (5) X-Pro and X-Ala sequences are established processing sites, and that the other sites discussed in this paragraph are purely hypothetical.

Do all the novel ("certain") processing sites (Fig. 16) reflect the existence of different, novel processing enzymes? This question is difficult to answer as long as we have not isolated and characterized the enzymes corresponding to each new processing site. However, it is likely that one single enzyme, DPAP, will accept both N-terminal X-Pro and X-Ala sequences (Kreil, 1990). Similarly, it will probably be only one, but a different processing enzyme (perhaps an endoproteinase) that cleaves at the C-terminal side of Glu and Asp residues. Asn, Tyr, and Thr are structurally quite different amino acid residues, and it is likely that the corresponding processing enzymes are three different enzymes. However, it cannot be ruled out that a single enzyme, or an enzyme complex functioning as a relatively unspecific aminopeptidase, would be able to cleave at the C-terminal sides of Asn, Tyr, Thr, and eventually also Glu and Asp residues. This unspecific amino-

peptidase should not be able to cleave at the C-terminal side of Gln residues because the biologically active peptide sequences should not be degraded. Alternatively, the cyclization of an N-terminal Gln into an N-terminal <Glu residue could be relatively fast (Fischer and Spiess, 1987; Pohl *et al.,* 1991) in order to prevent further degradation. We have already pointed out that an N-terminal X-Pro-Pro or X-Pro-Hyp sequence (Table I) would also protect mature neuropeptides against further degradation by an unspecific aminopeptidase.

Let us take the Antho-RPamide II precursor to illustrate this second processing scenario, where DPAP and an unspecific aminopeptidase carry out the N-terminal trimming (Table IV): initial cleavage at mono and dibasic sites liberates the immature neuropeptides. Subsequently, C-terminal processing takes place in a classical way according to Fig. 8. Removal of the N-terminal extensions occurs by a concerted action of the unspecific aminopeptidase and DPAP. The trimming of the N terminus is stopped at free, N-terminal Gln residues (which are quickly converted into <Glu residues) or at N-terminal X-Pro-Pro sequences (which are resistant by themselves).

It could also be that the real situation is a combination of the two extremes mentioned above, i.e., that there exists DPAP, a few specific proteases (e.g., the one cleaving at acidic residues), and a peptidase that is relatively unspecific. Thus, there are probably between two and six novel processing enzymes in cnidarian neurons, in addition to the four to five classical processing enzymes already known from higher animals (Figs. 8 and 16).

In the yeast *Saccharomyces cerevisiae,* the α-mating factor precursor protein is processed by an initial, endoproteolytic cleavage at pairs of basic residues. The enzyme responsible for this cleavage (the Kex-2 gene product; Julius *et al.,* 1984; Fuller *et al.,* 1988) is structurally related to the neuronal processing enzymes PC1/PC3 and PC2 from mammals. The initial cleavage step is followed by removal of the C-terminal basic residues by a yeast carboxypeptidase B-like enzyme, which has also been characterized at the molecular level (the Kex-1 gene product; Dmochowska *et al.,* 1987; Fuller *et al.,* 1988). This processing pattern in yeast resembles very much the cleavage steps in prohormones and other proproteins of higher organisms, including cnidarians (Fig. 8). We have mentioned that the final trimming of the N-terminally extended yeast α-mating factors is carried out by DPAP (Fuller *et al.,* 1988; Kreil, 1990). DPAP has not yet been found in neurons of higher animals, but we have indicated in this review that a DPAP is likely to be involved in the processing of N-terminally extended neuropeptides in neurons of cnidarians. For the initial processing of the precursor of *a*-factor, which is a second mating pheromone from *Saccharomyces,* an internal cleavage at the C-terminal side of Asn residues takes place (Brake *et al.,* 1985). A similar cleavage occurs during the maturation of the precursor for M-factor, which is a different mating pheromone from the yeast *Schizo-*

saccharomyces pombe (Davey, 1992). Thus, some of the additional neuronal processing enzymes that we postulate in cnidarians (Fig. 16) might have their counterparts in yeasts and other lower eukaryotes. In this context, it is interesting to mention that the postulated novel processing enzyme specific for acidic residues might have the same substrate specificity as the V8 protease from *Staphylococcus aureus* (Drapeau *et al.*, 1972; Rydén *et al.*, 1974). Thus, the cnidarian processing enzyme specific for acidic residues might even have its counterpart in prokaryotes.

On the other hand, it might also be possible that the novel, neuronal processing enzymes that we postulate in cnidarians have their counterparts in higher animals. We have already mentioned that non-neuronal DPAP is involved in the N-terminal trimming of immature honey bee mellitin and the frog skin peptides caerulein and xenopsin (Kreil, 1990). In crustaceans, cleavage at the C-terminal side of Ser residues has been postulated for the maturation of the crustacean neuropeptide H (Newcomb, 1987). Finally, it was found in rats that biologically active, C-terminal gastrin fragments are generated from gastrin-17, by cleavage at the C-terminal side of acidic residues (Rehfeld *et al.*, 1995). Thus, some of the novel, neuronal processing enzymes that we now postulate in cnidarians might have their relatives both in lower eukaryotes and prokaryotes, and in higher animals. This would be a situation similar to that of subtilisin (from *Bacillus subtilis*), the Kex-2 gene product (from *Sacchoromyces cerevisiae*), and PC1/PC3, PC2 and furin (from higher eukaryotes), which are all members of the same family of serine proteases (Barr, 1991).

VIII. Perspectives

Cnidarians are the lowest group in the animal kingdom having a nervous system. When we started to work on the nervous systems of these animals about 15 years ago, we expected that the cnidarian nervous system would be simple, having perhaps only a few neurotransmitter substances. Today we know that a single cnidarian species, e.g., a sea anemone, produces more than 30 different neuropeptides. It is our personal belief that these 30 neuropeptides are only the tip of an iceberg, and that many more neuropeptides await to be isolated, also from other cnidarian species, such as *Hydra*. These peptides will be neurotransmitters or neuromodulators acting at neuroneuronal or neuromuscular junctions, but also neurohormones regulating cell division, cell differentiation, and other developmental processes. Little or nothing is known about the endocrinology of cnidarians. However, cnidarians have a regulated reproduction and a regulated life cycle and we can expect that here also peptides will play a central role.

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M Cells in Peyer's Patches of the Intestine

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M cells are specialized epithelial cells of the mucosa-associated lymphoid tissues. A characteristic of M cells is that they transport antigens from the lumen to cells of the immune system, thereby initiating an immune response or tolerance. Soluble macromolecules, small particles, and also entire microorganisms are transported by M cells. The interactions of these substances with the M cell surface, their transcytosis, and the role of associated lymphoid cells are reviewed in detail. The ultrastructure and several immuno- and lectin-histochemical properties of M cells vary according to species and location along the intestine. We present updated reports on these variations, on identification markers, and on the origin and differentiation of M cells. The immunological significance of M cells and their functional relationship to lymphocytes and antigen-presenting cells are critically reviewed. The current knowledge on M cells in mucosa-associated lymphoid tissues outside the gut is briefly outlined. Clinical implications for drug delivery, infection, and vaccine development are discussed.
KEY WORDS: M cell, Gut-associated lymphoid tissue, Peyer's patches, Appendix, Infection, Immunization, Vaccination.

I. Introduction

The gut is permanently under surveillance by the immune system to prevent invasion by pathogens. This protective function is of general significance because the intestine has an enormous surface area, a relatively thin epithelium, and is heavily populated by various microorganisms. The intestinal barrier is permeable to substances of low molecular weight such as the monomers of many nutrients, e.g., amino acids, fatty acids and saccharides, but in healthy animals it is impermeable to macromolecules and particles

(Bjarnason *et al.*, 1995). The immune system, however, needs direct access to macromolecular epitopes of potential pathogens to generate specific immune responses. Therefore, such antigens are continuously sampled by highly specialized epithelial cells, the M cells. In the small and large intestines, M cells are exclusively present in the so-called dome areas that are associated with the submucosal lymphoid follicles of Peyer's patches and other sites of gut-associated lymphoid tissue (GALT). The M cells transport these antigens through the epithelium, closely interact with cells of the immune system, and thus have a key function in the initiation of immunological response and tolerance.

II. Lymphoid Cells in the Gut Wall

A. General Importance and Immunological Functions

The main function of the gut is to absorb nutrients. This is performed by a single layer of epithelial cells and facilitated by the enormous surface area of the gut mucosa. Due to the plicae, villi, and crypts, as well as the microvilli of the enterocytes, the total exchange area between the gut lumen and mucosa measures more than 100 m^2 (Pabst, 1987). This is about 60 times larger than the surface area of the skin, which uses its thick, stratified epithelium to effectively protect itself against invading microorganisms and toxins. Because of the large number of protozoa, bacteria, viruses, toxins, and nutritional antigens in the gut, the organism needs a local protective system to prevent invasion, tissue damage, and systemic infection. An understanding of this barrier is essential with respect to the research and clinical aspects of a multitude of problems in the fields of pediatrics, gastroenterology, immunology, and pathology. Some examples of current problems under investigation are the role of colostrum in the newborn, food poisoning, the development of food allergies or inflammatory diseases of the gut, protective immune reactions such as tolerance induction, and the development of oral vaccines. The cellular basis of the immunological barrier function of the gut is discussed in order to put the role of M cells into context. The numerous unspecific functions of the gut, for example, the functions of the salivary glands, the low pH of the stomach, gall, and pancreas secretion, gut motility, and the interactions of the gut content with microbial agents, which also play a role in the immune function, are not dealt with in this chapter.

B. Localization

1. Definition of Gut-Associated Lymphoid Tissue

Cells of the immune system are not only found in lymphoid organs like the spleen and lymph nodes, which possess a capsule and respond to antigens

delivered via the blood or lymph, but are also found in the gut wall as organized lymphoid structures: Peyer's patches in the small intestine, appendix vermiformis, colonic and rectal patches, and single lymphoid follicles. In birds a further lymphoid organ is associated with the gut wall—the bursa of Fabricius, which is a primary lymphoid organ for B cells (Oláh and Glick, 1992; Toivanen, 1992). The common features of these lymphoid tissues are that afferent lymphatics and a defined capsule are absent and a specialized epithelium covers the lymphoid tissue bulging toward the lumen (Fig. 1). This "dome epithelium" transports antigens from the gut lumen to the subepithelial tissue. After initiation of an immune reaction, primed B lymphocytes and lymphoblasts in Peyer's patches preferentially migrate as precursors of immunoglobulin-A (IgA) secreting plasma cells via the intestinal lymphatics, mesenteric lymph nodes, thoracic duct and peripheral blood to the lamina propria of the gut (Scicchitano *et al.*, 1986). Because of this preferential homing by lymphoblasts, the term "gut-associated lymphoid tissue (GALT)" was introduced. In further migration studies on IgA precursors, it was shown that gut-derived lymphoblasts also preferentially migrate into other organs lined with mucous membranes, e.g., the bronchial tract, salivary, lacrimal, and mammary glands, and the female genital tract. These data gave rise to the concept of the mucosa-associated lymphoid tissues (MALT) (Bienenstock *et al.*, 1978). Not only do B lymphocytes migrate from one part of the common mucosal system to the other but T lymphocytes also migrate, as demonstrated after immuni-

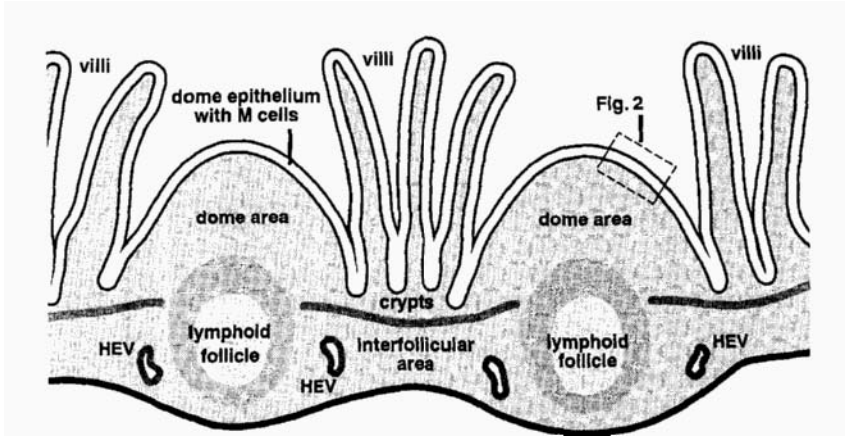

FIG. 1 Schematic drawing of a Peyer's patch in the small intestine, illustrating the general composition of gut-associated lymphoid tissue of various species. Lymphoid follicles lying in the submucosa are associated with dome areas that bulge toward the gut lumen. The dome areas are covered with a specialized epithelium, characterized by the presence of M cells and called the dome epithelium. While B lymphocytes prevail in the lymphoid follicles, the interfollicular areas are populated by T cells. Peyer's patches lack afferent lymphatics but can be entered by lymphocytes via high endothelial venules (HEV) in the interfollicular areas.

zation of Peyer's patches (Dunkley and Husband, 1987). Some authors include mesenteric lymph nodes in the GALT. Lymphoid and accessory cells dispersed in other compartments of the gut wall are often considered part of the GALT. In this chapter we restrict the term GALT to the organized lymphoid tissue of the gut wall. Lymphocytes interspersed throughout the gut epithelium are called intraepithelial lymphocytes (IEL). Large numbers of lymphoid cells are localized in the lamina propria of the gut wall and these are often called lamina propria lymphocytes (LPL). These components of lymphoid cells are mentioned first because they are an important part of the gut immune system in addition to the organized lymphoid structures like Peyer's patches, and second, the peculiarities of the Peyer's patch dome epithelium can be better understood in relation to the villus epithelium.

2. Peyer's Patches

More than 300 years ago Johann Konrad Peyer described aggregations of lymphoid tissue in the wall of the small intestine. The official anatomical term is "folliculi lymphatici aggregati," so there have to be at least two lymphoid follicles to form a Peyer's patch. The Peyer's patches are mainly localized opposite the mesentery. In humans, they develop well before birth and a considerable number (~50) can be identified from about 24 weeks of gestation on. In adolescence, more than 240 patches are found in the small intestine, and at an age of over 90 years about 50 patches are still seen (Cornes, 1965). Peyer's patches are not only present in the ileum but also in the duodenum and jejunum (Cornes, 1965). The size of an individual patch varies in humans, while Peyer's patches consist of only a few follicles in rodents. The size and location of each Peyer's patch seems to be genetically determined because the pattern of patches along the small intestine remains constant during the ontogeny of individual pigs (Rothkötter and Pabst, 1989). The transposition of gut segments or removal of a large portion of Peyer's patches does not influence the number of remaining patches (Rothkötter *et al.*, 1990).

In several species such as sheep (Reynolds and Morris, 1993), calves (Landsverk, 1987), and pigs (Binns and Licence, 1985; Rothkötter and Pabst, 1989), there are two types of Peyer's patch in the small intestine which differ in location, structure, cellular composition, and function: several discrete patches in the jejunum and upper ileum, which are comparable to human Peyer's patches, and a long, continuous patch in the terminal ileum, reaching about 1.5 m in sheep. The continuous ileal patch is well developed in sheep before birth and obviously influences the development of B lymphocytes to a great extent (Reynolds, 1987). This was shown by resecting the ileal patch in the sheep fetus, which resulted in severe B lymphocytopenia (Reynolds, 1987). The ileal patch regresses with age while

the Peyer's patches in the jejunum persist into old age (Reynolds and Morris, 1983). These major species differences have to be taken into account when functional and structural results on Peyer's patches are extrapolated from one species to another.

Peyer's patches are more than just aggregations of lymphoid follicles. They consist of definite compartments with a number of basic structural elements and specific compositions of lymphocyte subsets and accessory cells. The Peyer's patches and other sites of GALT can be divided into four compartments (Fig. 1). (1) The lymphoid follicles are localized below the muscularis mucosae. Proliferating B lymphocytes (centrocytes and centroblasts) form germinal centers which also contain tingible body macrophages that phagocytose remnants of lymphocyte nuclei. It has recently been shown that extensive apoptosis occurs in sheep ileal Peyer's patches, which might be essential for B cell selection (Motyka and Reynolds, 1991). (2) The follicles are surrounded by a corona of small lymphocytes, many of which express IgM and IgD on their cell surface. (3) The interfollicular area is characterized by high endothelial venules (HEV), which are surrounded by densely packed lymphocytes, most of which are T cells. Lymphocytes enter the Peyer's patches from the blood via these specialized venules with their typical "cobble-stone"-type endothelium. Therefore, this compartment has also been called the traffic area of Peyer's patches. (4) A mixture of lymphocytes is found on top of the follicle toward the gut lumen. It resembles a cap or the vault of a church and is called the dome area.

Blood vessels supplying the Peyer's patches were studied in mice and rats by scanning electron microscopy of corrosion casts (Bhalla *et al.*, 1981; Yamaguchi and Schoefl, 1983a). Two to four mural trunks contribute to an individual patch. In the dome area, a planar capillary plexus lies underneath the dome epithelium. Sparse networks of fine capillaries are found in the follicles, which are encircled by a dense venous network. The typical high endothelial venules are linked by anastomotic connections via short segments of fine vessels. The high endothelial venules play a central role for lymphocytes migrating into the Peyer's patch, as shown by transmission electron microscopy in mice (Yamaguchi and Schoefl, 1983c). The adherence and emigration of lymphocytes at these sites was demonstrated by *in vivo* experiments using fluorescinated lymphocytes (Yamaguchi and Schoefl, 1983b; Bjerknes *et al.*, 1986; Nagata *et al.*, 1994). The high endothelial venules are surrounded by fenestrated sheaths of collagen fibers, possibly enabling lymphocytes to migrate into the different compartments of the lymphoid tissue (Ohtani *et al.*, 1991; Ohtsuka *et al.*, 1992).

Scanning electron microscopy of sheep ileal Peyer's patches revealed that lymph sinuses are situated around the follicles. These sinuses are connected to lymph vessels, septal vessels, and to the deep mucosal

network that contains valves (Lowden and Heath, 1992). In pigs (Lowden and Heath, 1994) and similarly in rabbits (Ohtani and Murakami, 1990), the lymph vessels in the lamina propria are continuous with those in the interfollicular area, and lymph sinuses only partially surround the follicles. In a recent *in vivo* study, a dense plexus of lymphatic microvessels was identified in the perifollicular and interfollicular areas in rats (Nagata *et al.*, 1994). These lymphatics were densely filled with lymphocytes. It can be concluded that this is the route by which lymphocytes leave Peyer's patches.

3. Intraepithelial Lymphocytes

Lymphocytes situated basally above the basement membrane of the gut epithelium are called intraepithelial lymphocytes. These cells differ not only from peripheral blood lymphocytes but also from those in the lamina propria, so they have often been termed "curious," "unusual," or "peculiar" (Ernst *et al.*, 1985; Lefrançois, 1991; Croitoru and Ernst, 1992). These differences relate to morphology, surface phenotype, antigen receptors, ontogeny, differentiation, and functional properties (Shanahan, 1994; Sim, 1995).

The data on the frequency of intraepithelial lymphocytes in the human gut vary somewhat, e.g., 20 cells per 100 enterocytes (Ferguson, 1977) or 13 cells in the normal ileum and 5 cells in the colon per 100 enterocytes (Hirata *et al.*, 1986). Crowe and Marsh (1994) recently took the three-dimensional aspect into consideration and calculated a mean of 11 intraepithelial lymphocytes per 100 enterocytes (5–27 within a 95% confidence interval) for the healthy human jejunum. The intraepithelial lymphocytes lie among the epithelial cells in the basal part of the epithelium and are separated from the lumen by the junctional complex of the epithelial cells. There are no lymphoepithelial cell connections such as tight junctions, gap junctions, or desmosomes. The route by which the lymphocytes reach their intraepithelial position is unclear. A variable number of intraepithelial lymphocytes, almost exclusively T lymphocytes, contain cytoplasmic granules staining metachromatically with toluidine blue at a low pH and express the CD8 marker for T cytotoxic cells. Only about 5–10% have the phenotype of helper T cells ($CD4^+$). Most human intraepithelial lymphocytes have the memory cell phenotype ($CD45RO^+$) and less than 10% the virgin cell phenotype ($CD45RA^+$) (see Shanahan, 1994).

T lymphocytes recognize their antigen via the T-cell receptor (TCR), of which there are two types: $\alpha\beta$TCR and $\gamma\delta$TCR. While about 95% of T cells in peripheral lymphoid organs and the blood express the $\alpha\beta$TCR, a considerable proportion of intraepithelial lymphocytes in mice express the $\gamma\delta$TCR (Sim, 1995). In adult humans, normally less than 10% of intraepithe-

lial lymphocytes in the small intestine have the $\gamma\delta$TCR. In mice, about 40% of the intraepithelial lymphocytes are of thymic origin, as documented by marker and cell transfer studies as well as the frequency of intraepithelial lymphocytes in nude mice (Rocha *et al.,* 1994). The remaining 60% of the intraepithelial lymphocytes differentiate independent of the thymus: half of these express the $\alpha\beta$TCR and other half the $\gamma\delta$TCR. The CD8 molecule of this subset consists of α chains only (for references, see Croitoru and Ernst, 1992; Shanahan, 1994; Sim, 1995). So far it has not been conclusively shown whether such a dichotomy of thymus-dependent and thymus-independent intraepithelial lymphocytes also exists in humans.

Intraepithelial lymphocytes have various cytotoxic functions, e.g. classic, major histocompatibility complex (MHC)-restricted, antigen-specific, cytotoxic lymphocyte activity. The intestinal epithelial cells may play a role in presenting superantigens to mucosal T cells. Superantigens are secreted by bacteria, do not require an antigen-presenting cell, and are active in picomolar quantities (Aisenberg *et al.,* 1993). The data available on the production of cytokines by intraepithelial lymphocytes and their functional significance are still controversial (Sartor, 1994). Since intraepithelial lymphocytes respond poorly to mitogens, alloantigens, and immobilized anti-CD3 *in vitro* (Poussier and Julius, 1994; Shanahan, 1994), it has been argued that they are at the final stage of their development. Intraepithelial lymphocytes probably play a regulatory role in suppressing systemic immune responses to antigens in the gut lumen (Dobbins, 1986). Their life span, their (probably) local production in the epithelium, and their fate are areas for future research.

4. Intestinal Lamina Propria

The lamina propria is the layer of connective tissue between the epithelium and the muscularis mucosae. It contains a complex mixture of lymphoid and accessory cells which provide an immune response. The role of mast cells, neutrophils, and eosinophils cannot be discussed here but their relevance in immune reactions is beyond doubt. Valentich and Powell (1994) suggested that intestinal subepithelial myofibroblasts also play a role in mucosal immunophysiology, e.g., by secreting cytokines. Furthermore, several gut peptides, e.g., VIP, substance P, and somatostatin, might have a regulatory influence on the mucosal immune system via receptors on cells of the immune system (Chen and O'Dorisio, 1993).

In contrast to the epithelium, the lamina propria contains both major subsets of T lymphocytes. The ratio of $CD4^+$ to $CD8^+$ lymphocytes is about 2:1, which is similar to the situation in the blood. The expression of the activation marker CD25 [interleukin-2 (IL-2) receptor] is an early event in T-cell activation (see Brandtzaeg *et al.,* 1989). The $\alpha E\beta 7$ integrin (formerly

called HML-1) is a further activation marker expressed by nearly all intraepithelial lymphocytes and by 30–50% of lamina propria lymphocytes, but not by peripheral blood T cells (Shanahan, 1994). The lamina propria T cells express the $\alpha\beta$TCR and not the $\gamma\delta$TCR as intraepithelial lymphocytes. The predominant function of the $CD4^+$ T cells is to provide help for B cells in differentiation to produce immunoglobulin. In constrast to peripheral blood lymphocytes, most lamina propria lymphocytes have a high expression of the CD45RO antigen, which is considered to be a marker for "memory" T cells (see Brandtzaeg *et al.*, 1989). In a secondary response, these cells proliferate to recall antigens. The $CD45RA^+$ T cells seem to have a greater proliferation response to certain mitogens. T-helper cells can be divided into TH1 and TH2 cells. The TH1 cells produce the cytokines IL-2, interferon-γ and tumor necrosis factor-β, while the TH2 cells preferentially produce IL-4, IL-5, IL-6, and IL-10. The cytokines secreted by TH2 cells seem to be critical for IgA synthesis and the differentiation of B cells into IgA-producing plasma cells in the gut wall (for details, see McGhee *et al.*, 1992).

In the lamina propria, there are fewer small B cells than plasma cells (MacDonald *et al.*, 1987). The great majority of plasma cells produce IgA (~80%); about 18% secrete IgM and only ~3% IgG (Brandtzaeg *et al.*, 1989). IgA is the major immunoglobulin isotype in secretions and also in the gut fluid. The secretory IgA can agglutinate infectious microorganisms and prevent them from adhering to and penetrating the epithelia. It also neutralizes bacterial toxins without complement activation and therefore protects mucosal surfaces against inflammatory reactions (Biewenga *et al.*, 1993). In humans, there are the IgA_1 and IgA_2 subclasses. IgA_2 is the preferential subclass in the intestinal tract. The plasma cells in the lamina propria produce IgA as a monomer which is joined by the J chain to form a dimer. This dimeric form of IgA is said to have a higher capacity to bind and agglutinate antigens than the monomeric form (Brandtzaeg *et al.*, 1989). Only in dimeric form can IgA bind to the poly-Ig receptor (formerly called secretory component) which is necessary to transport the IgA molecule through the enterocytes into the gut lumen. Brandtzaeg *et al.* (1989) estimated that 70–80% of all Ig-producing cells are located in the mucosa of the intestines, and counted $\sim 10^{10}$ per meter of human gut. The Peyer's patches are important for the IgA-producing plasma cells in the lamina propria because the switch from IgM- to IgA-expressing B lymphocytes happens in the Peyer's patches (Cebra and Shroff, 1994). After Peyer's patches were excised in rats, the number of IgA-containing plasma cells in the lamina propria decreased but the number of IgM-containing cells remained constant (Enders *et al.*, 1988).

III. Dome Epithelium

A. Overview

The epithelium that covers the dome areas generally differs from both crypt and villus epithelium in its cellular composition, functions, and several histochemical, physicochemical, and ultrastructural properties. Since each dome area is associated with an individual lymphoid follicle, the epithelium has been termed follicle-associated epithelium (Bockman and Cooper, 1973). In the past few years the term "dome epithelium (DE)" has been used more widely and is used in this review.

The main structural characteristics of the dome epithelium are the presence of M cells and numerous intraepithelial lymphocytes and macrophages (Fig. 2). M cells can be easily identified by scanning and transmission

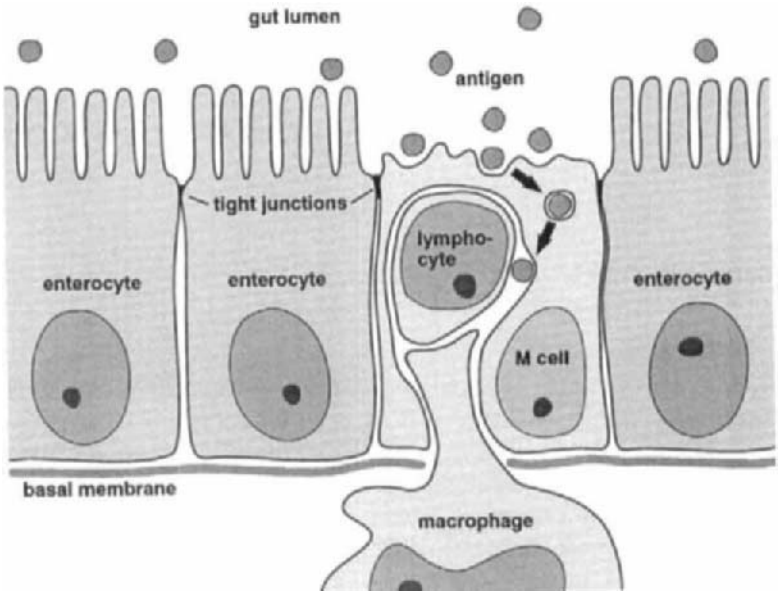

FIG. 2 General concept of antigen uptake by M cells in the dome epithelium. The dome epithelium is mainly composed of enterocytes and M cells. Lymphocytes and macrophages lie in the interstitial spaces of the epithelium. In contrast to enterocytes, the M cells possess a less regularly shaped apical surface with microplicae or small microvilli. The paracellular passage of antigens from the gut lumen to the lymphoid tissue is prevented by tight junctions between all epithelial cells. The antigens preferentially adhere to the apical membrane of M cells and are subsequently endocytosed by and transported through the apical cytoplasm. When exocytosed to the basolateral side of the M cells, the antigens come into contact with lymphocytes and macrophages, which initiate a specific immune response or systemic tolerance.

electron microscopy, but are difficult to distinguish from ordinary enterocytes in conventional light microscopic preparations. Therefore, several markers have been developed to identify M cells using light microscopy. These are outlined in the following section.

M cells possess some common characteristics, described in Section IV, A, 1, but vary considerably in number, structure, and histochemical properties, depending on species, location, and maturation stage (see Section IV, A, 2). The main function of M cells is to transport antigens from the lumen to the lymphoid cells lying in and beneath the dome epithelium. This has been studied with a multitude of tracers and microorganisms and is discussed in Section IV,B,1. Mechanisms that mediate the adherence of antigens to the M cell surface play a key role in our understanding of the M cell functions, and are important for clinical approaches to utilize M cells for vaccinations or drug delivery. These mechanisms are critically reviewed in Sections IV, B and VII.

The origin of M cells and the interrelationship of M cells with other epithelial and nonepithelial cells of the dome epithelium have been studied as part of an attempt to understand the complex immunological functions performed by the gut-associated lymphoid tissue (see Sections III, C and V). Outside the gut, M cells are found at other mucosal sites such as tonsils or bronchi. The available information on such M cells is outlined in Section VI.

B. Identification of M Cells

1. Ultrastructure

Although M cells have been studied for more than 20 years, clear-cut definitions for the identification of M cells independent of species and location do not exist. M cells were initially identified by thin-section electron microscopy in the rabbit appendix (Bockman and Cooper, 1973) and by scanning electron microscopy in human Peyer's patches (Owen and Jones, 1974). The M cells at these locations differed from neighboring enterocytes in the morphology of their apical surface and thus were recognized as a distinct cell type. Using ultrastructural characteristics (e.g., length and shape of the microvilli; see Section IV, A, 1), M cells have been described in the GALT of several other species, including mice, rats, guinea pigs, cattle, pigs, dogs, and horses, and also in the epithelia of several other mucosa-associated lymphoid tissues (MALT) (Owen, 1977; Owen and Nemanic, 1978; Chu *et al.,* 1979; Rosen *et al.,* 1981; Torres-Medina, 1981; Madara *et al.,* 1984; Rosner and Keren, 1984; Morfitt and Pohlenz, 1989; HogenEsch and Felsburg, 1990; Liebler *et al.,* 1991; Lowden and Heath, 1995). In most

cases, M cells possess an irregularly shaped apical surface often characterized by short microvilli or microplicae. The basolateral membrane is deeply invaginated, forming a pocket or envelope that engulfs intraepithelial lymphocytes and macrophages (Figs. 2 and 3). It should be noted that these nonepithelial cells have an intraepithelial but intercellular position that permits their passage into the dome epithelium and back to the subepithelial lymphoid tissue. Apart from this general pattern, the ultrastructure of M cells varies considerably and is discussed in detail in Section IV, A, 2.

At some locations of MALT, immature and intermediate-type M cells with characteristics of both M cells and enterocytes were observed. This caused speculation about the origin and development of M cells, and it was even suggested that M cells might represent a modified enterocyte rather than a distinct cell type (Smith and Peacock, 1980; Bhalla and Owen, 1982; Sicinski *et al.,* 1986; see Section III, C). To clarify these questions and

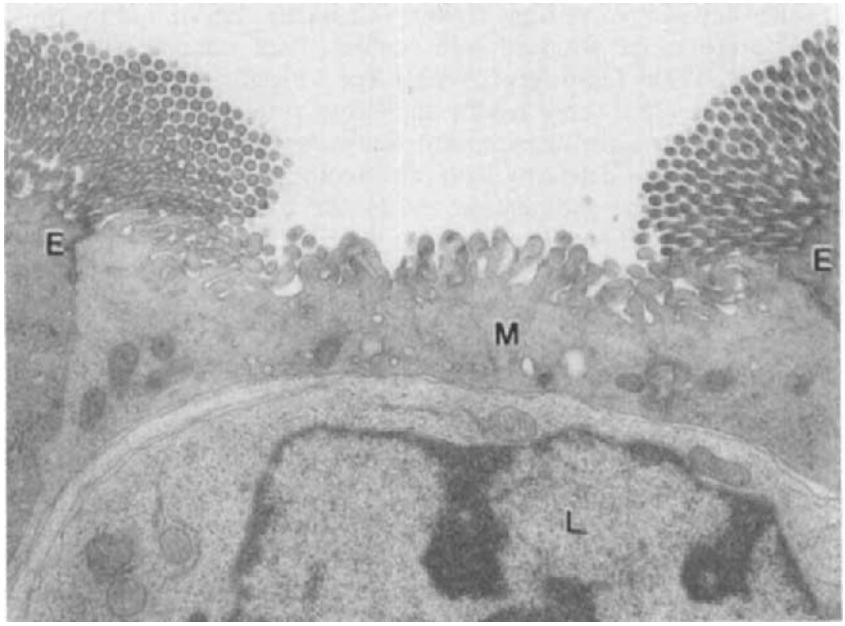

FIG. 3 Apical cytoplasm of an M cell (M) and adjacent enterocytes (E) in the Peyer's patch of a BALB/c mouse. M cells in the small intestine of rodents normally possess short stub-like microvilli on their apical surface. Intraepithelial lymphocytes (L) deeply invaginate the basolateral membrane and thereby induce an attenuated thin apical cytoplasm of the M cell. Small vesicles and/or a tubulovesicular system are present in this cytoplasm and are involved in the transcytosis of antigens from the lumen to the basolateral pocket. (Preparation and photomicrography by Anja Kracke.) Epon ultrathin section. ×13,000.

to study M cells on a light microscopic level, several groups established histochemical markers that detect cytoplasmic or membrane-bound molecules specific for M cells. Current markers include enzyme activities, unidentified proteins that are bound by monoclonal antibodies, cytoskeletal peculiarities, and certain terminal saccharides of glycoproteins and/or glycolipids.

2. Alkaline Phosphatase

A reduction or absence of alkaline phosphatase activity (AP) in the apical membrane of M cells compared with enterocytes was initially described by Schmedtje (1965) for the rabbit appendix and by Owen and Bhalla (1983a) for murine Peyer's patches. The method has been used as a "negative" marker by other groups not only in Peyer's patches of mice and rabbits but also in those of rats, dogs, and humans (Owen and Bhalla, 1983a; Brown *et al.*, 1990; HogenEsch and Felsburg, 1990; Jepson *et al.*, 1993c; Farstad *et al.*, 1994; Savidge *et al.*, 1994). Using the AP method, M cells could be identified in semithin sections (Owen and Bhalla, 1983a) and in whole-mount preparations observed with confocal laser scanning microscopy (Clark *et al.*, 1994a; Jepson *et al.*, 1993d). The AP method has the following disadvantages: (1) it is not readily applicable to thicker sections, (2) the relationship of the apical membrane to the associated cytoplasm and nucleus is often difficult to define by light microscopy, (3) goblet cells are also negative for alkaline phosphatase, and (4) AP activity occurs inhomogenously in the M cell population.

3. Monoclonal Antibodies

Since high percentages as well as high absolute numbers of M cells are present in rabbit Peyer's patches and appendix, M cell-enriched fractions of these organs were used to generate monoclonal antibodies specific for M cells. The antibodies produced by Pappo (1989; Pappo *et al.*, 1991) bound to epitopes on the basolateral membrane of M cells and to some other tissue components in Peyer's patches and appendix, but also to M cells of the sacculus rotundus and cecal lymphoid patch. One antibody generated by Roy *et al.* (1987) labeled an (unidentified) cytoplasmic epitope of M cells in the rabbit GALT and also a subpopulation of epithelial cells overlying the rabbit bronchus-associated lymphoid tissue (BALT). Several other tissue components, most of them of mesenchymal origin, were also recognized by this antibody, resulting in a labeling pattern very similar to that of antivimentin antibodies (see Gebert *et al.*, 1992; Jepson *et al.*, 1992; Section III,B,5). More specific and species-independent antibodies for the detection of M cells are still lacking.

4. Lectins

The polysaccharides of membrane-bound glycoproteins and glycolipids play an important role in the differentiation and interaction of epithelial cells (Damjanov, 1987; Falk *et al.,* 1994). Therefore, lectins specifically binding to terminal saccharides were used as tools to study the glycosylation of dome epithelial cells. Lectins detecting fucose or *N*-acetyl-galactosamine selectively labeled the apical membrane and the membrane of vesicles of M cells in the rabbit cecal patch (Gebert and Hach, 1993; Jepson *et al.,* 1993d; Fig. 4). Although large panels of lectins were applied to other sites of rabbit GALT, no M cell-specific glycoconjugates could be detected in the Peyer's patches, indicating that the glycosylation patterns are site-specific and possibly reflect the local microenvironment (see Section

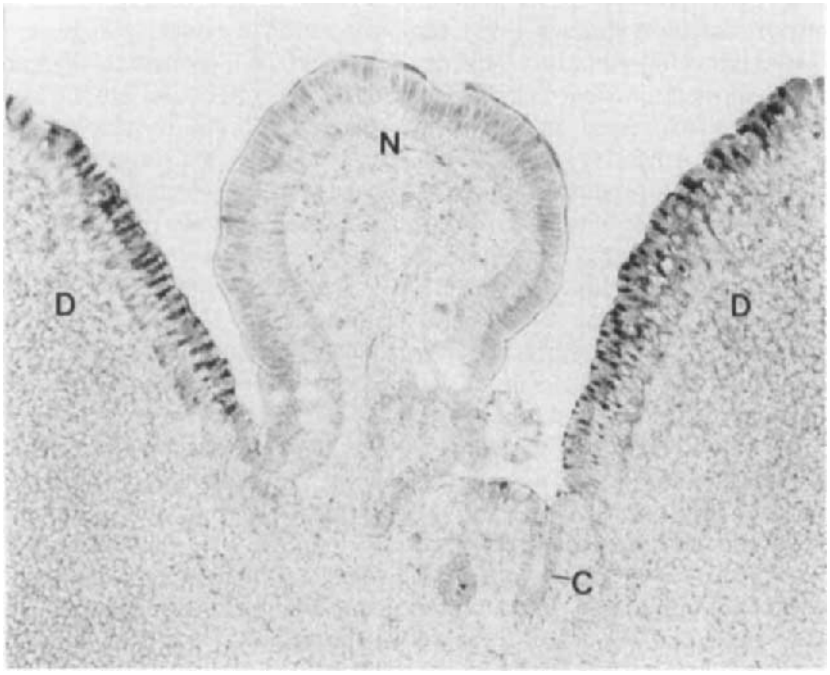

FIG. 4 Lymphoid patch of the rabbit cecum. Two dome areas (D) separated by a nondome region (N) are shown. The M cells at this location of GALT express large amounts of fucose and *N*-acetyl-galactosamine in the brush border and apical cytoplasm. Lectin histochemistry with the *Ulex europaeus* agglutinin detects these fucose residues, thereby identifying the M cells. Note that both crypt (C) and nondome epithelium lack M cells. Paraffin section; UEA-I-HRP; ×230.

IV,B,2). M cells in the Peyer's patches of BALB/c mice express high levels of fucose in the glycocalyx of their apical membrane and in their cytoplasm, which can be detected by the lectin of *Ulex europaeus* (UEA-1) (Clark *et al.,* 1993). M cells of the mouse cecal patch are selectively labeled by the same lectin but differ in the binding pattern of some other lectins (Clark *et al.,* 1994a; Giannasca *et al.,* 1994). In aldehyde-fixed Peyer's patches of other species (e.g., rats, guinea pigs, or cats), no glycoproteins specific for M cells were detected by lectin histochemistry (A. Gebert, unpublished observations). Thus, it is still unclear whether the detection of M cells by lectins can be generalized or is restricted to certain species and sites of GALT.

5. Cytoskeleton

M cells differ from enterocytes in shape, e.g., apical surface and basolateral pocket, and function, e.g., route of vesicular transport. Since the composition of the cytoskeleton is generally related to the cell type and to transport functions (Ingber, 1993), the composition of cytoskeletal proteins was studied by immunohistochemistry. Vimentin, an intermediate filament protein normally present in cells of mesenchymal origin and absent from epithelia, is expressed in rabbit M cells in addition to cytokeratins (Gebert *et al.,* 1992; Jepson *et al.,* 1992; Figs. 5 and 6). Vimentin therefore represents a cytoplasmic marker for rabbit M cells, which is applicable to cryo, paraffin, semithin, and ultrathin sections. Using vimentin immunohistochemistry, M cells were detected at all locations of GALT in the small and large intestine, in the GALT of newborn rabbits, and in the epithelia that cover the BALT and the tonsil crypt (Gebert and Hach, 1992; Gebert *et al.,* 1992; Jepson *et al.,* 1992; Gebert, 1995). Vimentin, however, is not expressed in M cells of other species, e.g., mice, rats, guinea pigs, cats, pigs, and humans (Jepson *et al.,* 1992; own observations). M cells of porcine Peyer's patches express high levels of cytokeratin 18 and therefore are easily detectable in cryo, semithin, and in formalin-fixed paraffin sections (Gebert *et al.,* 1994; Fig. 7). The specific functions of vimentin in rabbit and cytokeratin 18 in porcine M cells remain to be established. Nevertheless, these intermediate filament proteins represent the most suitable M cell markers available at the moment for further investigations in the rabbit and pig models.

6. Cross-Correlation of the Labeling Patterns

The labeling patterns of most of the markers listed above correlate highly with the ultrastructural appearance of the labeled cells (Owen and Bhalla, 1983a; Clark *et al.,* 1993, 1994a; Gebert and Hach, 1993; Jepson *et al.,*

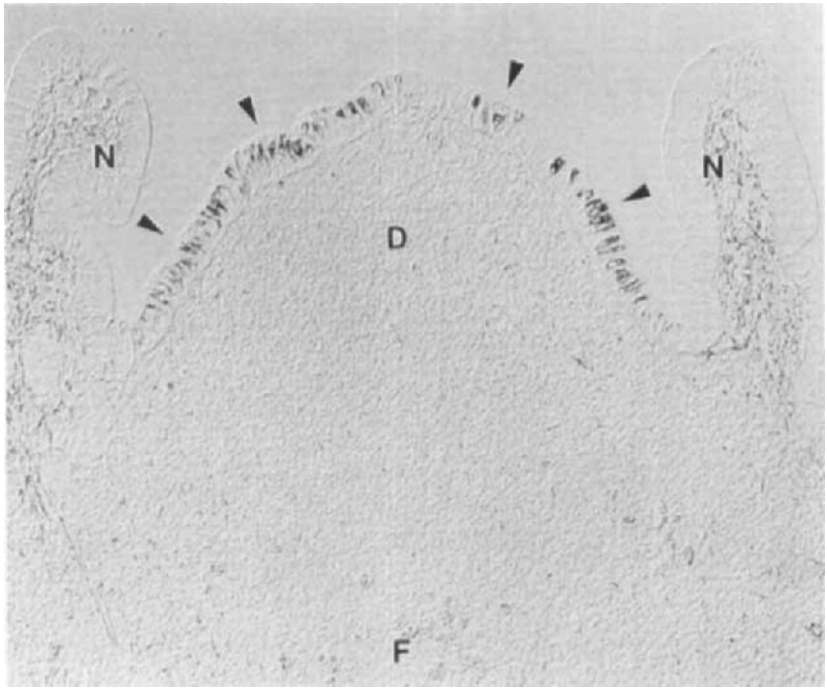

FIG. 5. Lymphoid patch of the rabbit cecum. The dome areas (D) are associated with lymphoid follicles (F) and bulge toward the lumen. The domes are separated by protrusions of cecal mucosa called the nondome region (N). The rabbit M cell marker vimentin has been used to identify the M cells immunohistochemically. Note that the M cells (arrowheads) are found all over the dome but are most numerous at the flanks. The M cells alternate with enterocytes and comprise about one third of all dome epithelial cells. Paraffin section; antibody V9; Nomarski interference contrast; ×150.

1993d). In addition, it has been demonstrated for some of these markers that they correlate with the *in vivo* transport function of the M cells (Pappo and Ermak, 1989; Jepson *et al.*, 1993b; Gebert *et al.*, 1994). Studies with these histochemical markers revealed distinctive patterns of labeled and unlabeled cells but only very few intermediate forms, suggesting that the M cell is a separate cell type rather than a modified enterocyte. This was further supported by the detection of immature M cells at the base of the dome near the mouths of neighboring crypts, even before these cells had acquired the morphological characteristics of M cells (Bye *et al.*, 1984; Pappo, 1989; Gebert *et al.*, 1992; Gebert and Hach, 1993; Fig. 8).

Although the histochemical markers for M cells have extended our knowledge about M cell functions, each of the markers is restricted to a

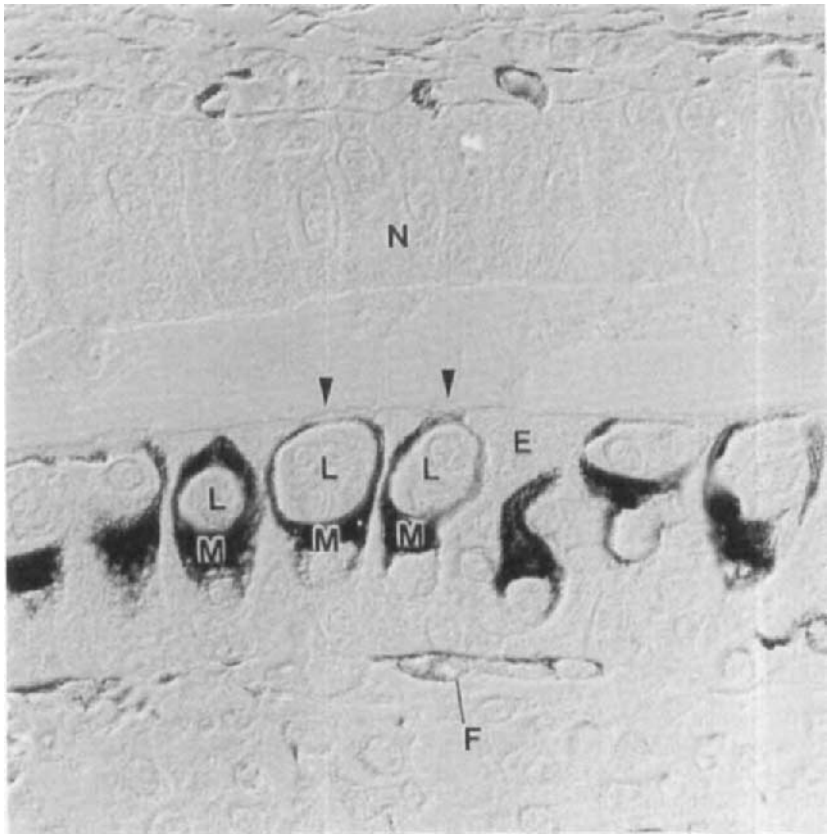

FIG. 6 Dome epithelium and adjacent nondome epithelium of the rabbit appendix. Vimentin filaments, present in the cytoplasm of rabbit M cells (M), are used to detect M cells by immunohistochemistry at a light microscopic level. The M cells engulf clusters of intraepithelial lymphocytes (L). The apical cytoplasm of the M cells has a membranous shape (arrowheads), separating the lymphocytes from the gut lumen. The enterocytes (E) of both dome epithelium and nondome epithelium (N) lack vimentin and thus remain unlabeled. Vimentin-positive cells (F), presumably fibroblasts or reticulum cells, lie beneath the basal membranes of both epithelia, but possess a flat shape in the dome epithelium. Paraffin section; antivimentin antibody V9; Nomarski interference contrast. ×900.

single or a few species, or even to a specific location of GALT, e.g., small or large intestine. These differences suggest that none of the markers described so far is of general significance for the specific morphology and function of M cells. The differences rather imply that M cells are locally modified according to site and/or species-specific conditions.

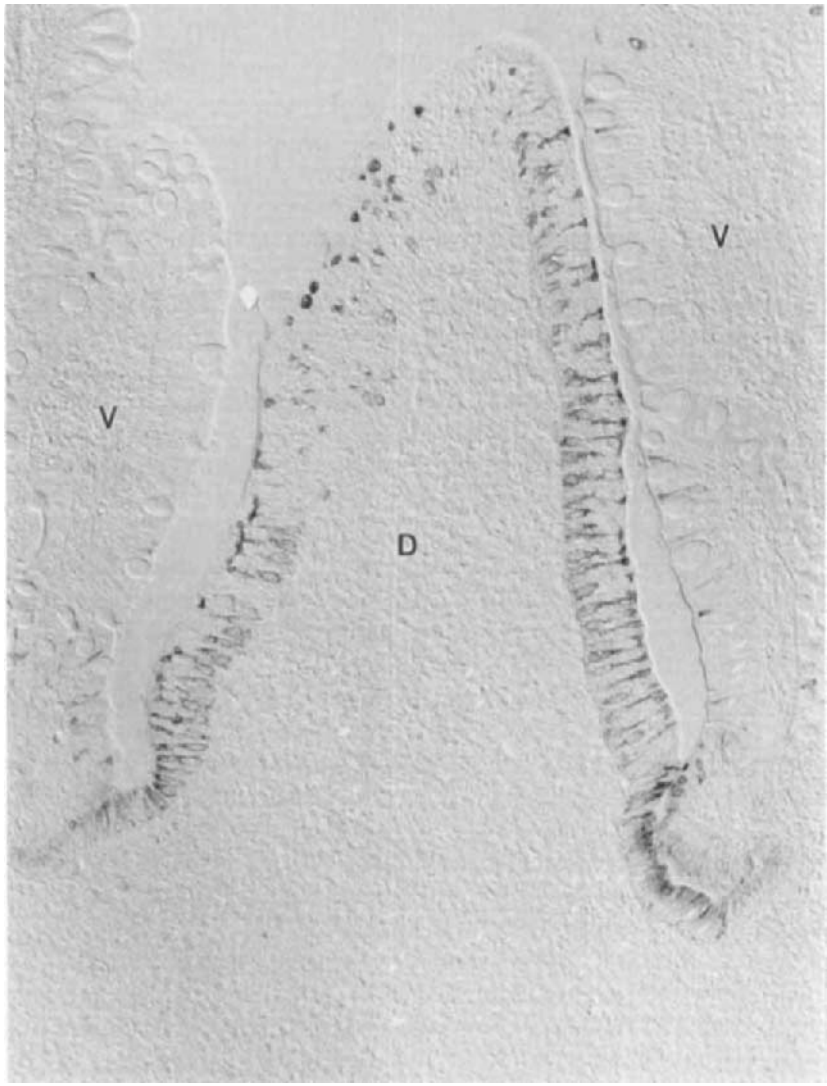

FIG. 7 Dome area (D) and adjacent villi (V) in the jejunal Peyer's patch of the pig. The M cells are labeled by immunohistochemistry for the intermediate filament cytokeratin 18. The M cells alternate with (unlabeled) enterocytes and comprise 20–50% of the dome epithelial cells. Paraffin section; antibody CY90; Nomarski interference contrast. ×250.

C. Origin, Differentiation, and Development of the Dome Epithelium

The dome epithelia of the GALT consist of enterocytes, M cells, intraepithelial leukocytes, in some species a few goblet cells, and a few other epithelial cell types (e.g., brush cells). The most characteristic of these cells, the M cell, unequivocally represents an epithelial cell, because it forms tight junctions and desmosomes with the neighboring epithelial cells (Madara *et al.*, 1984; Gebert and Bartels, 1991), contains large amounts of cytokeratins and villin (Gebert *et al.*, 1992, 1994; Jepson *et al.*, 1992; Kernéis *et al.*, 1995), and proliferates in the crypts similarly to enterocytes (Smith *et al.*, 1980; Bhalla and Owen, 1982; Bye *et al.*, 1984). Using [^{3}H]thymidine in a mouse model, Bye *et al.* (1984) showed that the dome epithelial cells (i.e., M cells and enterocytes) probably derive from stem cells which are located in the mid-third of crypts opening at the base of the dome area. The dome epithelial cells migrate to the lower flanks of the domes within 24 hr and differentiate to M cells or enterocytes (Bhalla and Owen, 1982). M cells of this region have been described as "immature," because they were tall and columnar, lacked a central cytoplasmic hollow, and showed a reduced ferritin uptake rate compared with mature M cells (Bye *et al.*, 1984). In this region, the dome epithelial cells come into contact with intraepithelial lymphocytes and macrophages. In mice, the M cells migrate within 60–72 hr to the upper parts of the dome (Smith *et al.*, 1980; Bhalla and Owen, 1982) where they attain the morphology of mature M cells and their full transport capacity (Bye *et al.*, 1984). Close to the tip of the dome area, M cells were found to bulge toward the lumen and to be disrupted (Schmedtje, 1980), indicating that the cells are sloughed off in a way similar to that described for small intestinal enterocytes at the tips of the villi (Gordon, 1989; Madara, 1990; Iwanaga *et al.*, 1993).

For over a decade it has been controversial whether M cells derive directly from undifferentiated stem cells of the midcrypt region (see Cheng and Leblond, 1974; Bye *et al.*, 1984) or whether at least one subpopulation develops from enterocytes (Smith and Peacock, 1980; Smith *et al.*, 1980; Bhalla and Owen, 1982). Lectin- and immunohistochemical studies of mice and rabbits revealed small, columnar cells that were labeled by M cell-specific lectins at the upper parts and the mouths of crypts neighboring dome areas (Pappo, 1989; Gebert and Hach, 1993; Giannasca *et al.*, 1994). In addition, the rabbit M cell marker vimentin was already present in a few small basal cells in the crypts of the rabbit appendix (Fig. 8). These observations strongly suggests that a subpopulation of undifferentiated crypt cells is predetermined as M cells before attaining their morphological and functional characteristics.

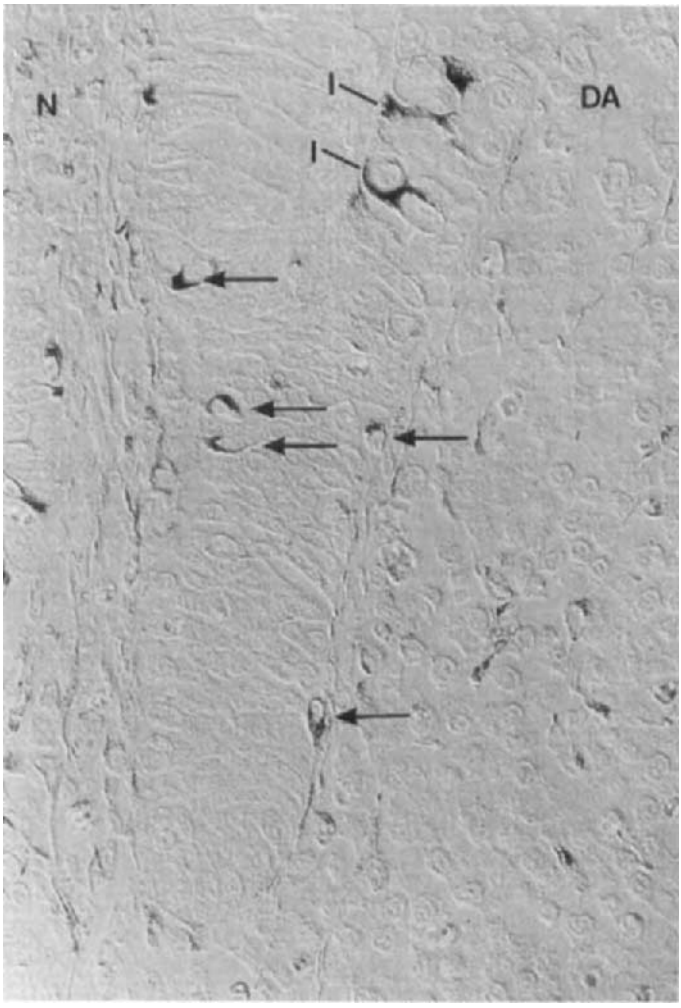

FIG. 8 Crypt located at the periphery of a dome area (DA) of the rabbit appendix, supplying both the dome epithelium and the opposite nondome epithelium (N) with epithelial cells. Vimentin immunohistochemistry detects immature M cells (I) at the lower flanks of the dome. Some of these immature cells are already in contact with intraepithelial lymphocytes but lack the large cytoplasmic pocket typical of mature M cells. In addition, vimentin-positive cells of round or ovoid shape (arrows) are found in the midregion of the crypt. Since such cells were absent from large intestinal crypts not draining to a dome, their presence in crypts associated with a dome suggests that they represent early developmental stages of M cells. Paraffin section; antivimentin antibody V9; Nomarski interference contrast. ×460.

The factors, however, that induce the formation of M cells in crypts draining to a dome area but not in normal crypts draining to villi or surface epithelium are still unclear. The following factors are feasible: (1) a direct induction of M cells by intraepithelial lymphocytes, (2) soluble factors produced by lymphocytes or macrophages, and (3) a specific composition of the basal lamina of the dome. Direct interactions of intraepithelial lymphocytes with undifferentiated cells have been postulated (Abe and Ito, 1978; Smith and Peacock, 1980, 1982; Bockman, 1983), but appear improbable since they would necessarily initiate the formation of M cells in the crypt zone, a region where intraepithelial lymphocytes are relatively rare. In addition, a morphometric study revealed no correlation between the number of intraepithelial lymphocytes and the appearance or distribution of immature M cells (Sicinski *et al.,* 1986). Soluble factors or mediators, which could be secreted by lymphocytes, macrophages, dendritic or reticulum cells of the lymphoid tissue underlying the dome epithelium, could be involved in the formation of M cells. The cellular composition of the gut epithelium along the crypt–villus axis and the biochemical composition of the basal lamina have been shown to correlate, suggesting that the basal lamina is involved in the composition and in compositional changes of the epithelium (Trier *et al.,* 1990; Beaulieu and Vachon, 1994). The few available studies on the extracellular matrix of the dome area revealed a similar biochemical composition of the dome and villus basal lamina, at least in the distribution of laminin, type-IV collagen, and fibronectin (Allan and Trier, 1991; Ohtsuka *et al.,* 1992). Observations by Fujimura and Kihara (1994) suggest the presence of intracellular adhesion molecule-1 (ICAM-1) associated with fibroblasts that lie directly beneath the basal lamina of the dome epithelium and with cytoplasmic projections that cross the basal lamina and are in contact with dome epithelial cells. Such interactions might induce or maintain the formation of M cells, the suppression of goblet cell differentiation, and the massive lymphocytic traffic between lymphoid tissue and dome epithelium (see Kedinger *et al.,* 1981; Haffen *et al.,* 1983). Recently, Kernéis *et al.* (1995) reported that structures similar to Peyer's patches were induced in the duodenum of mice by submucosal injections of lymphoid follicle cells from Peyer's patches. Since M cells were identified in these structures, the finding suggests that elements of the lymphoid tissue induce the formation of M cells directly or via other components.

The M cells of most species studied prevail at the flanks of the domes, and at the apex of the dome are reduced in number or are even lacking (Schmedtje, 1980; Roy *et al.,* 1987; Gebert and Bartels, 1991; Jepson *et al.,* 1993c; Figs. 5 and 7). The factors regulating this typical distribution are not known. It could be speculated that the life span of M cells is shorter than that of enterocytes and that the M cells are sloughed off before they

reach the apex of the dome. This view is supported by observations of disrupted M cells predominantly at the upper flanks (A. Gebert, unpublished observations). M cells also seem to be mechanically fragile (Owen and Jones, 1974) and could be mechanically damaged by the gut content at the upper rather than the lower dome. However, the lack of M cells at the apex of the dome could also be due to a lower migration speed of M cells compared with enterocytes. Future studies of M cell proliferation and migration using DNA precursors like bromodesoxyuridine or [^{3}H]thymidine could answer these questions.

IV. M Cells

A. Characteristics

1. General

M cells as highly specialized epithelial cells are exclusively found in the epithelia that cover mucosa-associated lymphoid tissues. General features of M cells that are independent of species and location include ultrastructural peculiarities and the transcytotic transport of antigens.

The shape of the apical surface of M cells normally differs from that of the adjacent epithelial cells (Figs. 3 and 9). Scanning electron microscopy revealed microplicae or microvilli that are less regular than and differ in length and diameter from those of enterocytes (Owen and Jones, 1974; Landsverk, 1981; Bockman and Boydston, 1982; Smith and Peacock, 1982; Bockman, 1983; Liebler *et al.,* 1991; Fig. 9). The glycocalyx on the apical surface of M cells is short and stub-like, as shown by thin-section electron microscopy and ruthenium red staining (Owen, 1977; Owen *et al.,* 1986b; Sanderson and Walker, 1993; Gebert and Bartels, 1995). The terminal web, formed by microvilli core rootlets and other aggregated microfilaments, is normally less developed in M cells than in enterocytes (Owen and Jones, 1974; Bye *et al.,* 1984) and therefore facilitates increased transcytotic traffic of vesicles. Many such vesicles normally lie in the apical cytoplasm of M cells (Figs. 3 and 10). They are round or ovoid, have an electron-lucent core, and some are coated with clathrin when they originate from or fuse with the apical or basolateral membrane (Neutra *et al.,* 1987). The basolateral membrane of M cells is usually invaginated, forming a pocket that is populated by intraepithelial lymphocytes, macrophages, and occasionally plasma cells. The cytoplasm between apical membrane and pocket membrane is often attenuated or "membranous," which led to the term "membranous (M) cell."

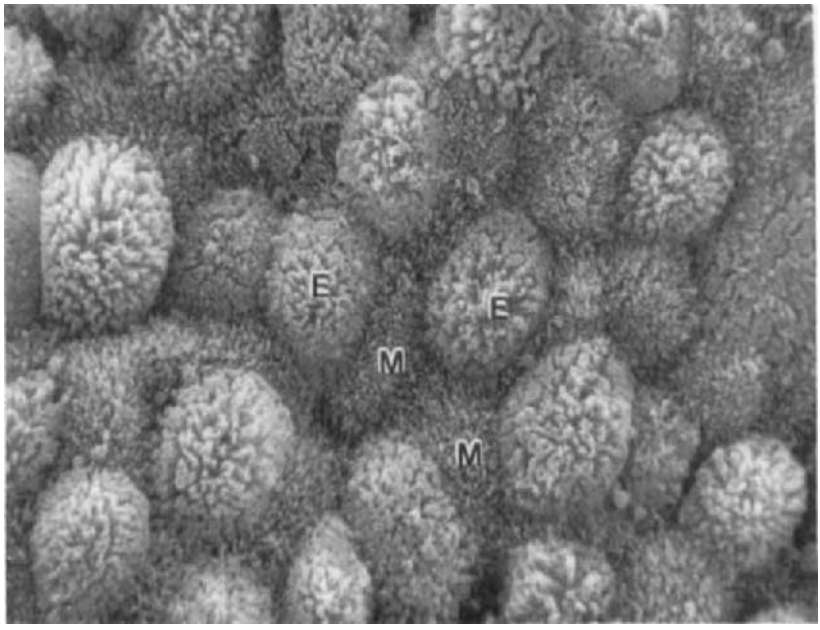

FIG. 9 SEM view of the rabbit Peyer's patch dome epithelium. An irregular patchwork of epithelial cells is formed by M cells (M) and enterocytes (E). SEM. ×2800.

Vesicular transport of soluble and solid antigens is common to all M cells and has been demonstrated using several tracers. Horseradish peroxidase (HRP), India ink, and ferritin applied to the gut lumen *in vivo* were found in the apical vesicles within minutes and in the intercellular space between pocket membrane and leukocytes within minutes or hours (Bockman and Cooper, 1973; Owen, 1977; Rosen *et al.,* 1981; Neutra *et al.,* 1987; Gebert and Bartels, 1995). Although the transcytosis of antigens has not yet been verified for M cells of all species and locations, it seems to be the central characteristic (for details, see Section IV,B,1).

2. Variations among Species and Locations

With the exception of the transport function for soluble antigens, all other characteristics and their number vary according to species and the location of the M cells in the small or large intestine or other MALT. The characteristics of M cells also vary within each location, reflecting different maturation stages or even subtypes.

a. Different Species Most studies on M cells used tissue taken from mice, rats, rabbits, and humans. Less information is available on M cells in guinea

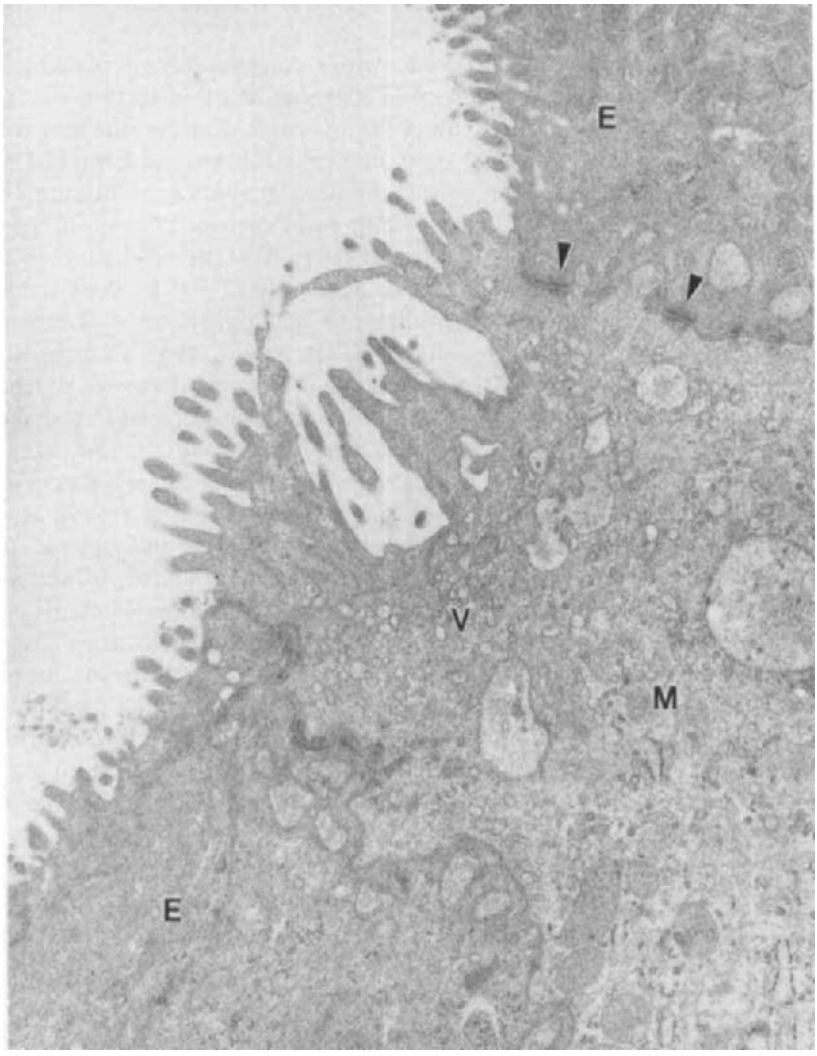

FIG. 10 Apical cytoplasm of an M cell (M) and two adjacent enterocytes (E) of the rabbit cecum. M cells of the large intestine have irregular microvilli of varying diameter and length which often overreach those of enterocytes. Note that numerous vesicles (V) are present in the apical cytoplasm of the M cell but are almost absent from that of the enterocytes. M cells and enterocytes are mechanically connected by desmosomes (arrowheads). Epon ultrathin section. ×16,000.

pigs, pigs, sheep, and cattle. There are only a few reports on M cells in dogs, monkeys, and chickens (Atkins and Schofield, 1972; Naukkarinen *et al.*, 1978; Beezhold *et al.*, 1983; Kato *et al.*, 1992; Davenport and Allen,

1995), and recently Hanger and Heath (1994) described M cells in the cecocolic lymphoid patch of the koala.

The number of M cells per dome varies considerably, depending on species and also on the site. As seen in scanning and thin-section electron microscopy, the M cells of the human Peyer's patch dome epithelium comprise less than 10% of all dome epithelial cells (Owen and Ermak, 1990; A. Gebert, unpublished observations). Similar numbers were reported for the rat and mouse Peyer's patches (Smith and Peacock, 1980; Smith *et al.,* 1980; Clark *et al.,* 1993, 1994a). A recent lectin histochemical study in the mouse model using the *Ulex europaeus* agglutinin (UEA-I) revealed that lectin-positive cells, which were assumed to be identical to M cells, comprised 8.2% of the surface of a dome (Clark *et al.,* 1993). Considerably higher numbers of M cells (30–50%) prevail in the rabbit Peyer's patches, as determined by electron microscopy and immunohistochemistry (Pappo *et al.,* 1988; Gebert *et al.,* 1992; Jepson *et al.,* 1993c; Fig. 9). Comparably high numbers of M cells were reported for the jejunal Peyer's patches of pigs using cytokeratin 18 as an M-cell marker (Gebert *et al.,* 1994).

M cells of the human Peyer's patches are covered with microplicae (microfolds), as detected by scanning electron microscopy (SEM) (Owen and Jones, 1974), which primarily led to the term M (microfold) cell. The M cells of other species also have an irregularly shaped apical surface, but in only a few cases do they possess microfolds. The M cells in the rat, mouse, and guinea pig Peyer's patches have short, stub-like microvilli (Smith and Peacock, 1980; Rosen *et al.,* 1981; Bhalla and Owen, 1982; Bye *et al.,* 1984; Madara *et al.,* 1984; Fig. 3). In contrast, the M cell microvilli in rabbit and pig Peyer's patches are relatively long and branched and of varying diameter (Chu *et al.,* 1979; Torres-Medina, 1981; Pappo *et al.,* 1988; Gebert and Bartels, 1991). It is reasonable to assume that the more irregular surface of M cells, compared with the enterocytes, facilitates the adhesion and endocytosis of antigens. Variations in the surface structure of M cells may also depend on osmolality during fixation or the rate of M-cell surface membrane turnover (Owen and Bhalla, 1983b).

b. Different Locations M cells of the small and large intestine differ considerably in their morphology and in the composition of glycoconjugates expressed on their apical membrane. While most Peyer's patch M cells have short microvilli or microplicae, those of the large intestine have longer microvilli, some of which even overreach those of adjacent surface epithelial cells (Liebler *et al.,* 1988, 1991; Morfitt and Pohlenz, 1989). The shape of M cells in the rabbit cecal patch is consistent with this pattern (Gebert and Bartels, 1995; Fig. 10), while those in the rabbit appendix have a flatish surface with only few extrusions (Bockman and Cooper, 1973; Gebert and Bartels, 1991), indicating that regional differences also exist within the large

intestine. Regional differences have also been described for the number and distribution of engulfed intraepithelial lymphocytes and the size and shape of the basolateral pocket (Snipes, 1978; Schmedtje, 1980; Gebert and Bartels, 1991). Due to these site-specific variations, it is sometimes difficult or even impossible to identify M cells by ultrastructural characteristics. In porcine Peyer's patches, only a few M cells can be detected by conventional thin-section electron microscopy, although about 30–50% of the dome epithelial cells are M cells as detected by tracer uptake and immunohistochemistry (Chu *et al.*, 1979; Gebert *et al.*, 1994). Therefore, the ultrastructural description of only one cell type prevailing in the dome epithelium of calves (Torres-Medina, 1981; Landsverk, 1987) does not necessarily indicate that all of these cells are M cells (or enterocytes). Further local specializations have been demonstrated for M cells of the rabbit cecal patch, which possess a deep invagination of the *apical* membrane that might serve as a gateway facilitating the uptake of antigens (Gebert and Hach, 1993; Gebert and Bartels, 1995).

Distinct site-specific variations of M cells are evident in the composition of terminal saccharides in the glycoproteins of the apical membrane. Using lectin histochemistry and confocal laser scanning microscopy, Clark *et al.* (1993) detected high levels of fucose in the apical membrane of M cells of murine Peyer's patches but only low levels in that of enterocytes and in the mucus of goblet cells. In contrast, the glycocalyx of M cells in the large intestine of BALB/c mice is not enriched in fucose (Clark *et al.*, 1994a), but is selectively labeled by certain lectins specific for galactose (Giannasca *et al.*, 1994). In the rabbit, however, the lectins UEA-I and HPA selectively label M cells of the large intestine (Fig. 11), but not those of the Peyer's patches (Gebert and Hach, 1993; Jepson *et al.*, 1993d). Although large panels of lectins specific for the most common terminal saccharides were applied to aldehyde-fixed Peyer's patches of rats, guinea pigs, and cats, no differences in the lectin-binding patterns were found between M cells and dome epithelial enterocytes (A. Gebert, unpublished observations). It is conceivable that M cell-specific glycoproteins will be clinically relevant for drug and vaccine targeting in the future, but so far no data are available on the carbohydrate composition of the apical membrane of human M cells. The site-dependent lectin binding patterns described for some species do not correlate with other characteristics such as ultrastructure or antigen transport capacity. The variations, however, indicate that M cells are possibly adapted to local gut content or microbial populations. In addition, the variations could reflect species-specific nutrition and pathogens, which probably enter the host via M cells (see Section IV,B,3).

c. Different Maturation Stages The morphological and histochemical properties of M cells change gradually from the base to the top of the

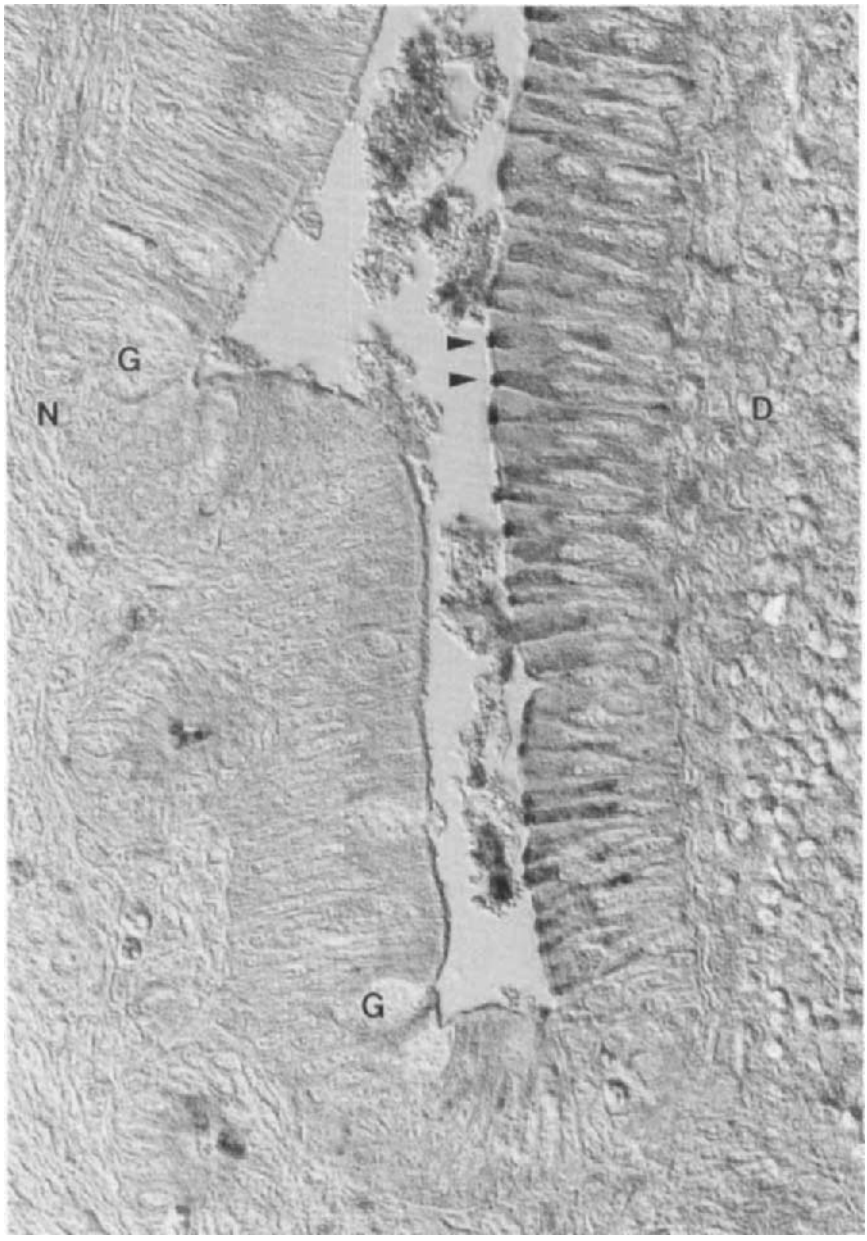

FIG. 11 Dome (D) and neighboring nondome region (N) of the cecal lymphoid patch of the rabbit. M cells (arrowheads), exclusively present in the dome epithelium, are selectively labeled by the lectin derived from *Helix pomatia* (HPA), which detects *N*-acetyl-galactosamine residues. Note that goblet cells (G) are unlabeled and absent from the dome epithelium. Paraffin section; Nomarski interference contrast. ×620.

dome. M cells at the base of the dome near the mouths of the crypts lack the central cytoplasmic pocket formed by an invagination of the basolateral membrane, are not in direct contact with intraepithelial lymphocytes, and have a more regular surface structure than M cells at the flanks (Smith and Peacock, 1980; Bye *et al.*, 1984). These cells were assumed to represent "immature" M cells: while possessing several characteristics of their mature siblings, they had a reduced transport capacity for antigens (Bye *et al.*, 1984; Sicinski *et al.*, 1986). Since a continuous spectrum of M cell structure ranging from immature to mature was demonstrated in these studies, a developmental line from undifferentiated crypt cells via immature to mature M cells is plausible.

The available markers for M cells (i.e., epitopes labeled by certain antibodies or glycoproteins labeled by lectins; see Section III, B) recognize the M cells as a homogeneous population. Giannasca *et al.* (1994) recently reported that in the mouse Peyer's patch, the lectins UEA-I, AAA, and EEA, as well as antibodies against the blood group antigen H-2 determinant, selectively recognized M cells but bound to different, overlapping subpopulations of M cells. Clark *et al.* (1994b) noted that the adhesion of *Salmonella typhimurium* to M cells at the same location occurred in a nonuniform pattern and they too proposed the existence of M cell subtypes. Our own experiments with fluorescein-labeled yeast showed that likewise the number of particles taken up by individual M cells varied considerably (A. Gebert, unpublished data). Therefore, the proposed subtypes might also reflect varying functional states.

B. Functions

Endocytotic uptake, transcytotic transport, and exocytotic release of luminal substances to the intercellular space of the epithelium are the central functions of M cells. Although these functions have been studied using various antigens and tracers, including soluble molecules, particles, viruses, and microorganisms, the mechanisms that mediate the transepithelial transport are still poorly understood.

1. Transport of Soluble and Solid Antigens and Tracers

The transport and diffusion of substances across epithelial barriers can be performed along the transcellular or the paracellular route, or both. In the normal gut epithelium, the tight junctions are closed, preventing macromolecules and particles from entering the gut wall (Madara *et al.*, 1980; Powell, 1981). Ussing chamber experiments with explanted Peyer's patches and horseradish peroxidase (HRP) revealed that the transepithelial transport

of this tracer could be significantly decreased by sodium fluoride, indicating that the transport is predominantly mediated by endocytosis (Ducroc *et al.*, 1983; Keljo and Hamilton, 1983). Temperatures of 25°C or less drastically reduced the transport rate—a finding that also supports the hypothesis of an active transport and a closed paracellular barrier. Freeze-fracture studies of rat, rabbit, and guinea pig GALT epithelia showed that the tight junctions, representing the structural correlates of the paracellular barrier, are closed in both villus and dome epithelium (Madara *et al.*, 1984; Gebert and Bartels, 1991; Fig. 12).

Depending on the physicochemical properties of the tracers and antigens, different mechanisms appear to be involved in their transcellular transport. Experiments by Keljo and Hamilton (1983) produced no evidence of saturation of the transport rate of HRP across Peyer's patch epithelium, indicating that this tracer is transported by unspecific (fluid-phase) endocytosis. This observation correlates with earlier findings by Owen (1977), who described the endocytosis of HRP by murine Peyer's patch M cells via nonclathrin-coated vesicles. Neutra *et al.* (1987) demonstrated that the membrane-adherent tracer wheat germ agglutinin was transported 50 times more avidly through rabbit Peyer's patch M cells than the nonadherent tracer bovine serum albumin (BSA). However, antigen–membrane interactions involved in such receptor-mediated endocytosis are still poorly understood (for details, see Section IV,B,2).

Several particulate antigens and tracers have been applied to the lumen over Peyer's patch domes (Table I). Most of these particles adhered to the apical membranes of M cells and were phagocytosed by them. The uptake of particulate tracers and entire pathogens by the epithelium of Peyer's patches was first described more than 70 years ago (Kumagai, 1922; Ogushi, 1925; Enticknap, 1953). In these studies, mycobacteria, India ink, carmine, and powdered red blood cells were applied to the gut lumen of rabbits and were seen to be taken up by the epithelium covering the lymphoid nodules of the gut wall. Kumagai (1922) noted that both active and inactivated mycobacteria "were taken up by the protoplasma of the epithelial cells" of Peyer's patches and appendix. Later electron microscopic studies with India ink confirmed that this particulate tracer was taken up by the M cells of the dome epithelium and not by the enterocytes (Bockman and Cooper, 1973). Studies with larger particles, e.g. 0.7 μm latex or copolymer microspheres or entire bacteria, showed that, in principle such tracers can be taken up by M cells (LeFevre *et al.*, 1978; Pappo and Ermak, 1989; Eldridge *et al.*, 1991a; Pappo *et al.*, 1991; Jepson *et al.*, 1993b,e; Ermak *et al.*, 1995; Fig 13). Since this transcytotic route could represent a gateway for the entrance of intestinal pathogens into the organism, various active and inactivated microorganisms have been tested (Table I).

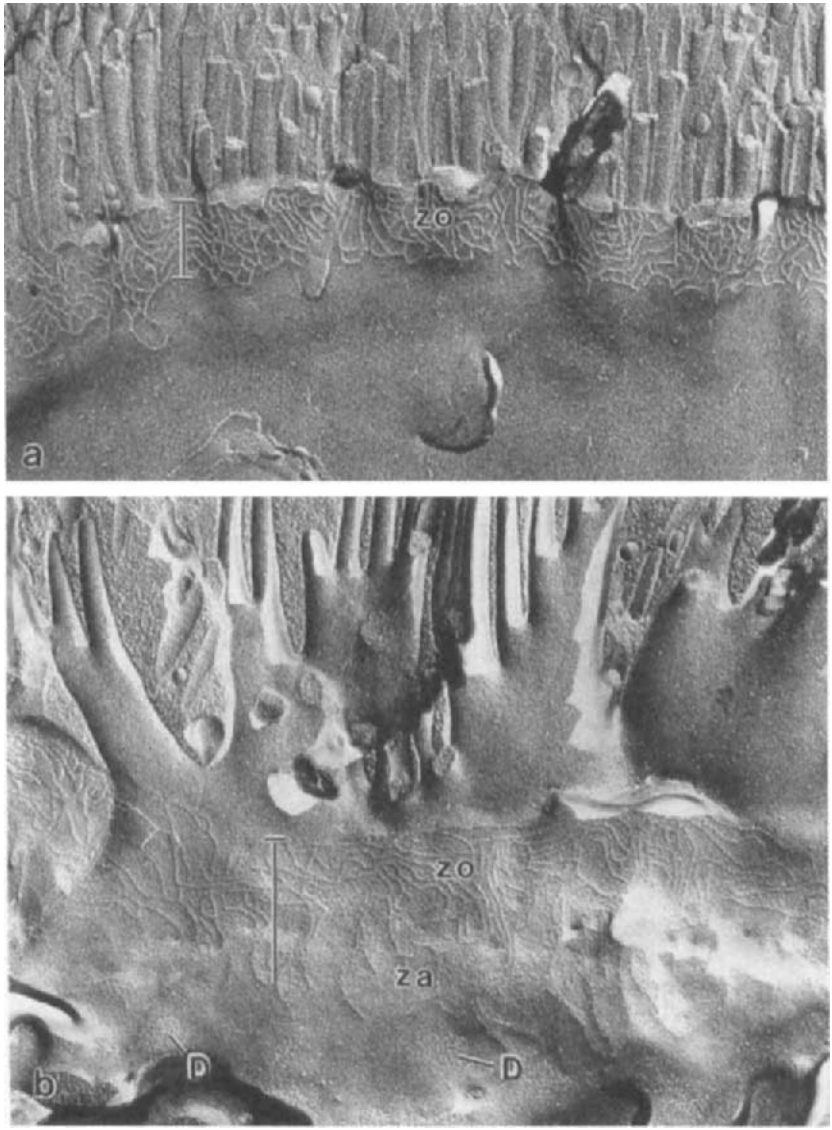

FIG. 12 Freeze-fracture replicas of villus and dome epithelium of the rabbit Peyer's patch. The strands of the zonula occludens (zo) between enterocytes of the villus epithelium (a) form a belt 0.3–0.4 μm in depth that is clearly demarcated on both apical and basolateral sides. In contrast, the zonulae occludentes formed between dome epithelial enterocytes and M cells (b) cross the region of the zonula adherens (za) and extend to the basolateral membrane. The depth of the zonula occludens in the dome epithelium exceeds that of the villus epithelium (vertical bars). Desmosomes (D) connect the M cells with the enterocytes. Note that the microvilli of the M cell in (b) are more irregular, branched, and of larger diameter than those of villus enterocytes. ×26,500.

TABLE I

Uptake of Soluble and Solid Tracers and Microorganisms by the Peyer's Patch Dome Epithelium

Soluble tracers		
Antivirus antibodies	Mouse	Weltzin *et al.* (1989)
Cholera toxin-binding subunit	Rabbit	Owen *et al.* (1986b)
Ferritin (native or cationized)	Rabbit, mouse, pig, cattle	Bockman and Cooper (1973); Bye *et al.* (1984); Neutra *et al.* (1987); Paar *et al.* (1992); Kracke and Bartels (1994); Liebler *et al.* (1995)
Horseradish peroxidase	Mouse, rabbit, pig	Owen (1977); Rosen *et al.* (1981); Keljo and Hamilton (1983); Ducroc *et al.* (1983); Gebert and Bartels (1995)
Lectins	Rabbit, mouse	Neutra *et al.* (1987); Giannasca *et al.* (1994)
Particulate tracers		
Carbon particles	Rabbit, mouse	Bockman and Cooper (1973); Joel *et al.* (1978)
Copolymer microspheres	Mouse, rabbit	Eldridge *et al.* (1991a,b); Ermak *et al.* (1995)
Hydroxyapatite	Mouse	Amerongen *et al.* (1992)
Latex microbeads	Mouse, rabbit	LeFevre *et al.* (1978, 1985); Pappo and Ermak (1989); Pappo *et al.* (1991); Porta *et al.* (1992); Jepson *et al.* (1993b)
Liposomes	Rat	Childers *et al.* (1990)
Viruses		
Astrovirus, bredavirus	Cattle	Woode *et al.* (1984)
HIV	Mouse	Amerongen *et al.* (1991a,b)
Mouse mammary tumor virus	Mouse	Neutra and Kraehenbuhl (1992)
Poliovirus	Man	Sicinski *et al.* (1990)
Reovirus	Mouse	Wolf *et al.* (1981, 1983, 1987); Bye *et al.* (1984); Bass *et al.* (1988); Amerongen *et al.* (1994)
Bacteria		
Bacillus Calmette-Guérin	Rabbit	Fujimura (1986)
Brucella abortus	Cattle	Ackermann *et al.* (1988)
Campylobacter jejuni	Rabbit	Walker *et al.* (1988)
Escherichia coli (RDEC-1)	Rabbit	Inman and Cantey (1983, 1984); Uchida (1987)
Mycobacterium paratuberculosis	Cattle	Momotani *et al.* (1988)
Salmonella typhi	Mouse	Kohbata *et al.* 1986
Salmonella typhimurium	Mouse	Jones *et al.* (1994); Clark *et al.* (1994b)
Shigella flexneri	Rabbit	Wassef *et al.* (1989); Perdomo *et al.* (1994)
Streptococcus pneumoniae	Rabbit	Regoli *et al.* (1995)
Vibrio cholerae	Rabbit, man	Owen *et al.* (1986b); Yamamoto *et al.* (1988)
Yersinia enterocolitica	Mouse	Hanski *et al.* (1989); Grützkau *et al.* (1990, 1993)
Yersinia pseudotuberculosis	Rabbit	Fujimura *et al.* (1989)
Other microorganisms		
Cryptosporidia	Guinea pig	Marcial and Madara (1986)
Baker's yeast	Pig	Gebert *et al.* (1994)
Giardia muris	Mouse	Owen *et al.* (1979)

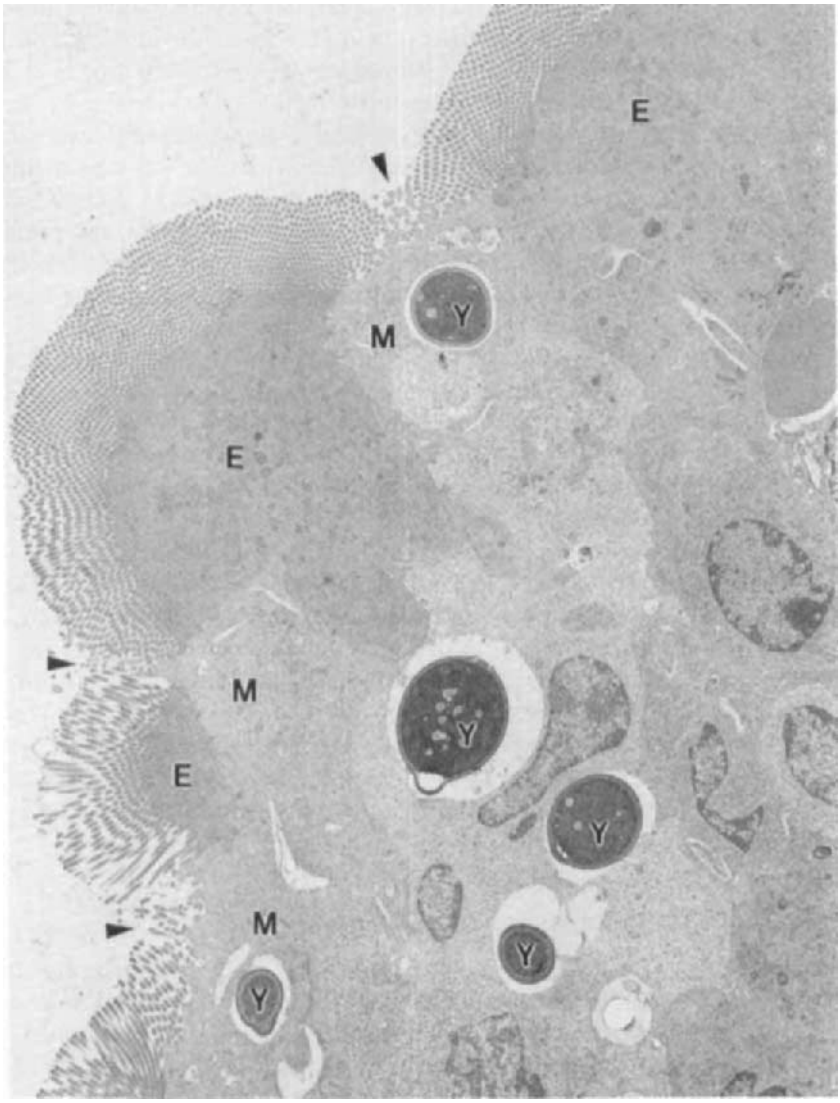

FIG. 13 Uptake of integer yeast cells by the dome epithelium of porcine Peyer's patches. M cells (M) alternate with dome epithelial enterocytes (E). While the latter possess a thick and regular brush border, the M cells have an irregular surface with only few thick microvilli (arrowheads). Yeast cells had been injected into the gut lumen 1 hr before resection of the Peyer's patch. In the apical part of the dome epithelium, yeast (Y) is found in cytoplasmic vacuoles of the M cells. The yeast cells are located outside the M cells, interspersed among nonepithelial cells, in a deeper region of the dome epithelium. (Preparation and photomicrography by Rita Beier.) Epon ultrathin section. ×3900.

Using the mouse model or explanted human Peyer's patches, it was demonstrated that reovirus, poliovirus, and HIV enter the host via M cells (Wolf *et al.*, 1981; Bye *et al.*, 1984; Sicinski *et al.*, 1990; Amerongen *et al.*, 1991a,b). A similar uptake of entire bacteria, including *Salmonella, Yersinia enterocolitica, Shigella flexneri, Vibrio cholerae, Campylobacter jejuni, Escherichia coli* strains and *Bacillus Calmette Guérin* (BCG) was documented in various studies (for details and references, see Table I). Marcial and Madara (1986) showed that some protozoa, like cryptosporidia, also preferentially bind to and actively invade M cells to gain access to the gut mucosa.

Despite the abundance of uptake studies, only very few quantitative data are available on the amount of antigen taken up by M cells. Pappo and Ermak (1989) determined that fluorescent latex particles were taken up by the rabbit Peyer's patch dome epithelium at a rate of about 2 μm/min, resulting in a total uptake of 2–3 $\times$ 10^5 particles per dome within 90 min, but they provided no data about intra- or interindividual variations. Our own experiments with fluorescence latex beads, baker's yeast, and bacteria [*Campylobacter jejuni, Escherichia coli* (HB101), *Actinobacillus pleuropneumoniae*] revealed considerable variation in the quantity of particles phagocytosed per dome, per patch, and per animal (A. Gebert, unpublished data). Since these variations apparently do not correlate with the mode of antigen application, the age of the animals, or the microbial population of the gut content, it can be assumed that the interaction of pathogen and M cell is complex and that possibly the phagocytotic activity of M cells is not constant but modulated by unknown factors. These possible influences and variations must be systematically elucidated in future studies, because they might prove advantageous for therapeutic approaches using M cells as a gateway for drugs or vaccines.

2. Interaction of Antigens and M-Cell Surface

It is likely that the interactions of antigens with the apical surface of the M cells play a crucial role in the initial step of intestinal and systemic immune responses or tolerances, since they could represent a filter selecting antigens to be forwarded to cells of the immune system (e.g., macrophages or dendritic cells). Detailed knowledge of the binding properties of M cell membranes could make it possible to develop orally delivered drugs and vaccines that would specifically enter the organism via M cells. In addition, the receptors that allow pathogenic microorganisms to adhere to the gut epithelium and to invade the tissue via M cells could be specifically blocked to prevent infection.

a. Preferential Adherence of Antigens Several soluble and particulate tracers as well as particles and bacteria of the normal gut content adhere

to the dome epithelium. Inman and Cantey (1983, 1984) demonstrated that RDEC-1 strain *Escherichia coli* preferentially adhered to the microvilli of M cells in rabbit Peyer's patches, but not to those of enterocytes. A similar preference was reported for other bacteria like *Vibrio cholerae* (Owen *et al.*, 1986b), *Shigella flexneri* (Wassef *et al.*, 1989), and *Campylobacter jejuni* (Walker *et al.*, 1988) in the rabbit model. In the mouse model, *Salmonella typhimurium* (Jones *et al.*, 1994) preferentially bound to the apical membrane of M cells. The same was shown for virions like reovirus type 1 (Wolf *et al.*, 1981, 1983; Bass *et al.*, 1988; Amerongen *et al.*, 1994) and HIV-1 (Amerongen *et al.*, 1991a,b) in the mouse. Sicinski *et al.* (1990) used explanted, unfixed human small intestine to demonstrate the selective adherence of poliovirus to the M cells of Peyer's patches. Unidentified, indigenous bacteria were also found adhering to the dome epithelium of Peyer's patches of different species (Owen and Nemanic, 1978; Gebert and Hach, 1993; Jepson *et al.*, 1993a; Gebert and Bartels, 1995). Although specific interactions of microorganisms with epithelial cells are likely and were confirmed as mediating the binding in some of the models (see Section III,B,2), inert particles like carbon or latex also preferentially bound to the M cells under certain conditions (Pappo and Ermak, 1989; Jepson *et al.*, 1993b), indicating that unspecific interactions are also involved. In addition, a few reports are available that describe an adherence of certain bacteria with no obvious preference for M cells or enterocytes (Yamamoto *et al.*, 1988; Grützkau *et al.*, 1990; Jepson *et al.*, 1993a).

b. Unspecific Mechanisms Goblet cells are absent from the dome epithelium of rabbits, pigs, cats, cattle, and sheep, and only rarely occur in that of rodents and humans. The paucity of goblet cells in the dome epithelium is assumed to reduce the thickness and/or modify the composition of the mucus layer covering the dome epithelium. Therefore, this should facilitate the direct contact of all components of the gut content with the surface of the domes. In addition, it was shown by ruthenium red staining that the mucus layer that directly covers the epithelial cells is drastically reduced over M cells compared with the neighboring enterocytes (Owen *et al.*, 1986b).

The brush border of normal enterocytes is dense and regular, and therefore inhibits the binding of bacteria to larger membrane domains. In contrast, the irregular or even flat surface of M cells could facilitate the association of potential antigens with the M cell surface. Electron microscopy revealed that the glycocalyx of M cells, compared with enterocytes, is thin and stub-like (Owen, 1977; Owen *et al.*, 1986b; Sanderson and Walker, 1993; Gebert and Bartels, 1995), and has a reduced enzymatic activity of alkaline phosphatase (Schmedtje, 1965; Owen and Bhalla, 1983a). Since cationized ferritin binds equally to M cells and enterocytes of mouse Peyer's

patches (Owen and Bhalla, 1983a; Bye *et al.*, 1984; Neutra *et al.*, 1987), anionic binding could be generally involved in the adherence of antigens to M cells, but at least in mice, it does not mediate preferential adherence to the apical membrane of M cells. The adhesion of inert particles to M cells appears to depend strongly on the physicochemical properties of the particle surface. Jepson *et al.* (1993e) reported that adherence and uptake of polystyrene microspheres exceeded that of poly (DL-lactide-coglycolide) microspheres by one order of magnitude. Future studies using tracers with well-defined surface properties are needed to determine the factors (e.g., local surface pH, membrane potential, electrostatic or hydrophobic interactions) involved in binding inert particles to the glycocalyx of M cells and making it sticky.

*c. **Specific Mechanisms*** Several observations suggest that the binding of antigens, especially bacteria and viruses, to M cell membranes is mediated by specific receptor–ligand interactions in addition to unspecific mechanisms. Inman and Cantey (1984) reported that *Shigella flexneri* (ShD15) and *Escherichia coli* (RDEC-1) adhered to rabbit M cells when the bacteria contained certain pilus plasmids of 85×10^6 Da. Wolf *et al.* (1983) showed that the adherence of reovirus to mouse M cells is determined by the viral hemagglutinin (σ_1 protein) and depends on the virus type. In most cases of selective adherence of microorganisms to M cells, however, the molecular mechanisms and the receptors on the apical M cell surface that mediate the adherence are unknown. Mechanisms that might be responsible for the selective binding of antigens include lectin–sugar interactions, IgA-mediated adherence, and possibly M cell-specific receptor molecules.

Lectin-like adhesins on the outer membrane of bacteria could play a key role in the first step of bacterial invasion. The adherence of *Vibrio cholerae* O1 to dome epithelial cells is obviously related to a fucose-specific adhesin on the outer surface of bacteria because L-fucose but not D-mannose inhibits adhesion (Yamamoto *et al.*, 1988). Recently Wennerås *et al.* (1995) reported that enterotoxigenic *E. coli* binds with its fibrillar CS3 adhesin to the carbohydrate sequence GalNAcβ1–4Gal on the rabbit intestinal brush border. Several studies showed that despite the relatively thin glyococalyx of M cells, their apical membrane is rich in glycoconjugates. Using lectin histochemistry, Owen and Bhalla (1983a) demonstrated that the terminal saccharides mannose, galactose, and *N*-acetyl-galactosamine are present in the glycocalyx of M cells of rat and mouse Peyer's patches. Neutra *et al.* (1987) reported that ferritin-conjugated wheat germ agglutinin avidly and selectively bound to the M cells of unfixed rabbit Peyer's patches and was endocytosed by coated pits. Systematic studies using panels of lectins with specificities for all sugars normally present in epithelia revealed that M cells of the rabbit cecum are rich in fucose and *N*-acetyl-galactosamine

(Gebert and Hach, 1993; Jepson *et al.*, 1993d; Fig 14). The same saccharides, however, were found on Peyer's patch M cells in only small amounts, indicating site-specific variations in the composition of the glycocalyx of M cells. Clark *et al.*, (1993, 1994a) reported that the apical membrane of M cells of mouse Peyer's patches is rich in fucose. In contrast, M cells of the large intestine of mice are characterized by terminal galactose but not by fucose (Giannasca *et al.*, 1994). The glycoconjugates could be targeted by bacterial adhesins, but they vary significantly with species and location of the lymphoid tissue. The variations have been interpreted as local adap-

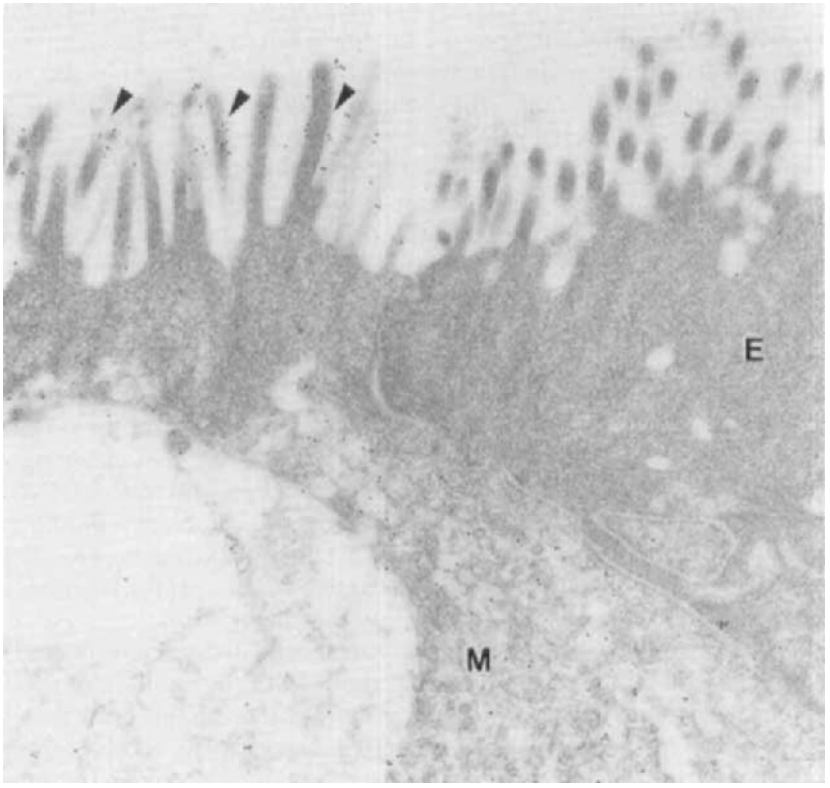

FIG. 14 Apical cytoplasm of an M cell (M) and an adjacent enterocyte (E) of the rabbit cecal lymphoid patch. Fucose residues are detected by the lectin derived from *Ulex europaeus* (UEA-I) conjugated to 10 nm colloidal gold. At this site of rabbit GALT, fucose is expressed in the glycocalyx of the microvilli of M cells (arrowheads) but not in that of enterocytes. While the terminal web region of both cells is devoid of fucose, this sugar is detected in the membrane of vesicles that are presumably involved in transcytosis. LR white ultrathin section; postembedding lectin-gold; ×20,000.

tations of the M cells so that they can take up bacterial surface antigens to induce specific immune responses against potential pathogens or specific immune tolerance for resident bacteria (Gebert and Hach, 1993). Our own unpublished experiments with aldehyde-fixed Peyer's patches of rats, guinea pigs, cats, and pigs revealed equal lectin bindings for M cells and enterocytes in these species. However, the *in vivo* accessibility of saccharides as well as other receptor molecules could differ between M cells and enterocytes. Giannasca and Neutra (1994) reported that rhodamine-conjugated cholera toxin bound to the apical membrane of M cells but not toxin immobilized on 15-nm gold particles. This could be due to the unique surface morphology, to the thin glycocalyx of M cells, or to the reduced activity of digestive enzymes on M cells as discussed earlier (Section IV,B,2).

In the normal gut mucosa, IgA is produced by plasma cells of the lamina propria, subsequently bound to the poly-Ig receptor of enterocytes, transported to the apical membrane by endocytosis, and secreted as secretory immunoglobulin A (sIgA) to the gut lumen (Brandtzaeg *et al.*, 1989). The sIgA neutralizes potential pathogens in the gut lumen and thereby hinders them from binding to the epithelial surface (Killian *et al.*, 1988; Kraehenbuhl and Neutra, 1992). It was shown that these immunoglobulins and also sIgA-antigen complexes adhere selectively to the apical membrane of M cells (Weltzin *et al.*, 1989; Kato, 1990; Porta *et al.*, 1992). This mechanism would facilitate the uptake of pathogens that had previously induced a humoral immune response, and could result in an enhanced, accelerated immune response. The receptor that binds sIgA to the M cell surface has not yet been defined but it probably differs from the macrophage Fc receptor, the suckling rodent epithelial Fc receptor, and the poly-Ig receptor (Weltzin *et al.*, 1989; Giannasca and Neutra, 1994). In contrast to the remaining gut mucosa, the dome epithelial cells, at least in rabbits and humans, do not express the poly-Ig receptor on their basolateral membrane (Bjerke and Brandtzaeg, 1988; Pappo and Owen, 1988). Although the significance of this peculiarity is not fully understood, re-secretion of sIgA–antigen complexes taken up by M cells could be reduced by this mechanism.

The abundance of tracers and microorganisms that selectively bind to M cells and the variations in the properties of M cells in different species and locations have led to the hypothesis that there is no universal "M cell receptor." A variety of unspecific bindings, specific receptor–ligand interactions, and combinations of these are probably involved in the adherence of antigens to the M cell surface.

3. Transcytosis of Antigens

The transepithelial transport of antigens and tracers in the dome epithelium of GALT is carried out along a transcellular rather than a paracellular route

(see Section IV,B,1). It is predominantly or even exclusively performed by the M cells. This transcellular transport can be divided into the following stages: (1) endocytosis at the apical membrane, (2) transport of endocytic vesicles to a tubulovesicular system called the endosomal compartment, and (3) exocytosis to the basolateral membrane (Neutra *et al.,* 1987). In general, the mode of endocytosis at the apical membrane depends on the size and properties of the antigen or tracer and on the receptor molecules present on the surface of the cell (Futter and Marsh, 1993). It was shown that several soluble tracers, e.g., horseradish peroxidase or ferritin, are endocytosed by M cells via fluid-phase endocytosis (Bockman and Cooper, 1973; Owen, 1977; Rosen *et al.,* 1981; Gebert and Bartels, 1995). These antigens are neither membrane bound, nor concentrated by the transcytotic process, and are released during exocytosis to the intercellular spaces of the epithelium. Other soluble antigens, like certain lectins, bind to yet-unidentified receptors on the apical membrane of M cells and are taken up by receptor-mediated endocytosis. Neutra *et al.* (1987) compared the transport efficiency of the soluble tracer BSA (conjugated to colloidal gold) with that of the membrane-bound tracer wheat germ agglutinin (conjugated to ferritin), and found that the latter was transported about 50 times more efficiently than the fluid-phase marker. While membrane-bound tracers are clearly taken up by clathrin-coated vesicles (Neutra *et al.,* 1987), it is not known to what extent the fluid-phase endocytosis in M cells is related to clathrin.

In addition to small soluble antigens, larger particles 0.3–4 μm in diameter are avidly taken up by M cells, as demonstrated with latex microspheres and various microorganisms (see Section IV,B,1; Table I; Fig. 13). Little is known about the mechanisms involved in the uptake of particles by M cells, but the mode seems to parallel that described for phagocytosis in macrophages. The initial step was described as direct contact of the particle (e.g., the outer membrane of a bacterium) with the glycocalyx of the M cell (Inman and Cantey, 1983; Inman *et al.,* 1986; Owen *et al.,* 1986b; Walker *et al.,* 1988; Gebert and Bartels, 1995). In contrast to the endocytosis of soluble antigens, the adherence of particulate tracers induces a reorganization of the cytoskeleton in the apical cytoplasm of the M cell. Actin has been reported as accumulating at sites where bacteria attach to dome epithelial cells (Jepson *et al.,* 1993a; Clark *et al.,* 1994b), a process similar to that described for the adherence of several bacteria to epithelial cells (Knutton *et al.,* 1987, 1989). Studies on the invasion of *Salmonella typhimurium* suggest a specific interaction of the bacteria with the M cell which reacts unspecifically, resulting in classic phagocytosis (Francis *et al.,* 1993; Jones *et al.,* 1994). It remains to be established whether the function of other cytoskeletal elements like intermediate filaments or microtubules is

also related to the transcytosis of soluble and to the phagocytosis of particulate antigens in M cells.

Endosomes of ordinary enterocytes and various other cells fuse with lysosomes, resulting in enzymatic degradation of ingested material (Blok *et al.*, 1981). Quantitative experiments with rat Peyer's patches revealed a drastically reduced activity of the lysosomal enzyme acid phosphatase in the M cells compared with neighboring enterocytes, and a 16-fold reduction in the volume fraction of the lysosomes in M cells (Owen *et al.*, 1986a). This suggests that, in M cells, the lysosomal compartment is bypassed and antigens are transcytosed without enzymatic degradation. Recently, Allan *et al.* (1993) detected acid phosphatase-enriched prelysosome-like and lysosome-like structures in rat M cells, and the major histocompatibility complex (MHC) class II determinants in the basolateral and lysosomal membranes. Together with the presence of cathepsin E in rat and human M cells, which is a characteristic proteinase of the lysosomal compartment of antigen-presenting cells (Bennett *et al.*, 1992; Finzi *et al.*, 1993), M cells may have the capacity to process and present endocytosed antigens to adjacent intraepithelial T lymphocytes (Allan *et al.*, 1993). However, it remains to be established whether M cells process and present antigens *in vivo*, and whether their capacity to do so exceeds that of ordinary enterocytes to present antigens by MHC class II molecules (Cerf-Bensussan *et al.*, 1984; Bland and Warren, 1986).

Although endocytosis at the apical membrane of M cells should continuously reduce the area of the apical membrane, little is known about membrane recycling from the basolateral membrane or from the endosomal compartment to the apical membrane. Bockman and Stevens (1977) injected HRP intravenously and observed that it was transported in the dome epithelium from the intercellular spaces to the lumen. Although it cannot be excluded that the HRP might have been secreted by the liver and taken up from the lumen (Lupetti and Dolfi, 1980), this bidirectional transport suggests a membrane recycling from the basolateral membrane to the apical membrane of M cells. In addition, by this mechanism antibodies and antibody–antigen complexes could be secreted by the dome epithelial cells to the gut lumen without the poly-Ig receptor.

4. Role of the Cytoskeleton

The elements and the composition of the cytoskeleton of eukaryotic cells are related to type, shape, and mechanical properties of the cell; and to the transport and positioning of vesicles and other organelles within the cell (Mooseker, 1985; Kirschner and Weber, 1989). M cells differ from ordinary enterocytes in both structure and transport function, suggesting that the cytoskeleton could also be involved in or even induce these charac-

teristics. Of the three systems in the cytoskeleton, i.e., microfilaments, intermediate filaments, and microtubules, the intermediate filaments of M cells have been studied best.

The epithelial origin and nature of M cells is confirmed by the presence of cytokeratins in the M cells of various species, including rats, guinea pigs, rabbits, pigs, cats, and humans (Gebert *et al.,* 1992; Jepson *et al.,* 1992; Farstad *et al.,* 1994; A. Gebert, unpublished observations). Similar to ordinary enterocytes, M cells of the rabbit small and large intestine contain the cytokeratins 8, 18, and 19, while cytokeratins characteristic for squamous epithelia are absent (Gebert *et al.,* 1992). In rabbit M cells, cytokeratins form a "skeletal disk" in the attenuated apical cytoplasm, which could help stabilize this fragile formation (Gebert *et al.,* 1992).

Distinct cytoskeletal peculiarities in the composition of the intermediate filaments have been described for rabbit and porcine GALT. Vimentin, the intermediate filament typical for mesenchymal cells, is present in rabbit M cells in addition to cytokeratins (Gebert *et al.,* 1992; Jepson *et al.,* 1992; Fig. 6). The vimentin is expressed at birth and can be used as a simple cytoplasmic marker for rabbit M cells (see Section III,B,5). Although the presence of vimentin in all rabbit M cells, including immature M cells lying at the mouth of the crypts (Fig. 8), suggests an essential role for vimentin, the specific functions in rabbit M cells remain to be established. No vimentin was detectable in dome epithelial cells of rats, guinea pigs, pigs, cats, or humans (Gebert *et al.,*1992; Jepson et al., 1992; A. Gebert, unpublished observations). The intermediate filament protein cytokeratin 18 is strongly expressed in M cells of porcine Peyer's patches (Gebert *et al.,* 1994). The cytokeratin 18 filaments can be used as a simple light microscopic marker in porcine M cells, since the filaments are easily detectable in conventional sections by immunohistochemistry, and since the marker correlates highly with the transcytotic function of the cells (Gebert *et al.,* 1994).

Little is known about the arrangement and composition of microfilaments and microtubules in M cells. The terminal web, normally composed of actin, actin-associated proteins and myosin, is less developed in M cells of the small intenstine (Owen and Jones, 1974; Bye *et al.,* 1984). This could be related to the irregular shape of the microvilli, which originate with their core rootlets in the terminal web. In addition, a less developed terminal web could facilitate vesicular trafficking from the apical to the basolateral membrane. However, in the large intestine, some M cells have a well-developed terminal web (Morfitt and Pohlenz, 1989; Gebert and Bartels, 1995). A submembranous aggregation of microfilaments was described at the adhesion sites of several bacteria to the apical membrane of M cells (Jepson *et al.,* 1993a; Clark *et al.,* 1994b). This suggests that actin filaments are involved and reorganized when large, particulate antigens are phagocytosed by M cells. Uptake experiments by Kracke and Bartels (1994) with

native ferritin showed that colchicine inhibits the endocytotic uptake of this soluble tracer, indicating that the microtubule system is involved in the fluid-phase transcytosis in M cells. In Ussing chamber experiments, Keljo and Hamilton (1983) found no effect of colchicine on the transepithelial transport of the fluid-phase marker HRP within 90 min, but stated that colchicine requires considerable preincubation before its effect on the formation of microtubules can be demonstrated.

V. Other Cells of the Dome Epithelium

The dome epithelium of most species is composed of enterocytes and M cells, and goblet cells are rare or completely lacking (Bhalla and Owen, 1982). A few brush cells (tuft cells, caveolated cells) are present in the dome epithelium of mice and rat Peyer's patches (Owen, 1977; Rosen *et al.,* 1981), but are absent from that of rabbits (A. Gebert, unpublished observations). It has been suggested that brush cells might serve as chemo- or volume receptors (Luciano and Reale, 1990; Kugler *et al.,* 1994), but their specific function in the dome epithelium is unknown. Enteroendocrine cells are normal constituents of the crypts and villi of the small intestine (Grube, 1986). According to a morphological study (Owen and Jones, 1974), these cells seem to be absent from the dome epithelia of mice, but no immunohistochemical studies have yet confirmed this. Factors and mechanisms that induce and maintain the unique cellular composition of the dome epithelium seem to be of general significance for understanding immunological functions, but they are still poorly defined (see Section III,C).

A. Role of Dome Epithelial Enterocytes

In various species and locations, the M cells comprise less than one third of the dome epithelial cells and are interspersed among a continuous layer of enterocytes. The two cell types form an irregular patchwork when viewed from the lumen (Owen and Nemanic, 1978; Smith and Peacock, 1980; Bockman, 1983). Normally M cells border on enterocytes and direct contact between two M cells is rarely found (Owen and Jones, 1974; Bockman and Boydston, 1982; Clark *et al.,* 1993; Gebert and Hach, 1993; Jepson *et al.,* 1993d). In some cases, the reverse situation can be seen with a confluent layer of M cells and single, interspersed enterocytes (Fig. 9). Since patchworks of the two epithelial cell types prevail in various species and locations, this arrangement seems to be important for structure and/or function, and

suggests that the enterocytes are necessary elements of the dome epithelium. The enterocytes might help stabilize the M cells and their thin cytoplasmic rims at the apical and basolateral membrane. This assumption is supported by the presence of numerous desmosomes connecting the basolateral membranes of M cells with those of enterocytes (Owen and Jones, 1974; Chu *et al.,* 1979; Gebert and Bartels, 1991; Figs. 10 and 12).

Several morphological and histochemical observations show that the enterocytes of the dome epithelium differ significantly from those of ordinary crypts and villi. The dome epithelial enterocytes of various species have substantially shorter microvilli than those of the villus epithelium (Madara *et al.,* 1984; own observations). Nevertheless, the density of intramembranous particles in the apical membrane (probably representing integral membrane proteins) is comparable between dome and villus enterocytes (Madara *et al.,* 1984; Gebert and Bartels, 1991).

The activity of digestive enzymes in the brush border varies, depending on the position of the individual enterocyte along the crypt-villus axis (Nordström *et al.,* 1967; Smith, 1985). Besides this process of maturation, Smith (1985) demonstrated that dome epithelial enterocytes express lower levels of lactase and α-glucosidase, but higher levels of alkaline phosphatase than villus enterocytes. In addition, the dome epithelial enterocytes are much less able to absorb valine than enterocytes of adjacent villi (Smith and Syme, 1982).

Lectin histochemistry revealed that, in the rabbit GALT, the dome and villus enterocytes differ significantly in the composition of terminal saccharides in the glycocalyx. In both rabbit Peyer's patch and appendix, the dome epithelial enterocytes were shown to express galactose in their brush border, while those outside the dome expressed only low levels of this saccharide (Gebert and Hach, 1993; Jepson *et al.,* 1995). The absence of the poly-Ig receptor in the dome epithelium (Bjerke and Brandtzaeg, 1988; Pappo and Owen, 1988) indicates that the dome epithelial enterocytes differ from their siblings on the villi not only in the composition of the apical membrane proteins but also in that of the basolateral membrane proteins.

The peculiarities of dome epithelial enterocytes described above suggest that these cells have a reduced absorptive function, with the exception of the well-expressed alkaline phosphatase activity. The exact role of these enterocytes in the immunological function of the dome epithelium, however, remains to be clarified.

B. Composition and Function of Nonepithelial Cells

1. Role of Macrophages

The accessory cells for immune responses, often also called antigen-presenting cells, can be subdivided into macrophages and dendritic cells.

These cells play a critical role not only in direct cell–cell contact with lymphoid cells, but also in secreting a number of cytokines and chemokines (new ones are still being discovered) in a paracrine-type regulation of immune reactions (Fiocchi *et al.,* 1994). The macrophages, which can be identified by enzyme histochemistry and antibodies against surface antigens, phagocytose particulate material and cell debris, and consist of heterogeneous cell populations (LeFevre *et al.,* 1979; Rutherford *et al.,* 1993; Harvey and Jones, 1991). The dendritic cells are characterized by extended cell processes, called dendrites, by strong expression of MHC class II antigens, and by specific surface antigens (Steinman, 1991; Panja and Mayer, 1994).

Macrophages have been identified in different compartments of Peyer's patches and their heterogeneity in the gut wall has been documented. However, no specific functions could be attributed to the subpopulations of macrophages in different areas of Peyer's patches. Macrophages were identified by their acid phosphatase activity in the subepithelial layer of the dome of rat Peyer's patches, but these macrophages were less intensely stained than those in germinal centers (Sminia *et al.,* 1983). The heterogeneity of macrophages was shown by using monoclonal antibodies and enzyme histochemistry in rats (Dijkstra *et al.,* 1985; Sminia and van der Ende, 1991). In mice, the monoclonal antibody Mac1 identified macrophages in the dome epithelium, while none were seen in the villus epithelium. The same monoclonal antibody was used to show macrophages in the dome and it clearly demarcated this region from the follicle (Ermak and Owen, 1986). Heterogeneous macrophage populations were also identified in human Peyer's patches by detection of acid phosphatase activity and monoclonal antibodies: some subsets were abundant in the dome and others were restricted to the lamina propria of villi (Mahida *et al.,* 1989). The expression of the myelomonocytic antigen (CD68) and the presence of calprotectin in human material were also used to characterize macrophages. Macrophages expressing only CD68 were preferentially found directly underneath the dome epithelium but not in the villus epithelium (Bjerke *et al.,* 1993). Jarry *et al.* (1989) used EM and immuno-EM in rats and found a frequency of $6 \pm 2\%$ macrophages in the cells of the dome epithelium while no intraepithelial macrophages were found in the villus epithelium. These macrophages were close to M cells or to lymphocytes that were associated with M cells.

The highly potent antigen-presenting dendritic cells were identified in the rat gut wall by their strong expression of MHC class II antigen (Wilders *et al.,* 1983). They were localized in and beneath the dome epithelium in close contact to M cells. The authors postulated that these dendritic cells migrate from the epithelium to the interfollicular area and via lymphatics to mesenteric lymph nodes. In a recent study, up to 15 times more dendritic cells were found in the gut lymph of rats 10 hr after an intravenous injection of endotoxin. The number of dentritic cells in the lamina propria was

decreased while the frequency of dendritic cells in Peyer's patches was unaffected (MacPherson *et al.,* 1995). This observation suggests antigen presentation in mesenteric lymph nodes after antigen uptake in villi but local initiation of an immune response in Peyer's patches. Spencer *et al.* (1986) showed cells with S100 protein-positive, long dendrites in the dome of human material. In other studies, no distinct population of dendritic cells could be detected by monoclonal antibodies in the subepithelial region of human Peyer's patches (Mahida *et al.,* 1989).

In contrast to the villus epithelium, the dome epithelium contains "professional" antigen-presenting cells that can also produce many cytokines to influence lymphoid cells in the M-cell pockets. Many more experiments are needed, combining the uptake of particulate material by Peyer's patches with surface markers, to identify in detail the macrophage and dendritic cell subsets that play a role in phagocytosis and presentation of the different types of antigens in the epithelium or subepithelial compartment.

2. Role of Lymphocytes

Specific lymphocyte subsets are essential for various immune reactions and have to lie in close vicinity. Therefore, it is important to know the subset composition of lymphocytes in the Peyer's patch dome epithelium. Here, per 100 μm of epithelium, 3.6 lymphocytes were seen in specific pathogen-free mice and 11.3 lymphocytes in conventional mice (Rell *et al.,* 1987), indicating the stimulating effect of microbial antigens. In adult rats, two times more lymphocytes were found in the epithelium of the domes than in that of the villi (Jarry *et al.,* 1989). In humans, an even higher difference was documented, but with a greater interindividual variation, e.g., a median of 3.0 lymphocytes (range 2.1–5.3) per unit area for the dome epithelium in contrast to 1.2 (range 1.0–1.6) for the villus epithelium (Bjerke *et al.,* 1988). In rats, the lymphoid cells associated with M cells were distributed as follows: 15 $\pm$ 3% small lymphocytes, 24 $\pm$ 3% medium lymphocytes, 16 $\pm$ 4% immunoblasts, and 5 $\pm$ 1% plasma cells. There are obviously more plasma cells and activated lymphocytes in the dome epithelium than in the villus epithelium, indicating B-cell differentiation in the M-cell pockets (Jarry *et al.,* 1989). Lymphocytes identified between the enterocytes of the dome epithelium often contain intracytoplasmic granules, as described for intraepithelial cytotoxic lymphocytes of the villi. However, lymphocytes in the pockets of M cells lack such granules (Jarry *et al.,* 1989).

As described in Section II,B,1, the intraepithelial lymphocytes of ordinary villi are predominantly T cells. In the pockets of M cells, however, equal numbers of T and B lymphocytes were documented. Most B lymphocytes expressed IgM on their surface and often coexpressed IgD. This combination of surface markers is comparable to B cells in follicle centers ($sIgD^+$,

sIgM$^+$) and to those in the marginal zone of the spleen (sIgD$^-$, sIgM$^+$) (Farstad *et al.*, 1994). In this study, in which Peyer's patches from 10 patients were studied, wide variations were documented for all markers, e.g., a median of IgM$^+$, IgD$^+$ 34% with a range of 6–60%. The reason for this variability is not known. In the same study, cells with cytoplasmic IgA and IgM were occasionally found while in a different study (Spencer *et al.*, 1986) no cells with cytoplasmic IgA were identified in the dome epithelium. The presence of IgA-producing plasma cells in the dome epithelium is surprising because the poly-Ig receptor for the transport of IgA into the lumen is absent here (Bjerke and Brandtzaeg, 1988; Pappo and Owen, 1988). The ratio of T-helper to T-cytotoxic lymphocytes is 0.6:10 in the villus, but 4:10 in the dome epithelium, which indicates a much higher frequency of T-helper cells in the dome epithelium (Bjerke *et al.*, 1988).

In a more recent study by the same group, nearly three quarters of all T cells in the dome epithelium were found to be T-helper cells (CD4$^+$; range 40–90%) (Farstad *et al.*, 1994). A similar pattern of T-helper and B lymphocytes was found in the dome epithelium of mice (Ermak and Owen, 1986). The T lymphocytes of the dome epithelium generally have the phenotype of memory T lymphocytes (Farstad *et al.*, 1994). T lymphocytes with the γ/δ T-cell receptor could not be identified near M cells while in the dome epithelium without contact with M cells, 4.4% (range 0.5–31%) of the lymphocytes expressed the γ/δ T-cell receptor (Farstad *et al.*, 1993).

Little is known about the kinetics of dome epithelial lymphocytes. Some T and B lymphocytes (range 1–10%) expressed the proliferation marker Ki67 (Farstad *et al.*, 1994), which indicates some local proliferation. In mice, splenic T and B lymphocytes were labeled with [^{3}H]adenosine and their immigration and localization in the Peyer's patch tissue compartments was studied by light microscopic and electron microscopic (EM) autoradiography (Bhalla and Owen, 1983). At 17 and 68 hr after cell transfer, the frequency of T and B cells associated with M cells did not differ, but at 40 hr about four times more B than T cells were found. The absolute numbers, however, were rather low: ~1% of the B cells were labeled at 40 hr, but this is still higher than in the villus epithelium. These data indicate a continuous but low influx of T and B lymphocytes into the dome epithelium. It is unknown whether lymphocytes return from the dome epithelium to the underlying lymphoid tissue. With regard to rare EM findings of lymphocytes migrating from the dome epithelium into the gut lumen (Heatley and Bienenstock, 1982; Owen and Heyworth, 1985; Regoli *et al.*, 1994), it is possible that this route might be taken by dome epithelial lymphocytes. However, adhesion molecules on lymphocytes and/or on dome epithelium which could regulate the influx and efflux of lymphocytes have not yet been investigated.

Several of the above experiments need to be taken with reservation, as the M cells were defined by the reduced expression of alkaline phosphatase,

or in some cases clustered lymphocytes in the dome epithelium were assumed to represent M-cell pockets. Only few data were obtained by EM techniques, clearly demonstrating M cells. The following aspects remain to be elucidated: the functional relevance of the higher frequency of lymphocytes in the dome epithelium, of the higher ratio of T-helper to T-cytotoxic cells, and of the presence of B lymphocytes and plasma cells in this compartment. It can be assumed from the available data that a unique microenvironment might exist within this part of the gut epithelium with all the cellular elements needed to initiate an immune reaction. The high variability among different individuals might reflect a varying degree and/or mode of stimulation.

VI. M Cells at Locations Outside the Gut

Lymphoid tissue is found along the entire gastrointestinal tract, including the stomach, small intestine, cecum, appendix, colon, rectum and, in birds, the bursa of Fabricius. In addition, mucosa-associated lymphoid tissue occurs in the oropharynx, larynx, upper and lower airways, conjunctiva of the eye, and minor salivary gland ducts. The skin and urogenital tract, however, lack organized lymphoid tissue (Streilein, 1983; Morris *et al.*, 1986). Thus care should be taken when applying the concept of mucosa-associated lymphoid tissues to all inner and outer surfaces.

A. Tonsils

The palatine, pharyngeal, lingual, and tubal tonsils form a circular band of lymphoid tissue in the oro- and nasopharynx of primates called Waldeyer's ring. The epithelia covering the tonsils are interspersed with lymphoid cells. While palatine and lingual tonsils are covered by a squamous stratified epithelium, the pharyngeal and tubal tonsils are covered by a respiratory epithelium (Winther and Innes, 1994). At sites where the submucosal lymphoid tissue of the tonsils comes into contact with the oral cavity, e.g., the crypts of the palatine tonsils, the epithelium is heavily infiltrated by lymphoid cells and its cellular composition is modified. There are indications that these epithelia contain specialized epithelial cells with the function and, to a certain extent, the morphology of intestinal M cells.

Most studies have focused on the palatine tonsils because of their accessibility. Using scanning electron microscopy (SEM), a subpopulation of epithelial cells characterized by microvilli instead of microplicae was found in the crypt epithelium of the rabbit palatine tonsil (Oláh and Everett, 1975;

Gebert, 1995). Similar cells covered with irregular microfolds have recently been described in the palatine tonsil of the dog (Belz and Heath, 1995). Experiments with India ink and horseradish peroxidase in pigs and rabbits revealed that the lymphocyte-rich crypt epithelium but not the ordinary surface epithelium takes up these tracers (Oláh *et al.*, 1972; Williams and Rowland, 1972; Gebert, 1995). Transmission electron microscopy showed that tracer uptake occurs in the microvilli-covered cells of the crypts (Gebert, 1995). In addition, the presence of the rabbit M-cell marker vimentin (Gebert, 1995; Gebert *et al.*, 1995), the close contact with intraepithelial lymphocytes, and the membranous shape of the apical cytoplasm of these cells are all indications that these are indeed M cells.

Although Howie (1980) described similar cells in the human palatine tonsil using SEM, several other SEM and thin-section studies failed to detect a separate epithelial cell type in humans (Owen and Nemanic, 1978; Nair and Rossinsky, 1985; Perry *et al.*, 1988; Perry, 1994). Therefore, it is not clear at present whether cells with the function and/or structure of intestinal M cells generally exist in the tonsil epithelium.

B. BALT

In contrast to Peyer's patches, appendix, and tonsils, the frequency of bronchus-associated lymphoid tissue (BALT) is variable and largely species-dependent. The development of BALT depends on microbial stimulation and can be induced by vaccinations or infections (Iwata and Sato, 1991; Delventhal *et al.*, 1992b). While BALT is found in almost all rabbits and in most rats, it is found only rarely in healthy adult humans (Pabst and Gehrke, 1990; Pabst, 1992; Gould and Isaacson, 1993). Differences are also seen in the composition of the lymphoid tissue: in some species no typical lymphoid follicles are formed and no definite compartments of T and B lymphocytes are detectable (Sminia *et al.*, 1989).

The epithelium that overlies the lymphoid aggregations is called lymphoepithelium and it differs in several aspects from the normal respiratory epithelium. Goblet cells are rare, the number of ciliated cells is reduced, but large numbers of lymphocytes and macrophages lie among the epithelial cells (Bienenstock *et al.*, 1973; Rácz *et al.*, 1977). As in Peyer's patches, BALT lymphoepithelium lacks the poly-Ig receptor (Gehrke and Pabst, 1990). In rabbit BALT there is some evidence that a separate type of epithelial cell resembling intestinal M cells occurs in the lymphoepithelium. These cells possess irregular microvilli, are found close to intraepithelial lymphocytes, and take up intraluminally administered horseradish peroxidase more avidly than the remaining cells of the bronchial epithelium (Myrvik *et al.*, 1979; Tenner-Rácz *et al.*, 1979). Since peroxidase preferen-

tially adheres to the apical membrane of these cells, it is likely that their glycocalyx is modified (Rácz *et al.*, 1977). The presence of rabbit M-cell markers in the rabbit BALT lymphoepithelium (Roy *et al.*, 1987; Gebert and Hach, 1992) supports the concept of M-cell-mediated antigen uptake here.

The BALT lymphoepithelium of rats also takes up macromolecular tracers and virus particles more avidly than the ordinary respiratory epithelium (Fournier *et al.*, 1977; Gregson *et al.*, 1982; Morin *et al.*, 1994). Although the morphology and cellular composition of the lymphoepithelium bears some resemblance to that found in rabbits (Chamberlain *et al.*, 1973; van der Brugge-Gamelkoorn *et al.*, 1986), there is no clear evidence that a separate cell type exists in the rat BALT which could mediate the uptake of aerogenic antigens (Fournier *et al.*, 1977; Gregson *et al.*, 1982). Since the airway epithelium is much more permeable for foreign soluble and particulate substances than the gut epithelium (Richardson *et al.*, 1976; Ito *et al.*, 1992), it might be that antigen sampling by specialized epithelial cells is not necessary in the airways, or that dendritic cells take on this task in lung immune reactions (Delventhal *et al.*, 1992a; Pabst and Tschernig, 1995).

C. NALT/DALT/CALT

Lymphoid tissue underneath the respiratory epithelium of the nasal cavity has been termed nasal-associated lymphoid tissue (NALT). In the rat, NALT is situated on both sides of the septal opening to the pharyngeal duct (Spit *et al.*, 1989). Similar aggregations of lymphoid tissue have also been observed in mice, hamsters, rabbits, and monkeys (Loo and Chin, 1974; Harkema *et al.*, 1987; Kuper *et al.*, 1992; own observations). The lymphoepithelium covering NALT resembles that of the BALT with its reduced numbers of goblet and ciliated cells, and numerous intraepithelial B and T lymphocytes and macrophages (Spit *et al.*, 1989; Kuper *et al.*, 1990). It has been reported that the nonciliated cells in the lymphoepithelium overlying rat NALT are morphologically modified (Spit *et al.*, 1989) and take up horseradish peroxidase–gold complex particles (Kuper *et al.*, 1992). Further studies are needed to elucidate whether these cells represent a separate epithelial cell type similar to the M cells of the GALT.

So-called duct-associated lymphoid tissue (DALT) has been described in the wall of minor salivary gland ducts of primates (Schroeder *et al.*, 1983; Nair and Schroeder, 1986). DALT is composed of follicular and parafollicular areas that are covered by a lymphocyte-rich cuboidal epithelium (Nair and Schroeder, 1985). Bacteria in the lumen of the minor salivary duct as well as tracer experiments with a mixture of HRP, ferritin, and India ink suggest that DALT normally comes into contact with antigens of the oral cavity (Nair and Schroeder, 1983, 1985). Further experiments

are needed to find out whether uptake of antigens and initiation of immune responses are performed by DALT, and whether DALT is also present and plays an immunological role in species other than monkeys.

Submucosal lymphoid tissue is a normal constituent of the rabbit conjunctival epithelium, concentrated at the opening of the nasolacrimal duct and termed conjunctiva-associated lymphoid tissue (CALT) (Chandler and Axelrod, 1980). Similar aggregations of lymphoid tissue are normally present in guinea pigs (Stock *et al.,* 1987; Latkovic, 1989), but are only found in one third of healthy humans (Wotherspoon *et al.,* 1994). Immunological functions analogous to those of the Peyer's patches have been postulated for CALT (Chandler and Axelrod, 1980; Franklin and Remus, 1984). Stock *et al.* (1987), however, found no selective uptake of horseradish peroxidase by the lymphoepithelium covering the CALT of guinea pigs. Using scanning, thin-section, and freeze-fracture electron microscopy and vimentin immunohistochemistry, we were unable to define a distinct population of epithelial cells in rabbit CALT as conjunctival M cells (A. Gebert, unpublished data).

VII. Clinical Relevance and Perspectives

Detailed knowledge of M cells and their functions is essential for understanding intestinal infections and immune responses to enteric pathogens. The main function of M cells, i.e., the transport of antigens from the gut lumen to the lymphoid tissue, might be exploited for oral vaccinations and oral drug delivery in the future. Some oral vaccinations, e.g., against poliomyelitis and typhoid fever, have already been used successfully for several years (Levine *et al.,* 1987; Zhaori *et al.,* 1988), but it is still unknown whether M cells play a central role in the uptake of these vaccines. Several other bacterial and viral antigens have been applied enterically and it was thought that the M cells might play a role in the protocols but this has not been proven so far. Furthermore, little is known about the relevance of M cells in intestinal infections, oral vaccinations, and the induction of intestinal immune responses.

A. M Cells as Targets for Enteropathogenic Microorganisms

Several studies in which pathogenic bacteria were instilled into the gut lumen support the hypothesis that the M cells in the dome epithelium of Peyer's patches are a primary entry site for host invasion (Walker *et al.,* 1988; Wassef *et al.,* 1989; Jones *et al.,* 1994; see Section IV,B,1). However,

the intestinal surface area not covered by M cells is many times larger than that of the dome epithelium and therefore might also be of quantitative importance. Alternative entry sites for pathogens in these nondome regions might be the tips of the villi where epithelial cells are sloughed off (see Madara, 1990), the crypt regions where the paracellular barrier is incomplete (Madara *et al.*, 1980), and small lesions of the mucosa caused by sharp-edged particles in the gut content (compare Moore *et al.*, 1989). Therefore, quantitative data are needed to compare the total amount of antigen taken up in the domes with that taken up in normal villi and crypts. However, the number of lymphoid cells in the domes by far exceeds that in normal villi and crypts, suggesting that the generation of a specific immune response is accelerated in the organized lymphoid tissue. Thus, uptake of antigens and pathogens in the gut is not restricted to the GALT, and the quantitative relevance of GALT versus ordinary gut epithelium remains to be established.

The mechanisms and pathways of microorganisms used to sustain the infection after entering the M cells vary and depend on the specific pathogen. After migration across the dome epithelium, *Salmonella* spp. start to colonize the subepithelial lymphoid tissue and cause severe typhoid fever or enteritis (Carter and Collins, 1974; Hohmann *et al.*, 1978). In contrast, *Shigella flexneri* and also *Listeria monocytogenes* are taken up by M cells and probably spread along the epithelial barrier across the basolateral membranes of M cells and enterocytes (Makino *et al.*, 1986; Vasselon *et al.*, 1991; Perdomo *et al.*, 1994). Other bacteria, e.g., several *E. coli* strains, colonize the epithelium without destroying M cells (Inman and Cantey, 1983).

Pathogenic bacteria damage the host tissue not only by invasion but also by the production of enterotoxins. The toxin produced by *Vibrio cholerae* specifically binds via the GM1-monosialoganglioside to enterocytes and induces increased fluid transport into the gut lumen (Holmgren *et al.*, 1975; Ljungström *et al.*, 1980). It was demonstrated that the cholera toxin-binding subunit is taken up by M cells (Owen *et al.*, 1986b), but it is not known whether this is of significance for intestinal infection with *Vibrio cholerae.* The heat-labile enterotoxin of *E. coli* also binds to the brush border of enterocytes and is transcytosed to the basolateral side (Lindner *et al.*, 1994). More of this enterotoxin was detected in the epithelium of domes than in that of villi (Lindner *et al.*, 1994), but whether this uptake is performed by M cells is yet unknown.

B. M Cells as Potential Entry Sites for Oral Vaccines

M cells are involved in the uptake and transport not only of pathogenic microorganisms during infections but probably also of vaccines. Although

various oral vaccination protocols have been used in clinical experiments, little is known about the role of M cells in these immunizations. It is assumed that the vaccine is taken up by M cells, but experimental evidence is rare. Many details not relating to M-cell function, but of special interest for oral vaccinations, have recently been reviewed (McGhee *et al.*, 1992; Shalaby, 1995).

Vaccines for oral application need to combine several features to obtain maximum effects in initiating systemic immune responses. The passage of the vaccine through the acidic milieu of the stomach can be achieved without adverse effects by using enteric-coated capsules (Levine *et al.*, 1987). Particulate substances are preferable to soluble material in oral vaccinations since the former more often induce immunity, whereas the latter more often induce immune tolerance (Clements *et al.*, 1988; O'Hagan *et al.*, 1991; Mowat *et al.*, 1993). Since mobile strains of *S. typhimurium* were more efficient in infecting the gut wall, mobile candidate vaccines probably have a better chance of reaching the epithelium (Jones *et al.*, 1992). Repeated application in multiple doses also results in a more effective induction of immunity (Levine *et al.*, 1987; Ferreccio *et al.*, 1989).

Several oral vaccination protocols have been used during the past few decades in practical medicine, but only those against poliomyelitis and typhoid fever are discussed here. The different methods used for these immunizations have been reviewed in detail (Salk, 1980; Ivanoff *et al.*, 1994). Both live and inactivated polio viruses induce the production of neutralizing antibodies (Ogra and Faden, 1986; Zhaori *et al.*, 1988), but the mechanisms that lead to this immunity are not understood yet. At least polio virus type 1 (Sabin strain) is taken up by M cells, as demonstrated *in vitro* by Sicinski *et al.* (1990). However, whether the uptake of attenuated or wild polio virus strains during vaccination and infection is restricted to M cells or also occurs in regions outside the GALT is still unclear.

Attenuated strains, which are unable to develop within the host, are used for *Salmonella* vaccinations. Most of these strains have mutations in their metabolic regulation, e.g., defects in the cell wall lipopolysaccharides in *Salmonella typhi* Ty21a (Germanier and Fürer, 1975). Since *Salmonella* spp. are transported across the dome epithelium by M cells (Kohbata *et al.*, 1986; Clark *et al.*, 1994b; Jones *et al.*, 1994), it might be expected that strains used for oral vaccinations against typhoid fever would also take this route. Current protocols for oral vaccination against typhoid fever preferentially use the Ty21a mutant, and have an efficiency of up to 75% according to clinical trials (Ivanoff *et al.*, 1994). High efficacy is obtained by a single dose of vaccine containing about 3×10^9 attenuated bacteria in enteric-coated capsules and can be increased by four such doses (Levine *et al.*, 1987; Ferreccio *et al.*, 1989). Intervals of 1 day between the individual doses are more efficient than intervals of 14 days (Levine *et al.*, 1987).

Many aspects of a successful oral vaccination, e.g., dose, single or repetitive application, and time intervals, have been arrived at by trial and error and not because the pathomechanisms are understood.

In addition to the vaccinations against typhoid fever described above, further attempts have been made to improve oral vaccinations during the past decade. Attenuated salmonella strains with genetically engineered mutations, e.g., in the biosynthesis of aromatic metabolites, have been used as carriers in experimental models (Tacket *et al.*, 1992). Cloned vectors have been introduced into these carrier strains, e.g., various toxins and surface antigens of bacteria and viruses (Guzmán *et al.*, 1991; Walker *et al.*, 1992). Little is known about the interactions of these so-called "construct vaccines" with the gut wall and its lymphoid tissue. It is assumed (but has not yet been demonstrated) that these carriers enter the gut wall via M cells in a way comparable to that shown for wild strains of *Salmonella* (Kohbata *et al.*, 1986; Clark *et al.*, 1994b; Jones *et al.*, 1994). The processes that regulate whether immunity, oral tolerance, or even infection of the host is generated by the immune system are still unknown.

Various antigen-delivering systems have been developed and tested for oral vaccination protocols. These artificial antigen carriers release the vaccine within the lymphoid tissue over a long period, resulting in prolonged stimulation compared with the application of a single dose of pure antigen (Eldridge *et al.*, 1991b; Maloy *et al.*, 1994). Using antigen-delivering systems, it is even possible to obtain intestinal immunity against macromolecules that normally induce oral tolerance (O'Hagan *et al.*, 1989; Mowat *et al.*, 1993).

Liposomes (phospholipid-artificial membrane vesicles) have been used to induce mucosal and systemic immunity against streptococci (Gregory *et al.*, 1986; Wachsmann *et al.*, 1986), and it has been demonstrated that such liposomes are taken up by M cells (Childers *et al.*, 1990). Feeding ovalbumin normally results in oral tolerance. After application of lipophilic immune-stimulating complexes (ISCOMS) containing ovalbumin, antiovalbumin antibodies were detected in the gut (Mowat *et al.*, 1993). However, the entry site of such ISCOMS, e.g., via M cells, has not yet been studied. Polyacrylamide microparticles and poly(D-L-lactic coglycolic acid) (PGLA) microspheres have been used as antigen-delivering carriers (O'Hagan *et al.*, 1989; 1991; Eldridge *et al.*, 1991a). Using light microscopy, PGLA microspheres were detected in murine Peyer's patches 24 hr after intraluminal application and were found deep in the lymphoid tissue, mesenteric lymph nodes, and spleen (Eldrige *et al.*, 1991a). In another study using rabbits, PGLA microspheres were instilled into intestinal loops containing Peyer's patches; 7% were found in the subepithelial area of the dome 45 min after application, suggesting a rapid transport by the dome epithelium (Jepson *et al.*, 1993e). The uptake of such microspheres is performed by M cells, as demonstrated by electron microscopy (Ermak *et al.*, 1995). PGLA micro-

spheres were used to encapsulate ovalbumin and staphylococcal enterotoxin B toxoid (Eldridge *et al.*, 1991b; Maloy *et al.*, 1994). The production of antigen-specific antibodies was induced in both studies, whereas cytotoxic immune responses were detected in only one of the models (Maloy *et al.*, 1994). It has to be determined whether encapsulation of the macromolecules is necessary for effective presentation by macrophages in the dome area (Mahida *et al.*, 1989; Soesatyo *et al.*, 1990).

Intestinal vaccinations can be improved by substances that provide adjuvant function, e.g., cholera toxin or heat-labile *E. coli* toxin (Clements *et al.*, 1988; Vajdy and Lycke, 1992). It is not known how these enterotoxins enhance the intestinal immune response or whether M cells are involved. With cholera toxin as an adjuvant, the use of soluble antigens that normally induce oral tolerance (e.g., ovalbumin) resulted in the induction of immunity (Clements *et al.*, 1988; Vajdy and Lycke, 1992). Further research is needed to understand the role of M cells in intestinal immune responses under the adjuvant function of cholera toxin or heat-labile *E. coli* toxin.

C. M Cells and the Induction of Intestinal Immune Responses

Antigen uptake by intestinal mucosa is not restricted to M cells but also occurs in the ordinary epithelium outside the GALT. Macromolecules such as ovalbumin, horseradish peroxidase, or albumin (molecular weights 40–69 kDa) permeate the epithelium paracellularly (Ma *et al.*, 1992). Keljo and Hamilton (1983) demonstrated that horseradish peroxidase was transported across normal intestinal epithelium. However, the transport capacity of the Peyer's patches was threefold higher than that of the ordinary epithelium, suggesting a much more effective transport by M cells than by other gut epithelial cells or along the paracellular route. So far it is not known whether the less efficient uptake of macromolecules by the ordinary gut epithelium plays a specific role in the development of intestinal immune responses in addition to antigen uptake by M cells.

Antigens taken up by the gut epithelium induce either specific immunity or tolerance. Specific immunity comprises cellular immunity and/or the production of secretory immunoglobulins (Brandtzaeg *et al.*, 1989). When tolerance has developed, the immune system does not react to the antigens present in the gut on subsequent exposure (Elson, 1985). A switch from tolerance induction to the induction of an immune response has also been achieved by adjuvants like cholera toxin or heat-labile *E. coli* toxin, or by encapsulation of the antigen in copolymer microspheres (Clements *et al.*, 1988; Eldridge *et al.*, 1991a,b; Vajdy and Lycke, 1992; Maloy *et al.*, 1994).

The mechanisms that determine whether immune response or tolerance is developed remain to be elucidated, but in the future they could play a significant role in vaccinations, immune suppression, and protection against infections. M cells could influence the immunological response by interacting directly with lymphoid cells or by producing humoral factors. The latter has been shown in M-cell preparations made from the Peyer's patches of rabbits where interleukin-1, an inflammatory cytokine, was secreted after stimulation with lipopolysaccharide (Pappo and Mahlman, 1993). This result has to be confirmed, and future research has to clarify how the secretion of this cytokine or other humoral factors is integrated into the induction of an immune response in the dome area.

So far it is not known whether the number of M cells plays a role in the regulation of intestinal immunity. There is evidence that larger amounts of antigens and/or intraepithelial lymphocytes increase the number of M cells per dome (Wolf *et al.*, 1987; Savidge *et al.*, 1991). In contrast, the number of M cells in rabbit Peyer's patches and appendix decreased as a result of systemic treatment with cyclosporin-A or dexamethasone (Savidge and Smith, 1990; Roy and Walsh, 1992). A reduced number of M cells in the inflamed ileum, as reported by Cuvelier *et al.*, (1994) for patients with spondylarthropathy, suggests that M cells could also be involved in the initiation and/or maintenance of inflammatory bowel diseases. M cells may induce intestinal immune reactions by presenting antigens to lymphoid cells. There is some evidence that M cells express MHC class II molecules and are capable of presenting antigens to lymphoid cells (Allan *et al.*, 1993; see Section IV,B,3). In enterocytes, MHC class II is expressed in the apical cytoplasm and on the basolateral membrane, but is lacking on the apical membrane (Mayrhofer and Spargo, 1990). A comparable pattern of MHC class II expression in M cells would concentrate the molecules in regions where the M-cell membrane faces the immune cells in the basolateral pockets. However, many more morphological and functional studies are necessary to elucidate whether M cells are involved not only in antigen transport but also in antigen presentation.

Future research on M cells would benefit from methods to effectively isolate M cells while preserving their unique phenotype for *in vitro* studies. In such systems, their functions could easily be studied and they could be selectively stimulated or inhibited. The interrelationship of M cells with other components of the GALT might be investigated in animals that lack M cells. This could be achieved by drugs that target and subsequently destroy the M cells, or by surgical resection of the Peyer's patches, but the isolated follicles should be kept in mind. In addition, genetically modified animals with defects in their GALT structure or function could help elucidate the complex interactions of M cells with antigens on the one hand and the immune system on the other.

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pp125^{FAK} in the Focal Adhesion

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Integrins are a large superfamily of transmembrane adhesion molecules. In many types of cultured cells, integrins are concentrated in specialized sites called focal adhesions. Integrins are capable of transducing signals to the inside of the cell, which can effect cell migration, differentiation and growth, but the signaling mechanism of integrins has been obscure because their short cytoplasmic domains do not possess endogenous catalytic activity. The recent discovery of a tyrosine kinase called pp125^{FAK} (for focal adhesion kinase) has led to a proposed model in which the binding of integrins to extracellular ligands activates FAK, which then generates a tyrosine phosphorylation cascade within the cell. Data both for and against this model have been obtained, and the precise function of FAK in cultured cells and organized tissues is still not clear. However, many interesting features (its unusual molecular structure, its functional and physical association with integrins, and its potential for participating in multiple signaling pathways) suggest that FAK may play a pivotal role in conveying information from the membrane to the inside of the cell.

KEY WORDS: Phosphotyrosine, Cytoskeleton, Signal transduction, Tyrosine kinase, Integrin, Actin, Extracellular matrix.

I. Introduction

The focal adhesions of cultured cells have been recognized for decades as sites of tight structural attachment of the cell membrane to the underlying substrate. These sites have also been called "adhesion plaques" or "focal contacts," names that describe both the function and the distinctive appearance of these specialized membrane domains. The focal adhesion serves two important structural roles in the cell: attachment of the membrane to the substrate, and anchoring and stabilization of the actin cytoskeleton

(Burridge *et al.*, 1988). Furthermore, it is now recognized that the focal adhesion acts as an important site of signal transduction (Damsky and Werb, 1992; Schwartz, 1992; Juliano and Haskill, 1993; Lo and Chen, 1994; Clark and Brugge, 1995). Information that is conveyed from the outside to the inside of the cell at the focal adhesion can affect such cell behavior as migration, proliferation, programmed cell death, and differentiation. One of the continuing challenges in this area of research is to understand both the structural and the signaling roles of the many molecules that are concentrated in the focal adhesion.

A. Organization of the Focal Adhesion

At the molecular level, the organization of focal adhesion is far from simple. On the outside of the membrane, extracellular matrix (ECM) components such as fibronectin, vitronectin, laminin, and collagen are found. The primary transmembrane components of focal adhesions are the integrins (Hynes, 1992). Members of this protein superfamily act as transmembrane receptors for ECM components on the outside of the cell, and interact with cytoskeletal and cytoplasmic components of the focal adhesions on the inside of the cell (Sastry and Horwitz, 1993; Pavalko and Otey, 1994).

For the past decade, much attention has been focused on identifying the many proteins that are concentrated at the cytoplasmic face of the focal adhesion, and several models have been proposed to describe the molecular links that anchor actin to the membrane at these sites. Currently, it is thought that multiple mechanisms may exist to connect the actin cytoskeleton to integrins, as illustrated in Fig. 1. These models are based on the accumulated results of *in vitro* protein-binding assays, and many of the links represented here have not yet been shown to occur in living cells. The diagram in Fig. 1 illustrates potential links that may exist in parallel in the same focal adhesion, or that may form sequentially in focal adhesions as they mature. Only the structural proteins of the focal adhesion are illustrated, and additional minor constituents also exist.

Talin is a major structural element of the focal adhesion, and it is also the first cytoplasmic protein that was shown to bind to integrin (Horwitz *et al.*, 1986). Besides binding to integrin, talin also binds to actin (Muguruma *et al.*, 1990; Goldman and Isenberg, 1991; Kaufmann *et al.*, 1991) and to vinculin (Burridge and Mangeat, 1984; Gilmore *et al.*, 1993; Johnson and Craig, 1994). Vinculin appears to occupy a central location in the microarchitecture of the focal adhesion because this protein has a large number of binding partners. In addition to talin, vinculin can interact *in vitro* with α-actinin (Belkin and Koteliansky, 1987; Wachsstock *et al.*, 1987), paxillin (Turner *et al.*, 1990), and tensin (Wilkins *et al.*, 1986). Recently, a cryptic

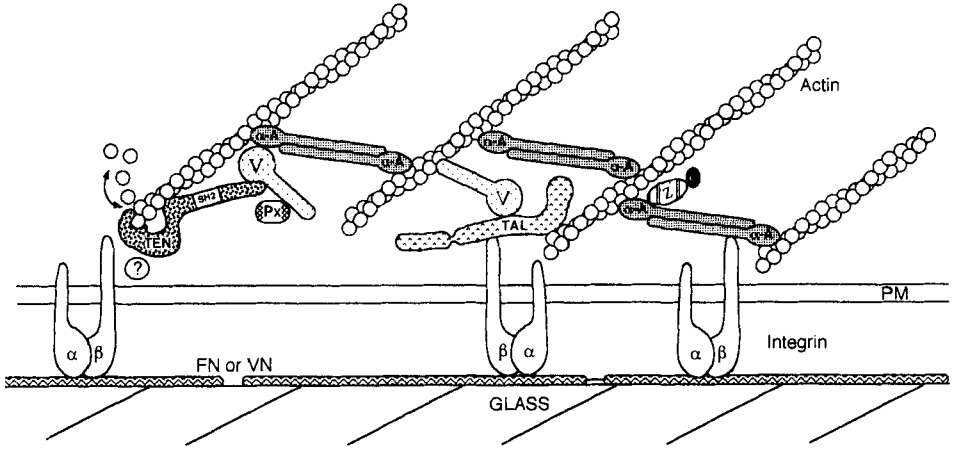

FIG. 1 Diagram illustrating the major structural proteins found in a focal adhesion of a cultured cell that is adhering to a glass coverslip coated with either fibronectin or vitronectin. Key to abbreviations: α-A, α-actinin; C, cysteine-rich protein; FN, fibronectin; Px, paxillin; PM, plasma membrane; TAL, talin; TEN, tensin; V, vinculin; VN, vitronectin; Z, zyxin. (Figure contributed by Dr. Susanne Bockholt, Dept. of Biology, Univ. of Utah.)

binding site for actin was located on vinculin (Johnson and Craig, 1995), suggesting that vinculin can also bind directly to actin when its conformation is favorable.

One of vinculin's binding partners is α-actinin, an actin-cross-linking protein that has been shown to bind to the cytoplasmic tail of the β-subunit of integrin (Otey *et al.*, 1990). Zyxin is a third binding partner for α-actinin (Crawford *et al.*, 1992). Zyxin also binds to a smaller protein called cysteine-rich protein (cCRP), and both zyxin and cCRP contain conserved motifs called LIM domains, which promote protein–protein interactions (Sadler *et al.*, 1992). Two additional focal adhesion proteins, paxillin and tensin, are discussed in more detail in a subsequent section on potential FAK substrates. Both paxillin (Turner *et al.*, 1990) and tensin (Wilkins *et al.*, 1986) have been shown to bind to vinculin *in vitro*, and tensin also binds to actin (Lo *et al.*, 1994).

Although significant progress has been made both in identifying the structural components of the focal adhesion and in characterizing the interactions of these proteins with one another, it is still not known how these many complex links are regulated by the cell. Most cultured cells undergo cycles of rounding up for mitosis and subsequent respreading, so that the cell must necessarily possess a mechanism for regulating the disassembly and reassembly of the focal adhesions. Integrins play a prominent role in this regulation, but we are just beginning to understand the signals that

trigger the formation of these complex protein links when integrins in the membrane bind to their extracellular ligands.

In addition to triggering the assembly of focal adhesions, the binding of integrins to the ECM results in a variety of rapid responses within the cell, including both proximal changes (such as Ca^{2+} influx, rapid pH change, increased tyrosine phosphorylation of intracellular proteins) (Schwartz, 1992) and also distal changes (such as suppression of apoptosis and changes in gene expression) (Juliano, 1994; Ruoslahti and Reed, 1994; Ashkenas *et al.*, 1995). Integrins do not possess any endogenous catalytic activity, so in order for them to function in signal transduction, their cytoplasmic domains must be interacting with catalytic molecules that can then propagate signals to the inside of the cell. For many years, the signaling mechanism of integrins remained a mystery. For this reason, the recent discovery of a tyrosine protein kinase called $pp125^{FAK}$ has generated great excitement in the field of integrin research because this kinase has the potential to be a key member of an integrin-mediated signal transduction cascade.

B. The Discovery of $pp125^{FAK}$

$pp125^{FAK}$ (or FAK, which stands for focal adhesion kinase), was discovered independently by several labs in the years 1990–1992. In 1990, a protein called p120 was studied by Steve Kanner and co-workers in Parsons' lab as a possible substrate for $pp60^{src}$. It had been known for some time that expression of the tyrosine kinase $pp60^{src}$ led to transformation of chicken embryo fibroblasts. Along with the many morphological changes observed in src-transformed cells (including the disruption of the actin cytoskeleton and the cellular adhesions), an increase in the phosphotyrosine content of a number of cellular proteins was also observed. Monoclonal antibodies to the phosphotyrosine-containing proteins of transformed cells were obtained by Kanner and co-workers (Kanner *et al.*, 1990), and one of these antibodies was then used to screen an expression library. This led to the cloning, sequencing, and further characterization of "p120" by Schaller and co-workers in Parsons' lab, who gave this protein its current name, $pp125^{FAK}$ (Schaller *et al.*, 1992).

At the same time, Guan and Trevithick, working in Hynes's laboratory, described a 120-kDa protein that localized to focal adhesions but that was distinct from any other known focal adhesion protein (Guan *et al.*, 1991). This pp120 was phosphorylated on tyrosine in cells that had spread on fibronectin, but when the cells were detached by trypsinization, pp120 was rapidly dephosphorylated. Since the phosphorylation state of pp120 was dependent on the adhesion state of the cell, it appeared likely that integrins were involved in regulating pp120 phosphorylation. This was tested by

incubating cells with antibodies to the β_1 integrin subunit, and then cross-linking the anti-integrin antibodies with secondary antibodies so that the integrins in the membrane were artificially clustered. This clustering had the effect of upregulating the phosphorylation of pp120. In control experiments, attachment of cells to a surface such as polylysine (which does not cluster integrins) had no effect on pp120 phosphorylation. Thus, tyrosine phosphorylation of pp120 was dependent on integrins. It was then discovered that the pp120 described by Guan *et al.* and the pp125^{FAK} cloned by Schaller *et al.* were in fact the same protein (Guan and Shalloway, 1992). Related work was being performed also in Juliano's lab on a protein called pp130 (Kornberg *et al.*, 1991) and in Patel's lab on a protein called FadK (for focal adhesion kinase) (Hanks *et al.*, 1992). Both pp130 and FadK were later shown to be identical to pp125^{FAK}.

Although the downstream function of FAK within the cell was not obvious, several features of this molecule (its localization to focal adhesions, its phosphorylation upon clustering of integrins, and its tyrosine kinase activity) hinted that FAK might be one member of a signaling cascade that could communicate between integrins and the inside of the cell. It was suggested that FAK might play a regulatory role in the assembly of the multiprotein complexes that connect integrins to the actin cytoskeleton. Although the precise function of FAK in living cells is still not known at this time, a great deal has been learned in the past few years about the structure of the FAK molecule, the regulation of FAK activity, and the interactions of FAK with other molecules in the focal adhesion. FAK has been studied in a number of different cell types, but this chapter focuses on the function of FAK in the focal adhesions of adherent cells, and therefore the literature on FAK in platelets and other circulating cells is not reviewed here. Readers interested in the role of FAK in platelets should see the excellent review by Shattil *et al.* (1994).

II. Structure of FAK

The molecular structure of FAK is unusual in two regards. Unlike many other nonreceptor tyrosine kinases, FAK lacks SH2 and SH3 domains, which are protein interaction motifs. FAK is also unusual in that the kinase domain is in the middle of the protein (Fig. 2). The kinase domain occupies roughly one-third of the protein, with large C-terminal and N-terminal domains on either side of the catalytic domain (Schaller *et al.*, 1992).

The functional roles of the N-terminal and C-terminal domains of FAK have been studied using fusion proteins and mutational analysis, and it is now clear that the C-terminal domain plays a role in recruiting FAK to

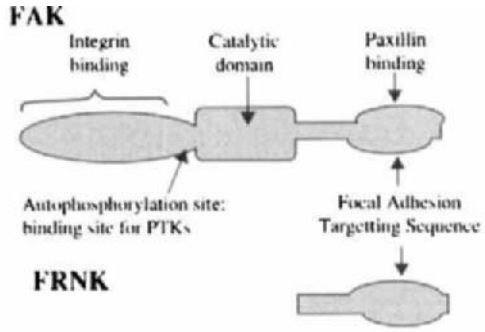

FIG. 2 Diagram of the domains of FAK and FRNK. (Contributed by Dr. Michael Schaller, Dept. of Cell Biology and Anatomy, Univ. of North Carolina at Chapel Hill.)

the focal adhesions. Hildebrand *et al.* (1993) identified a region in the C-terminus of FAK, specifically in residues 856–1012, that was essential for efficient localization of FAK to adhesion sites. Variants of FAK that contained mutations in this region, termed the "focal adhesion targeting sequence," failed to localize properly in cultured fibroblasts. Additional compelling evidence that this sequence is involved in focal adhesion targeting came from experiments in which the sequence was inserted into a cytosolic protein unrelated to FAK, which then efficiently localized to the focal adhesions (Hildebrand *et al.*, 1993).

These results were further supported by the finding that certain cells express a truncated form of FAK, which has been named FRNK (for FAK-related non-kinase). FRNK is identical to the C-terminal domain of FAK (Schaller *et al.*, 1993). It lacks a kinase domain, and is thus not catalytically active. However, FRNK also localizes to focal adhesions. Together with the mutational analysis of Hildebrand *et al.* (1993), these data argue strongly that the C-terminus is important in targeting FAK to sites of adhesion. The FAK C-terminal domain may be interacting with one or more structural proteins of the focal adhesion that function in FAK recruitment, but these proteins have not yet been identified conclusively.

Recently, a second function has been attributed to the C-terminal domain of FAK. Hildebrand *et al.* (1995) showed that FAK forms complexes with the focal adhesion protein paxillin. These authors used fusion proteins to represent the C-terminal and N-terminal domains of FAK, and asked which of the fusion proteins could bind paxillin in immunoprecipitation assays or in blot-overlay experiments. Both assays demonstrated that the C-terminal domain of FAK binds to paxillin, but the N-terminal domain does not. Interestingly, the paxillin-binding site appears to be distinct from the focal-adhesion targeting sequence in FAK, suggesting that paxillin is not the

protein responsible for recruiting FAK to the focal adhesion. A similar experimental approach was used to demonstrate a third role for the C-terminus of FAK: binding to talin. FAK was shown to coimmunoprecipitate with talin from lysates of cultured cells and also to bind cellular talin in blot-overlay assays, and the binding site for talin was mapped to the C-terminal domain of FAK using deletion mutants (Chen *et al.*, 1995). It is not yet known if talin plays a role in recruiting FAK to the focal adhesion.

The interactions of FAK with focal adhesion proteins such as talin and paxillin may play a role in mediating the activation of FAK by integrins, perhaps by communicating with the actin cytoskeleton. Several lines of evidence (discussed in a later section) suggest that an intact actin cytoskeleton is required for FAK activation. Thus, the C-terminus of FAK serves an important function in targeting FAK to focal adhesions and in interacting with cytoskeleton-associated proteins. The N-terminal domain of FAK has also been shown to have an important role in regulating FAK activity. First, an autophosphorylation site has been mapped to the N-terminal domain, at tyrosine 397 (Schaller *et al.*, 1994). The role of autophosphorylation in regulating the kinase activity of FAK is discussed in a subsequent section.

The N-terminal domain of FAK appears to be important for the binding of FAK to integrins. A recent study asked if FAK binds directly to integrins, or if FAK is instead activated through an intermediary protein or second messenger. To address this question, a synthetic peptide was used to mimic the cytoplasmic domain of β_1 integrin, an approach that had been used previously to investigate interactions between integrins and cytoskeletal proteins (Otey *et al.*, 1990). The peptide was coupled to microbeads and used to precipitate integrin-binding proteins from lysates of cultured fibroblasts (Schaller *et al.*, 1995). FAK was found to precipitate efficiently with the intact β_1 cytoplasmic tail, and also with a shorter peptide representing the membrane-proximal region of the β_1 tail. In order to determine if the binding of FAK to integrin was direct or if other proteins were required, the peptide-coated beads were incubated with purified fusion proteins representing the N-terminal and C-terminal domains of FAK. In these experiments, the N-terminal domain of FAK coprecipitated with the integrin peptide, but the C-terminal domain of FAK did not. These results suggest that FAK binds directly, through its N-terminal domain, to a membrane-proximal site in the cytoplasmic tail of β_1 integrin. It is unlikely that integrin binding alone is sufficient to activate FAK, since several lines of evidence suggest that the membrane-distal portion of the β_1 integrin cytoplasmic tail is also required in order to upregulate FAK phosphorylation (Guan *et al.*, 1991; Akiyama *et al.*, 1994). One possibility is that the membrane-proximal region of the β_1 tail is used as a docking site for FAK, but that conformational changes that occur upon integrin clustering are communicated

through the membrane-distal β-integrin tail in order to activate kinase activity of FAK.

FAK is a ubiquitously expressed protein, and it is also highly conserved structurally. It has been cloned from avian (Schaller *et al.,* 1992), murine Hanks *et al.,* 1992), human (Andre and Becker-Andre, 1993; Choi *et al.,* 1993; Whitney *et al.,* 1993), and amphibian (Hens and DeSimone, 1995) sources, and the amino acid sequences of these four species of FAK are 90–95% identical (Hens and DeSimone, 1995). In humans, a truncated brain-specific isoform of FAK has been identified (Andre and Becker-Andre, 1993), but it is not known if this tissue-specific isoform serves a specialized function. However, the staining pattern of FAK mRNA in *Xenopus* embryos revealed a high level of FAK expression in the developing brain and spinal cord (Hens and DeSimone, 1995).

III. Regulation of FAK Activity

A. Role of Integrins

One of the earliest studies on integrin-mediated signaling reported that the phosphotyrosine levels of FAK increased when integrins were bound to their ECM ligands (Guan *et al.,* 1991). Since then, a number of authors have confirmed that when cells are grown in suspension or on substrates such as polylysine (which allows cells to attach, but does not activate integrins), the amount of phosphotyrosine in FAK remains low, but if cells are allowed to spread on fibronectin or other matrix proteins, the phosphotyrosine content of FAK increases dramatically (Burridge *et al.,* 1992; Guan and Shalloway, 1992; Hanks *et al.,* 1992; Kornberg *et al.,* 1992). It is often the case that the enzymatic activity of tyrosine kinases is modulated by autophosphorylation, and this possibility was tested for FAK by Guan and Shalloway (1992). *In vitro* kinase assays were performed using FAK immunoprecipitated from cells grown on either fibronectin or polylysine. FAK from fibronectin-adhered cells was found to be 2.5 times more catalytically active than FAK from unspead cells attached to polylysine. This result suggests an appealing model in which the binding of integrins to the ECM results in the autophosphorylation of FAK, thus upregulating the enzymatic activity of FAK and generating a tyrosine phosphorylation cascade. However, recent results have indicated that the situation is not this straightforward. The principal autophosphorylation site on FAK has been mapped to tyrosine 397, but mutation of tyr397 to phenylalanine has only a modest effect on the enzymatic activity of FAK as measured by *in vitro* kinase assays. This suggests that autophosphorylation of FAK on tyrosine residues

may not be the critical event in activating FAK's enzymatic activity. One explanation for these results could be that FAK is phosphorylated by another kinase when integrins bind to the ECM, so that phosphorylation by this additional kinase is more important in activating FAK than the observed autophosphorylation.

Recent experimentation has focused in detail on the integrin-dependent phosphorylation of FAK. Researchers have asked if occupation of the ligand-binding site on integrin is sufficient to stimulate the tyrosine phosphorylation of FAK, or if clustering of the integrin is also required. These experiments have asked which subunit of integrin, α or β or both, is responsible for FAK activation.

It was mentioned in a previous section that clustering of integrins was found to be a requirement for FAK activation. The pioneering paper by Guan *et al.* (1991) reported that the synthetic peptide Arg-Gly-Asp (which occupies the ligand-binding site on integrin but does not cluster neighboring integrin molecules) was not sufficient to stimulate FAK phosphorylation. However, when these cells were incubated with antibodies to the β_1 subunit of integrin, and the anti-integrin antibodies were then cross-linked with secondary antibodies, the resultant clustering of integrins was sufficient to upregulate FAK phosphorylation. A related study by Kornberg *et al.* (1991) demonstrated that the effect was subunit-specific: treatment with antibodies to the β_1- or α_3-integrin subunits stimulated FAK phosphorylation, but antibodies to other α subunits did not.

A recent study extended these results by comparing the roles of integrin clustering and integrin occupation using a microbead system (Miyamoto *et al.,* 1995). The beads were coated with either intact fibronectin, or with the synthetic peptide Arg-Gly-Asp, or with antibodies to integrin. The antibodies were of two types: inhibitory antibodies (which occupy the ligand-binding site on the integrin extracellular domain, and thus interfere with cell adhesion) or noninhibitory antibodies (which do not occupy the ligand binding site, but which can be used in conjuction with secondary antibodies to cluster integrins). In this system, immunofluorescence staining was used to determine if FAK colocalized with the coated microbeads. Miyamoto *et al.* (1995) found that FAK colocalized with clustered integrins even when the ligand-binding site on the integrin was not occupied.

Pelletier *et al.* (1995) have taken this question one step further by examining the importance of the "activation state" of the integrin in the upregulation of FAK phosphorylation. This study made use of antibodies to $\alpha_{IIb}\beta_3$, which bind to the integrin extracellular domain and "activate" the integrin; that is, the antibodies induce a conformational change in the integrin such that it becomes capable of binding fibrinogen. Both "activating" and "nonactivating" antibodies were used to cluster integrins on the cell surface, and Western blot analysis was then used to assay the phosphotyrosine

content of cellular FAK. It was found that clustering with nonactivating antibodies resulted in an increase in FAK phosphorylation, but clustering with activating antibodies did not. There results suggest that the conformational state of the integrin, in addition to integrin clustering at sites of adhesion, is important in mediating FAK activation.

Other researchers have focused on the related question: which parts of the integrin molecule are involved in activating FAK? Is the extracellular domain of integrin as well as the cytoplasmic tail required? Which regions of the cytoplasmic tail (near the membrane or near the C-terminus) are most closely associated with FAK activation? The first answers to these questions were obtained in one of the early studies on FAK. Guan *et al.* (1991) used integrin deletion mutants to show that the distal region of the cytoplasmic tail of the β_1-subunit was required to upregulate FAK phosphorylation in spreading cells. More recently, Akiyama *et al.,* (1994) used single-subunit chimeric molecules to confirm that the β subunit, but not the α-subunit of integrin is involved in stimulating FAK phosphorylation. The chimeric molecules contained integrin cytoplasmic domains coupled to the transmembrane and extracellular domains of the interleukin-2 receptor. Three different integrin cytoplasmic domains (β_1, β_3, and β_5) were able to stimulate FAK phosphorylation, but one β-integrin tail (β_{3B}) failed to activate FAK. The β_{3B} variant differs from the β_3 subunit only in the C-terminal half of the cytoplasmic tail, suggesting that this distal region of the β-subunit may be especially important in conveying conformational information from integrin to FAK.

B. Role of Cytoplasmic Proteins

A truncated form of FAK (called FRNK) may also play a role in regulating the activity of FAK. The name FRNK is an acronym for FAK-related nonkinase. FRNK is a 41–43-kDa protein identical in sequence to the C-terminal domain of FAK (Schaller *et al.,* 1993). Because FRNK lacks the kinase domain of FAK, it is not catalytically active. However, FRNK colocalizes with FAK in the focal adhesions since both contain the focal adhesion targeting sequence found in the C-terminal region of FAK. Recently, Richardson and co-workers in Parsons lab found that overexpression of FRNK decreased the tyrosine phosphorylation of FAK and delayed cell spreading on fibronectin (Richardson and Parsons, 1996). This raises the issue of how the activity of FRNK may be regulated. One possibility is that FRNK is regulated simply by the level of expression. It is interesting to note that FRNK has been detected, so far, only in embryonic fibroblasts. Alternatively, FRNK may be regulated by phosphorylation. Upon binding of integrins to the ECM, at the same time that FAK is phosphorylated on a

tyrosine, a concomitant phosphorylation of FRNK on serine residues was detected (Richardson and Parsons, 1996). The significance of this serine phosphorylation of FRNK has not yet been investigated fully, and it is not clear how FRNK acts to regulate the phosphorylation of FAK. There is no evidence that the two proteins interact directly. It seems more likely that both proteins interact with a common binding partner, so that overexpression of FRNK inhibits FAK by sequestering this protein. If the expression of FRNK is indeed limited to embryonic cells, it will be important to determine if another protein serves a similar regulatory role in nonembryonic cells.

C. Role of Nonintegrin Receptors: Signaling Crosstalk

An exciting concept that has emerged recently is the idea that FAK may be activated by a number of agents that act independently of integrins. It appears that FAK may be a component of more than one signaling pathway (Zachary and Rozengurt, 1992). Mitogenic peptides and growth factors such as platelet-derived growth factor (PDGF) (Rankin and Rozengurt, 1994; Knight *et al.*, 1995; Abedi *et al.*, 1995), lysoposphatidic acid (LPA) (Seufferlein and Rozengurt, 1994; Chrzanowska-Wodnicka and Burridge, 1994), bombesin (Leeb-Lundberg and Song, 1993; Sinnett-Smith *et al.*, 1993; Zachary *et al.*, 1993), vasopressin (Zachary *et al.*, 1993), endothelin (Zachary *et al.*, 1993), bradykinin (Leeb-Lundberg *et al.*, 1994), and angiotensin II (Polte *et al.*, 1994) have been shown to stimulate the tyrosine phosphorylation of both FAK and paxillin. In some cases, upregulation of FAK phosphorylation has been shown to require an intact actin cytoskeleton. For example, if the microfilament network is disassembled by treatment with cytochalasin D, the phosphotyrosine content of FAK does not increase in response to bombesin stimulation (Sinnett-Smith *et al.*, 1993). Since these agents require an organized actin cytoskeleton in order to activate FAK, it is likely that their effects also depend on integrin occupancy. However, it is also possible that these growth factors and neuropeptides might activate FAK independently of integrins, which would imply that FAK could be a point of convergence of several distinct signaling pathways.

This idea has gained support from recent studies on insulin and insulin-activated signaling. In one recent study, Rat-1 fibroblasts were treated with insulin, and it was found that insulin stimulates the dephosphorylation of FAK. This effect was dependent upon tyrosines 1328 and 1334 in the insulin receptor (Pillay *et al.*, 1995). In a related study using CHO cells, FAK was found to be dephosphorylated and actin stress fibers were reduced in number in the insulin-treated cells (Knight *et al.*, 1995). Both effects, however, were transient. The relationship between FAK dephosphorylation and insulin stimulation may be mediated by a tyrosine phosphatase called SHPTP2,

which has been implicated in the downstream signaling of insulin (Yamauchi *et al.*, 1995). Interestingly, platelet-derived growth factor had the opposite effect on CHO cells: PDGF treatment led to increased phosphotyrosine content in FAK and an increased number of stress fibers (Knight *et al.*, 1995).

Signal-transduction crosstalk is still an emerging field, and there are many questions that remain to be answered about the role of FAK in transmitting signals from growth factors and mitogens. Clearly, the effects of certain growth factors on FAK are complicated, and platelet derived growth factor is a good example. The effect of PDGF on FAK phosphorylation can vary depending on the isoform of PDGF, the dosage used, and the cell type. In rabbit aortic vascular smooth muscle cells, the PDGT-BB isoform stimulates an increase in the phosphotyrosine content of both FAK and paxillin even at high does (100 ng/ml PDGF-BB) (Abedi *et al.*, 1995). In contrast, PDGF-AA failed to induce FAK phosphorylation in vascular smooth muscle cells, but both isoforms of PDGF stimulated FAK phosphorylation in Swiss 3T3 cells. Interestingly, Swiss 3T3 cells responded to both PDGF-AA and PDGF-BB at low concentrations, but at higher concentrations, the effect on FAK was abolished (Abedi *et al.*, 1995).

IV. Downstream Effects of FAK

A. Substrates and Binding Partners of FAK

Although a great deal has been learned about the detailed structure of the FAK molecule, relatively little is known about its function in living cells. One approach to investigating the downstream effects that result from the activation of FAK has been to try to identify the substrate proteins of this tyrosine kinase. Thus far, two candidates have been suggested as potential substrates: tensin and paxillin. Tensin is a 215-kDa protein that binds to actin filaments (Lo *et al.*, 1994) and is concentrated in both cell–matrix and cell–cell adhesions (Wilkins *et al.*, 1986; Bockholt *et al.*, 1992). Tensin contains an SH2 domain (Davis *et al.*, 1991) and is phosphorylated by v-Src in RSV-transformed cells (Glenney and Zokas, 1989). Bockholt and Burridge (1993) have shown that the phosphotyrosine content of tensin increases when cells spread on fibronectin or laminin. The conditions that promote tensin phosphorylation (an intact actin cytoskeleton, clustering of integrins, cell spreading) are the same conditions that promote FAK activation, so it is possible that tensin is a substrate of FAK. However, there are currently no data that argue strongly for a direct relationship between FAK and tensin.

There are several lines of evidence that suggest that paxillin may be a substrate for FAK. First, paxillin and FAK become coordinately phosphorylated on tyrosine residues when integrins bind to the ECM (Burridge *et al.,* 1992) or when cells are stimulated with agents such as bombesin, LPA or PDGF (Zachary and Rozengurt, 1992). There is also evidence that paxillin and FAK are physically associated (Hildebrand *et al.,* 1995). Recently, the role of FAK in the phosphorylation of paxillin was examined using different variants of FAK (Schaller and Parsons, 1995). In one set of experiments, wild-type FAK was overexpressed in fibroblasts, and the phosphorylation state of paxillin was then assayed. It was observed that in cells expressing a tenfold excess of FAK, there was only a modest increase in the tyrosine phosphorylation of paxillin compared with normal cells. However, when the cells were also treated with vanadate to inhibit tyrosine phosphatases, the FAK overexpressors exhibited a significant increase in the level of tyrosine phosphorylation of paxillin. The authors also tested FAK deletion mutants in this system and found that FAK variants that failed to localize to focal adhesions also failed to induce paxillin phosphorylation. These data suggest that FAK must be localized to the focal adhesion in order for paxillin to become phosphorylated on tyrosine in response to cell spreading.

As was mentioned previously, there is some question whether autophosphorylation of FAK on tyrosine 397 is important in upregulating the catalytic activity of FAK. One model for FAK's role in the cell predicts that autophosphorylation of FAK results in an increase in its kinase activity and thus an increase in the phosphotyrosine content of FAK substrates. This model was tested by mutating tyrosine 397 of FAK to phenylalanine and overexpressing this variant of FAK using the system described above (Schaller and Parsons, 1995). Although mutation of Tyr397 did not have a significant effect on the kinase activity of FAK when measured *in vitro,* expression of this mutated variant rendered FAK defective in the induction of tyrosine phosphorylation of paxillin. These data indicate that when FAK is properly targeted to the focal adhesions, and when it is phosphorylated on Tyr397, then the overexpression of FAK results in increased tyrosine phosphorylation of paxillin. Although there are still no definitive data to demonstrate that FAK catalyzes the tyrosine phosphorylation of paxillin *in vivo,* all of these results taken together strongly suggest that the phosphorylation of paxillin is dependent on FAK. The possibility remains that another kinase is activated by FAK, and that the phosphorylation of paxillin is thus indirectly regulated by FAK.

It is intriguing that both of the candidate substrates for FAK in the focal adhesion (tensin and paxillin) have been shown to bind to vinculin *in vitro.* Compared with the other proteins found on the cytoplasmic face of the focal adhesion, vinculin appears to be involved in a particularly complex

web of protein–protein interactions. Vinculin binds to talin (Burridge and Mangeat, 1984; Gilmore *et al.,* 1993; Johnson and Craig, 1994), and talin binds to both integrin (Horwitz *et al.,* 1986) and actin (Muguruma *et al.,* 1990; Goldmann and Isenberg, 1991). Vinculin itself binds to actin (Menkel *et al.,* 1994; Johnson and Craig, 1995), and vinculin also binds to α-actinin (Belkin and Koteliansky, 1987; Wachsstock *et al.,* 1987), which binds to both integrin (Otey *et al.,* 1990) and actin. Thus, vinculin appears to occupy a critical position in the "center" of the focal adhesion. Possibly the recruitment of vinculin to the focal adhesion is an important early step in the assembly of the many complex protein–protein links that anchor actin to integrin. Tensin and paxillin may be involved in targeting vinculin to sites of cell–matrix interaction, and the tyrosine phosphorylation of tensin and paxillin may serve in part to regulate their interactions with vinculin when focal adhesions are assembled in newly spreading cells.

FAK may also affect cell behavior through its association with members of the Src family of tyrosine kinases. One of the first studies on FAK suggested that it could be a substrate for $pp60^{v\text{-}src}$ (Kanner *et al.,* 1990). More recently, FAK has been shown to form complexes with two members of the Src family: $pp60^{src}$ and $pp59^{fyn}$ (Cobb *et al.,* 1994; Schaller *et al.,* 1994; Xing *et al.,* 1994). These complexes are formed through the SH2 domains found in Src and Fyn. The association of FAK with Src has also been studied by coimmunoprecipitation of FAK from fibroblasts grown on either fibronectin or polylysine. One recent study demonstrated that when fibroblasts are adhered to fibronectin, FAK coprecipitates with c-Src (Seufferlein and Rosengurt, 1994). If cells are detached from fibronectin or grown on poly-L-lysine, the binding of FAK to c-Src is reduced. In transformed cells, v-Src and FAK are constituitively associated, independent of cell adhesion to fibronectin through integrins. This result is especially interesting, since changes in the actin cytoskeleton and in cell adhesion are commonly seen in Src-transformed cells. It is likely that Src-mediated phosphorylation of FAK may alter the regulation of FAK in transformed cells.

It appears that FAK may also be involved in communication between integrins and the nucleus by impinging on the mitogen-activated protein (MAP) kinase pathway. The MAP kinase family is involved in signal transduction from a large number of growth and differentiation factors. Recent data have hinted at a connection among integrins, FAK, and MAP kinase. It was demonstrated that adhesion of cells (Swiss 3T3 or REF52 fibroblasts) to ECM components such as fibronectin or laminin resulted in the activation of MAP kinases (Chen *et al.,* 1994). Adhesion of cells to polylysine had no effect on MAP kinase, suggesting that engagement of integrins was required. An intact actin cytoskeleton was also required: if the actin was disassembled by treatment with cytochalasin D, activation of MAP kinase was blocked (Chen *et al.,* 1994). A related study demonstrated a similar

effect in NIH-3T3 cells: adhesion to fibronectin stimulated the activation of MAP kinases, and this upregulation of MAP kinases was blocked by treatment with cytochalasin D (Zhu and Assoian, 1995).

Although these experiments did not address the mechanistic question of how integrins could be activating MAP kinase, another study has suggested that FAK may be involved. FAK has been shown to coimmunoprecipitate with the adaptor protein GRB2 (Schlaepfer *et al.*, 1994), and GRB2 is thought to mediate signal transduction from membrane receptors to the Ras/MAP kinase pathway. The connection between FAK and the MAP kinase pathway is, at this point, a tenuous one, but it seems possible that integrin engagement may promote the binding of FAK to GRB2, and this might explain how integrins could mediate the activation of MAP kinases. MAP kinase has been shown to translocate to the nucleus in response to growth factor stimulation (Lenormand *et al.*, 1993), which suggests a model in which integrins act on the nucleus to induce changes in cell differentiation, growth, and behavior through a pathway involving FAK, GRB2, and MAP kinase. This model is potentially very exciting because it describes a mechanism by which integrins could influence a wide range of cell behaviors, but many aspects of this model still remain to be tested in living cells.

B. *In Vivo* Effects of FAK

Relatively little is known about the function or even the localization pattern of FAK in organized tissues compared with cultured cells. In a study on membrane specializations of the sarcolemma, FAK was localized to the myotendinous junction but not the neuromuscular junction of *Xenopus* skeletal muscle (Baker *et al.*, 1994). Myotendinous junctions are sites of cell–ECM interaction and mechanical force transmission, so that these junctions are functionally analogous to the focal adhesions of cultured cells. FAK expression in developing *Xenopus* embryos was also detected in the intersomitic junctions (Hens and DeSimone, 1995), and these sites contain many of the same structural proteins found in focal adhesions (fibronectin, talin, etc.). Thus, data from both sectioned *Xenopus* adults and from whole *Xenopus* embryos suggest that FAK may serve a function in the specialized junctional sites of organized tissues which is similar to the function of FAK in the focal adhesions of cultured cells. High levels of FAK have been detected in the rat central nervous system, where it is found in all regions of the brain (Burgaya *et al.*, 1995), and FAK has also been localized to the focal adhesions of cultured astrocytes and to growth cones of developing neurons (Burgaya *et al.*, 1995). To date, it is not known if FAK serves a specialized function within the cells of the central nervous system, but it

has been suggested that FAK may provide a mechanism by which the ECM can regulate neurite outgrowth and neuronal plasticity.

Although several models for the function of FAK within cultured cells have been proposed, none have been tested exhaustively. One popular model suggests that FAK is involved in regulating the assembly of focal adhesions and stress fibers. It is difficult to specifically inhibit FAK directly in living cells, so this model has not been thoroughly tested, but several studies have generated interesting results that bear on this question. One early study made use of tyrosine kinase inhibitors to ask if tyrosine phosphorylation of cellular proteins was required in order for cells to spread. In REF52 cells treated with the tyrosine kinase inhibitor herbimycin A, there was a reduction in the extent of cell spreading, in the number of focal adhesions, and in the number of actin stress fibers (Burridge *et al.*, 1992). However, herbimycin A is a broadly effective inhibitor of tyrosine kinases, and it is not known if this drug acts on FAK directly.

Another tyrosine kinase inhibitor, tyrphostin, was used to ask if tyrosine phosphorylation was required in order for human umbilical vein endothelial cells (HUVEC) to spread and migrate on ECM substrates. When monolayers of HUVECs were "wounded" by scraping, the time required for the cells to migrate into the wound was doubled by treatment with tyrphostin (Romer *et al.*, 1994). Cell migration involves the constant assembly of new focal adhesions at the leading edge of the cell, so these results are consistent with a role for FAK in focal adhesion assembly.

One recent study argues compellingly that FAK activation is not required for focal adhesion and stress fiber assembly, at least in certain cell types (Wilson *et al.*, 1995). This study examined stress fiber assembly in mouse aortic smooth muscle cells (MASMC), which form focal adhesions and stress fibers when the cells are cultured on fibronectin substrates. FAK was localized to these adhesions by immunofluorescence staining, but when FAK was immunoprecipitated from lysates of spreading cells, it was not immunoreactive with an antiphosphotyrosine antibody. Since FAK activation is thought to correlate with an increase in the phosphotyrosine content of FAK, the authors concluded that the immunoprecipitated FAK in the MASM cells was not activated. This interpretation was verified by performing an *in vitro* kinase assay on the immunoprecipitated FAK, and FAK was found to be catalytically inactive in spreading MASM cells. Thus, in this cell type, stress fibers and focal adhesions were able to form in the absence of activated FAK. These data suggest that it may be premature to assign a role in focal adhesion assembly to FAK. However, since this result has been obtained to date only with mouse aortic smooth muscle cells, it is possible that the function of FAK may be different in these cells than in other cell types that express FAK, such as fibroblasts and epithelial cells.

The *in vivo* function of FAK in epithelial cells has been tested through the use of tyrosine phosphatase inhibitors. These drugs shift the intracellular equilibrium to favor tyrosine phosphorylation: since the tyrosine phosphatases are inhibited but the kinases are unaffected, the net result is an increase in the phosphotyrosine content of a number of proteins, including FAK. If it is correct that the kinase activity of FAK depends on tyrosine phosphorylation, then any drug that promotes phosphorylation would also promote FAK activation. A caveat that should always be borne in mind is that these drugs are not specific to FAK: the phosphotyrosine content of many different proteins is affected by phosphatase inhibitors.

One study made use of two different phosphatase inhibitors (vanadate and phenylarsine oxide), which were tested on epithelial cells (Defilippi *et al.*, 1995). The overall morphology of the vanadate-treated cells was found to be altered: the cells were more circular in shape, with a greater number of cell adhesions, mostly in a radial distribution. These results demonstrate an interesting correlation: conditions that increase the phosphorylation of FAK also increase the number of focal adhesions. This result is consistent with the idea that FAK regulates the assembly of focal adhesions. In addition, the authors examined the relationship between FAK phosphorylation and the integrity of the actin cytoskeleton. In these experiments, the actin stress fibers were disassembled by treatment with cytochalasin D, and the phosphotyrosine content of FAK was reduced. If the cells were treated with vanadate along with cytochalasin D, the phosphotyrosine content of FAK was maintained at a high level in spreading cells. Interestingly, the inclusion of vanadate also protected the stress fibers from disassembly by cytochalasin D. Therefore, another correlation has been demonstrated: when FAK phosphorylation is preserved, the integrity of the actin cytoskeleton is also preserved.

Another experimental approach to determine the role of FAK has been to knock out the expression of FAK in mice, and to examine the effect of FAK deficiencies in both FAK(−) embryos and in FAK(−) cells cultured from those embryos. Using this technique, Ilic *et al.* (1995) found that FAK(−) mice appear normal in the very early stages of development, but begin to exhibit mesodermal defects by embryonic day 8. These defects are pronounced by day 8.5. "Fibroblast-like" cells were obtained from the FAK(−) embryos, so it was possible to ask if the cells were capable of assembling focal adhesions and stress fibers, and of spreading on ECM substrates, in the absence of cellular FAK.

The FAK(−) cells did not spread fully on fibronectin, and they had some reduction in their ability to migrate. Surprisingly, however, the FAK(−) cells actually had more focal adhesions than FAK(+) cells, and an abundance of stress fibers. Furthermore, both paxillin and tensin were tyrosine phosphorylated in the FAK(−) cells. These data could mean that some of

the functions attributed to FAK (such as the phosphorylation of paxillin and tensin, and the assembly of focal adhesions and stress fibers) are in question. However, it is possible that the cells obtained from the FAK(−) embryos were not truly fibroblasts, but were actually smooth muscle precursor cells, since smooth muscle cells grow out from embryonic explants almost as readily as fibroblasts. If this were the case, then the results would be consistent with those of Wilson *et al.* (1995) who found that FAK activity was not necessary in order for aortic smooth muscle cells to assemble stress fibers and focal adhesions. It will be important in the future to characterize these FAK(−) cells more fully and to determine exactly how "fibroblast-like" they are. Also, as functional redundancy is a common theme in biology, it is possible that a different protein substitutes for FAK in the FAK(−) cells. At this time, perhaps the most important message to be learned from the FAK knockout experiments is that this is a lethal mutation, which emphasizes the point that FAK is important in normal embryonic development.

Another recent model has suggested that FAK may play a role in anchorage-dependent cell growth. It has been known for some time that many adherent cell types fail to grow if they are maintained in suspension, and this dependency on substrate attachment has been termed "anchorage dependence." Recently, several studies have shown that integrins are involved in the regulation of anchorage-dependent growth. Adherent cells such as endothelial and epithelial cells must have their integrins bound to ECM ligands in order for the cells to survive (Meredith *et al.*, 1993; Frisch and Francis, 1994; Re *et al.*, 1994). If these cells are maintained in suspension, or are plated on a nonadhesive substrate, the cells undergo programmed cell death, which is also called apoptosis. FAK may be involved in suppressing apoptosis: as long as integrins are occupied and FAK is activated, cell death is prevented (Ruoslahti and Reed, 1994). This model remains to be tested directly in living cells, but it has strong intuitive appeal. Since anchorage-dependent cell growth depends upon communication between integrins and the nucleus, it is possible that FAK is part of a signaling cascade functioning primarily to inform the nucleus of the occupation state of the integrins.

V. Concluding Remarks

In summary, $pp125^{FAK}$ is an intriguing tyrosine kinase that has generated tremendous interest in integrin-mediated signal transduction. A great deal has been learned about this protein at the molecular level, including many details of its functional domains. Currently, less is known about the regula-

tion and function of FAK in living cells and organized tissues, although a number of models for FAK function have been proposed. The role of FAK in the assembly of focal adhesions is still being tested, as is its role in suppressing apoptosis. In addition, other members of a FAK family of kinases may exist, and these could have specialized, tissue-specific functions. Fortunately, many of the necessary tools are at hand, and it is expected that rapid progress will be made in answering these remaining questions regarding pp125^{FAK}.

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Feedback Inhibitors in Normal and Tumor Tissues

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Negative feedback represents the principal mechanism for regulating growth in biological systems. Over the past 20 years, our understanding of the role played by inhibitory factors governing this process has advanced considerably. This is particularly well illustrated in the field of experimental hematology with the recognition of hemopoietic progenitor cell proliferation inhibitors, an expanding group of unrelated peptides that act to limit proliferation in hemopoietic precursor cells. The characterization and subsequent production of these molecules by chemical synthesis or recombinant DNA technology has enabled investigators to explore their role in normal hemopoiesis and define a potential role in clinical medicine. A number of inhibitory factors, including macrophage inflammatory protein-1α (MIP-1α) and the tetrapeptide AcSDKP appear to share a relative specificity fo hemopoietic progenitor cell subsets. Others, such as interferon and tumor necrosis factor, have a more complex action and their hemopoietic effects are likely to be indirect and nonspecific. In addition to the role of inhibitors in normal steady state, it has become increasingly evident that loss of sensitivity to the normal feedback inhibitory signals may be of central importance in carcinogenesis and tumor promotion. This presumably represents a developmental strategy that allows the neoplastic cell to maintain a growth advantage over its normal cell counterpart. The underlying mechanisms that terminate in inhibitor-resistance are yet to be elucidated, but in some instances they may be associated with aberrant tumor suppressor gene function.

KEY WORDS: Hemopoiesis, Feedback inhibition, MIP-1α.

Introduction

The stability of the "milieu interieur" is recognized as an essential requirement of all living organisms, ensuring a state of permanent mass and func-

tion. At its most basic, it defines a steady state between the rate of cell loss and cell gain and is particularly relevant when discussing control at supracellular levels such as temperature or blood glucose regulation. Despite the term, however, homeostasis is not a static process but a dynamic interplay in which the organism must adapt to changing requirements. This adaptation is made possible by the development of numerous complex sensory monitoring systems that use a series of feedback loops and thus allow fine tuning and resetting of the equilibrium.

Our understanding of the underlying mechanisms remains in its infancy but considerable insight has come from the investigation and observation of the regulatory pathways governing hemopoiesis and epithelial cell proliferation. Within these tissues, considerable emphasis has been placed on the role of stimulatory polypeptides in normal growth regulation. Until relatively recently, and despite their obvious importance, the counterbalancing inhibitory factors have been largely neglected. A similar scenario has developed in the field of cancer research. The recognition that stimulatory signals are enhanced in certain forms of cancer has led to the identification of numerous proto-oncogenes that are linked to growth regulatory pathways. After a considerable delay, it is somewhat ironic that we are only just beginning to speculate about the place of inhibitory signals in malignant disease and appreciate their importance in carcinogenesis. This has been particularly highlighted by the work on tumor suppressor genes (Friend *et al.,* 1987; Donehower and Bradley, 1993) and studies that have revealed altered growth kinetics in chronic myeloid leukemia (CML) resulting, at least in part, from a resistance to a number of hemopoietic feedback inhibitory molecules (Eaves *et al.,* 1993a; Cashman *et al.,* 1994).

This chapter does not endeavour to define all aspects of growth regulation in biological systems. Instead it focuses on hemopoiesis and hemopoietic stem cell inhibitors as representative models. The loss of feedback inhibition in tumor growth is considered and its therapeutic potential is discussed. The rapidly expanding field of hemopoietic growth factors is not discussed further unless it is directly relevant.

The basic cybernetic principle of negative feedback has been exploited by mankind for many centuries in both science and technology. Despite this, the concept was not considered seriously, in physiological terms, until 1957 when Weiss and Kavanau published their general theoretical growth model. The theory, illustrated in Fig. 1a, outlined a system of templates (stimulatory factors) and antitemplates (inhibitory factors) that regulate growth by negative feedback.

> Each specific cell type reproduces its protoplasm by a mechanism in which key compounds (templates) characteristic of the particular cell type act as catalysts. Each cell also produces specific freely diffusable compounds antagonistic to the former (antitemplates) which can block and thus inhibit the

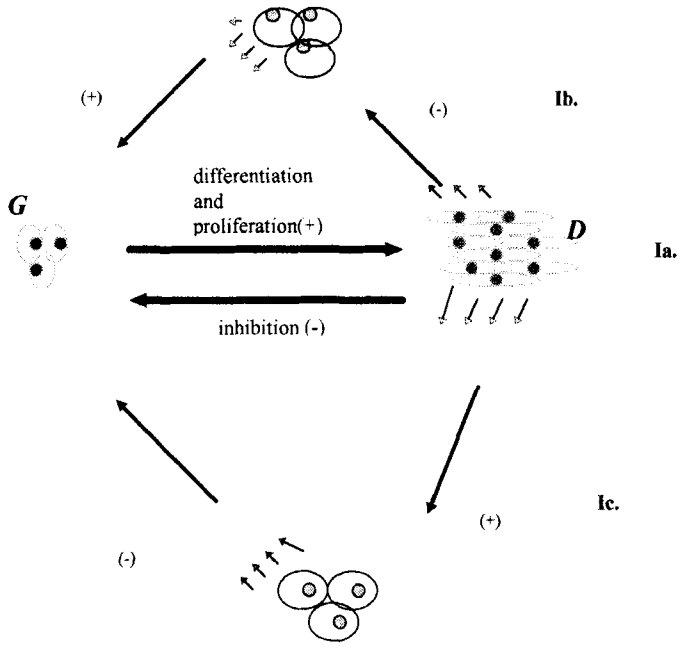

FIG. 1 Principles of feedback inhibition G, generating mass (stem cell population; D, differentiating mass (progenitor cell compartment); (−), inhibitory signal; (+), stimulatory signal.

reproductive activity of the corresponding templates. The antitemplate system acts as a growth regulator by a negative feedback mechanism in which increasing populations of antitemplates render ineffective an increasing proportion of homologous templates, resulting in a corresponding decline of the growth rate.

This hypothesis, initially formulated to explain the growth curve of chickens, was taken up enthusiastically by several investigators and proved particularly fruitful when applied to regulation of epithelial growth. In 1960 Iverson developed the idea further and published corroborating evidence from his work on skin carcinogenesis (Iverson, 1968). In the same year, Bullough and Lawrence (1960) devised a particularly innovative model based on the mouse ear (Fig. 2). In their experiment, a 3-mm^2 area of superficial epidermis and dermis was removed and mitotic activity in the undamaged, contralateral epidermis was analyzed. If a stimulating wound hormone were produced by the damaged epidermis (Fig. 2, upper diagram), two regions of increased mitotic activity corresponding to the damaged edges would develop. Alternatively, if the concentration of epidermal inhibitor were reduced (lower diagram), a continuous zone of mitotic activity spanning the wound zone would be expected. The result, confirmed in Iverson's experiment, was consistent with the hypothesis that epithelial proliferation

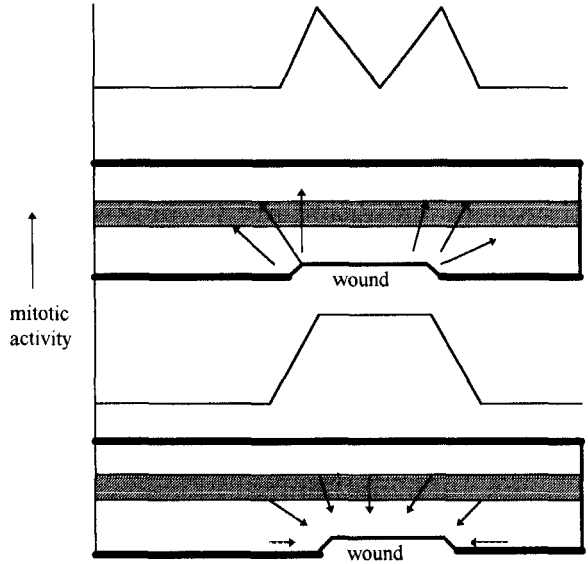

FIG. 2 Bullough's ear model (Bullough and Lawrence, 1960) showing the hypothetical mitotic activity in undamaged ear epidermis opposite a 3 mm^2 area from which the epidermis and superficial dermis have been removed. The upper diagram shows the proliferative activity assuming the damaged tissue releases a stimulator. The lower diagram shows the proliferative response assuming that an epidermal inhibitor is reduced in the neighborhood of the wound.

was controlled by a locally acting, freely diffusible negative feedback regulator.

In 1962 Bullough introduced the term "chalone" for substances acting as tissue-specific mitotic inhibitors. (Chalone is derived from the Greek word originally meaning "to slow down the speed" or "to reef in the sails"). These molecules were assumed to be synthesized locally and were expected to be evolutionarily conserved, thus showing cross-species activity. Following the demonstration of the epithelial inhibitor in tissue extracts, a variety of other chalones were soon being discussed (Voaden and Leeson, 1969; Forcher and Houck, 1973). Rytomaa and Kiviniemi (1968a) were the first to report a hemopoietic chalone that was produced from a granulocyte extract that inhibited myelocyte proliferation. More recently Paukavits and Laerum (1982) characterized the active component from human leukocytes and synthesized an acidic pentapeptide (pEEDCK) whose range of activity extended to reversible inhibition of lineage-committed granulocyte-macrophage colony-forming cells (GM-CFC), multipotent colony-forming cells–spleen (CFC-S) and possibly primitive (pre-CFU-S) hemopoietic stem cells (Laerum and Paukovits, 1984; Paukovits *et al.*, 1993).

The rapid rise to fame of chalones in the 1960s was only paralleled by their speedy decline. The crude extracts produced were easy targets for criticism and much of the experimental work was viewed with considerable scepticism. The theory was certainly appealing but there were (and remain) considerable problems in producing convincing "negative" experimental data.

Considering the difficulties, it is worth noting that a number of investigators did persevere and were finally rewarded for their toil. The most fruitful avenues of investigation continued to be in the fields of epithelial biology and experimental hematology. Many of the originally described tissue extracts have since been disregarded. Several, however, represented the forerunners of a number of highly purified and genetically engineered products that are now available and are currently entering clinical trials in hematology and oncology.

II. Feedback Inhibition

A. Principles

The model described by Weiss and Kavanau, and illustrated in Fig. 1a, represents the simplest form of negative feedback regulation. The expanding, differentiated mass (D) elaborates an inhibitory factor (I) that acts directly on the generating mass (G), thus limiting its output. However, it is also possible that the feedback factors act via intermediary cell populations. In this scenario the feedback inhibitory signal may suppress a stimulator that acts on the generating mass (Fig. 1b). Alternatively, the differentiating population may produce a positive feedback signal that enhances an intermediary cell-produced inhibitor (Fig. 1c). In all cases the principal regulatory mechanism must involve an inhibitory signal. Positive feedback, when viewed in isolation, is not a tenable concept because it would rapidly lead to instability and ultimately, exhaustion of the system.

B. Hemopoietic Structure and Feedback Inhibitors

The diversity and amplification of hemopoietic progenitor cells is made possible by factors that regulate the cellular options of self-renewal, differentiation, and proliferation. The role of the hemopoietic growth stimulatory factors has historically been emphasized in dictating these proliferation and differentiation decisions (Metcalf, 1990). More recently, the role of

hemopoietic negative feedback factors has been appreciated and our understanding, particularly of stem cell regulation, has expanded dramatically.

Hemopoiesis can be viewed as a three-part structure of developing cell populations (see Fig. 3). A relatively small number of self-renewing and pluripotent stem cells give rise to an increasingly lineage-committed progenitor cell population and ultimately the mature functional cells that are morphologically identifiable. The murine spleen colony-forming unit assay described by Till and McCullough (1981) has traditionally been used to define a multipotent, self-renewing cell population in the mouse. A more primitive pre-CFU-S is now well recognized [variously termed "marrow repopulating cells" (MRA), "long-term reconstituting cells" (LTR), "long-term culture initiating cells (LTC-IC)] and the stem cell is more appropriately visualized as a continuum of cells with an age distribution and decreasing self-renewal capacity (Schofield, 1978; Hodgson and Bradley, 1979). The human stem cell remains particularly elusive, although quantitative and qualitative data can be obtained from *in vitro* growth in long-term bone marrow culture (LTBMC) (Dexter *et al.*, 1978). Using this system, a long-term culture-initiating cell (LTCIC) has been described that gives rise to unipotential and multipotential clonogenic cells for as long as 5–8 weeks in culture (Sutherland *et al.*, 1990). Limiting dilution analysis has shown

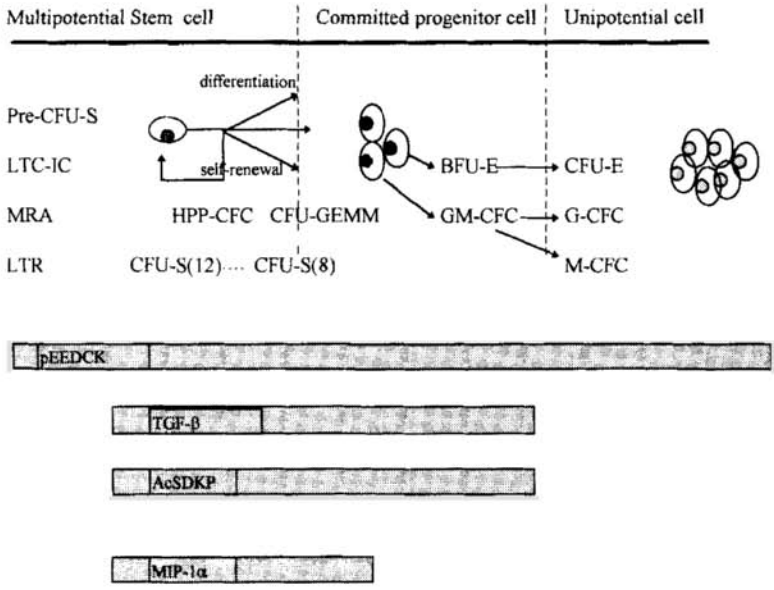

FIG. 3 The hemopoietic hierarchy, including the proposed inhibitory spectrum of pEEDCK, TGF-β, AcSDKP, and MIP-1α.

that the frequency of LTC-ICs in unfractionated bone marrow is approximately 1–2 per 10^4 cells (Sutherland *et al.*, 1990).

Immunophenotypic analysis has also been used in attempts to purify and quantitate primitive hemopoietic stem cells. Monoclonal antibodies to cell surface antigens such as CD34, HLA, and Sca-1 are important markers in several enrichment protocols, but none of these completely distinguishes the most primitive stem cell from CFU-S and other progenitor cells (Sprangrude *et al.*, 1988; Andrews *et al.*, 1989). The maturing progenitor populations remain morphologically indistinguishable but can be assessed in colony assays under appropriate *in vitro* conditions. The characteristics of these colonies depend upon the culture conditions, the growth factors present (and their source), and the timing of readout. In semisolid media, some bone marrow cells can form multilineage colonies. The CFC-mix or CFU-GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte) identifies a cell with the potential to form granulocytes, erythrocytes, monocytes, and megakaryocytes. The cells detected in this assay share some properties with the day-8 CFU-S. A CFC with a high proliferative potential can also be detected in murine and human bone marrow (McNiece *et al.*, 1989). These are considered to be among the most primitive progenitor cells grown *in vitro* and are closely related to the day-12 CFU-S [which are also equated with colony-forming unit-A (CFU-A) colonies grown *in vitro* (Lorimore *et al.*, 1990). Besides the multipotential progenitor cells, a number of unipotential- and bipotential-committed progenitor cells can also be recognized. These include the granulocyte-macrophage CFC, the erythroid burst-forming unit (BFU-E), and the more mature erythroid progenitor cell (CFC-E).

Rytomaa first described a granulocytic extract that regulated myelocyte proliferation (Rytomaa and Kiviniemi, 1968a). Inhibitors have since been reported for other lineage-committed hemopoietic subsets in granulopoiesis and erythropoiesis (Fetsch and Maurer, 1987; Kivilaasko adn Rytomaa, 1971; Axelrad *et al.*, 1987; Guigon *et al.*, 1990). These molecules act to limit the proliferation rates of the differentiated progeny and it is probable that appropriate inhibitors exist at all levels of cellular differentiation and in all hemopoietic lineages.

The recognition that hemopoietic stem cells are proliferatively quiescent (Becker *et al.*, 1965), as opposed to the proliferatively active progenitor cell population, suggests that the major control point for proliferation regulation exists within the stem cell compartment. Utilizing an ingenious model in which mice were irradiated with one hind limb shielded, stem cell regulation was found to be locally controlled (Gidali and Lajtha, 1972). The proliferative behavior of the CFU-S in the shielded limb was found to be independent of that in the unshielded limb. This suggested localized CFU-S proliferation control. As a consequence of these experiments, Lord *et al.*, (1976) were able to obtain a conditioned medium from normal bone marrow

(NBME-IV) that blocked entry of CFU-S into DNA synthesis. The inhibitory fraction largely conformed with Bullough's definition for a chalone in that it was locally produced, tissue specific (CFU-S inhibition with no effects seen in *in vitro* assays for mixed CFC and lineage-committed CFCs), and was evolutionarily conserved with cross-species activity demonstrated (Lord *et al.*, 1976; Tejero *et al.*, 1984).

Using a variety of sorting techniques, a subpopulation of marrow macrophages was found to be the source of the inhibitory activity (Wright *et al.*, 1980). A second subpopulation of macrophages synthesized a CFU-S stimulator (Wright *et al.*, 1982). Analysis of the stimulator to inhibitor interaction revealed that the two activities function via an on/off switch mechanism with the presence of the opposing factor required for proliferation reversal (Lord *et al.*, 1977a) (unlike the transit cell proliferation inhibitors, whose effect can be reversed simply by washing). It was another 14 years before the active component of the NBME-IV molecule was isolated and characterized as macrophage inflammatory protein 1α(MIP-1α) (Graham *et al.*, 1990); the clinching fact was the neutralization of NBME-IV by antibody to MIP-1α.

Although their interrelationships have yet to be elucidated, three further inhibitors of hemopoietic stem cells have been recognized, sequenced, and synthesized. These four factors share common actions, suggesting a degree of overlap and redundancy. Three of them, MIP-1α, AcSDKP (Gorolatide), and pEEDCK have now entered clinical trial protocols. A fourth, transforming growth factor-β (TGF-β) is in the latter stages of preclinical studies.

It is unlikely that inhibitory regulation is confined to hemopoietic tissue. Comparative studies indicate, however, that other renewing cell systems have a similar hierarchical structure (Potten and Hendry, 1983) and it is probable that equivalent regulatory processes are also operative.

III. Cell Proliferation

A. The Cell Cycle and Its Regulation

Cell cycle regulation is both a highly complex and rapidly expanding field of cell biology. Although a full discussion is beyond the scope of this review, a brief introduction is considered appropriate because manipulation of cell cycling represents a common final pathway for all feedback signals by inhibitory regulators.

In 1953 Howard and Pelc defined the four classical components of the cell cycle as consisting of stages of DNA synthesis (S) and mitosis (M) separated by two gaps, designated G_1 and G_2. In most mammalian cells, S

typically lasts 6–10 hr; G_2, 3–5 hr; and M, 1 hr. By contrast, the duration of G_1 shows great variability. For example, in culture, Chinese hamster lung cells do not have a definable G_1 (Robbins and Scharff, 1967), while in other quiescent populations there does not appear to be an upper limit on time. The great variability in G_1 suggests that this is where the main control points for cycle progression exist.

Observations on proliferatively quiescent populations that have a very low cycling rate (normal hepatocytes, uterine epithelium of oophorectomized mice) suggest that cells can also reside in a separate, out-of-cycle G_0 state (Lajtha, 1963).

G_1 itself is not a single entity but more likely a series of stages that a cell must complete before proceeding into S. Restriction points in G_1 determine whether a cell will progress to S or enter quiescence. Once cells have reached a point late in G_1, referred to as the R point and analogous to START in yeast (Pardee, 1989), cells will automatically proceed to S, even in the absence of nutrients. DNA synthesis is probably programmed late in G_1 and will normally proceed, in the absence of any artificial block, automatically to G_2. The decision to enter G_0 is dependent on the concentration of mitogens or inhibitors in the local environment. Cell concentration is also recognized to be important and may in fact determine the levels of mitogens and/or inhibitors. Once a cell has entered G_0, it can remain in this state until it recognizes an alteration in the feedback messages. Reentry into the cell cycle occurs at some point before S although the precise position in the cell cycle is unknown and may be different for different cell types. Kinetic studies on CFU-S show that the movement of cells from G_0 to the S phase is very rapid so that G_0 in this population must lie very close to the onset of DNA synthesis (Lord, 1981).

It is not surprising, given that control points exist predominantly in G_1, that inhibitory hemopoietic regulators are thought to act at this stage of the cell cycle. NBME-IV(MIP-1α) acts at the G_0–S switch with loss of sensitivity to the inhibitor as the cell approaches the G_1–S interface and complete loss of sensitivity in the S phase (Lord *et al.*, 1979). Similar conclusions were drawn for the tetrapeptide, AcSDKP, which acts on CFU-S in G_0 or early G_1 only (Frindel *et al.*, 1992). By contrast, epidermal growth factor (EGF)-stimulated keratinocytes can be inhibited with TGF-β at any time during G_1 up to the G_1–S boundary (R. J. Coffey *et al.*, 1988; Pietenpol *et al.*, 1990).

The downstream events that follow inhibitor–receptor binding are poorly understood; there are very little published data on MIP-1α, AcSDKP, or pEEDCK. They may function directly or indirectly via the recently recognized tumor suppressor genes. The product of the retinoblastoma susceptibility gene (pRB) for example, has properties of a cell cycle regulator factor (Chen *et al.*, 1989; Goodrich *et al.*, 1991). Regulation of pRB

occurs at the level of phosphorylation and there is strong evidence to suggest that it is the unphosphorylated form of pRB that is responsible for the repression of cellular proliferation (Goodrich *et al.*, 1991; Buchkovitch *et al.*, 1989; Ludlow *et al.*, 1990). The phosphorylation state of pRB is regulated by TGF-β. The addition of TGF-β to lung epithelial cells in mid- to late G_1 prevents the phosphorylation of pRB and leads to cell cycle arrest in G_1 (Lahio *et al.*, 1990). The addition of TGF-β in late G_1 when pRB is already phosphorylated or during S has, however, no effect (Lahio *et al.*, 1990). Further observations indicate that pRB mediates TGF-β regulation of *c-myc* gene expression, and growth inhibition (Pietenpol *et al.*, 1990).

In addition to the G1 restriction points, there is some evidence for regulatory points both in G_2-fission yeast for example (Forsburg and Nurse, 1991), and in DNA synthesis itself (Axelrad *et al.*, 1981). Washing erythroid burst-forming units, which normally proliferate slowly, rapidly increases the proportion synthesizing DNA (Axelrad *et al.*, 1981). Conversely, DNA synthesis falls precipitously (within 20 min) following the addition of negative regulatory protein (NRP) (Axelrad *et al.*, 1983). These experiments suggest that control of DNA synthesis can also take place after cells have entered the S phase.

In conclusion, inhibitory regulators act principally in the G_1 phase of the cell cycle. Within this period, the inhibitors may act by switching cells from G_1 to G_0 and subsequently maintain them in this holding reservoir. This appears to be the case with the active component of NBME-IV (MIP-1α). Alternatively, cell progression through G_1 to S may merely be delayed, with inhibition reversed simply by washing away the inhibitory factor. At present there is no evidence that hemopoietic inhibitory regulators act in G_2 and, apart from the special case of NRP, there is no evidence for effect on cells already in DNA synthesis.

B. Microenvironmental Influences and Self-Renewal

A discussion of proliferation regulation would be incomplete without briefly introducing two further concepts—regulation of growth by self-renewal and microenvironmental influences. Both these concepts are fundamentally involved with stem cell regulation and are intimately related to inhibition of stem cell proliferation.

Despite an apparent random distribution of cells in the bone marrow, it has become increasingly evident that the hemopoietic system is organized along lines similar to other self-renewing tissues, e.g., epithelium (Lord and Hendry, 1972; Lord and Schofield, 1980). This organization ensures that the more primitive hemopoietic progenitors will lie in close proximity to the stromal components. The relationship between hemopoiesis and its

microenvironment has been highlighted in the LTBMC model (Dexter *et al.,* 1978) in which hemopoiesis can be maintained in the absence of exogenously added growth factors. In this system, the cultured marrow first develops an adherent stromal layer in which the more primitive hemopoietic progenitors reside and remain proliferatively quiescent by virture of locally produced feedback regulators, including MIP-1α and TGF-β (Eaves *et al.,* 1993a; Cashman *et al.,* 1990). Movement of cells away from this local environment is associated with enhanced proliferation and differentiation. Similar conclusions can be drawn for epithelial stem cell regulation in relation to their location in the base of the intestinal crypt and the basal layer of the epidermis (Potten and Hendry, 1983).

The importance of the bone marrow microenvironment for growth regulation is not unexpected given the known relationship and interactions of growth factors with extracellular matrix (ECM). This interaction is even more apparent with the proliferation inhibitor, TGF-β, which enhances ECM formation and upregulates the cell adhesion receptors (Ignotz and Massagué, 1987b; Ignotz *et al.,* 1989). Some insight into the link between the microenvironment and stem cell inhibition has come from experimental work on CML grown in LTBMC. There, primitive hemopoietic progenitors are proliferatively quiescent and adhere to the extracellular matrix component, fibronectin, partly through the integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ and the CD44 receptor (Verfaille, 1994).

By contrast, CML ph^{1+} progenitors proliferate rapidly with reduced stromal adherence despite a normal complement of CD44 receptors and α4, α5, and β1 integrins. Verfaillie (1994) hypothesized that dysfunctional activity of one of these receptors may account for the defective adhesion and excess proliferation observed. Activation of the β1 integrin by 8A2, which recognizes and activates CD29, restored adhesion to fibronectin, normalized α5 integrin-dependent adhesion, and restored integrin-independent CD44-dependent adhesion to the CML cells. Interestingly, this upregulation was also associated with reduced proliferation of the CML progenitor populations. Furthermore, adhesion of CML progenitors was enhanced by the addition of TFG-β or MIP-1α (Bhatia *et al.,* 1994). However, no cycling analysis was performed in this experiment. These findings suggest that proliferation regulation of primitive hemopoietic progenitor cells occurs via stromal cell interactions. While MIP-1α may restore adhesive properties to CML cells, however, Eaves *et al.* (1993a) showed that CML progenitor cells are resistant to the proliferation inhibitory actions of MIP-1α. Nevertheless, both MIP-1α and TFG-β inhibit purified normal bone marrow progenitor cell subsets, in the absence of stromal cells (Keller *et al.,* 1994), suggesting that both these factors are products of the stromal cell population, which is important in maintaining the normal quiescent state.

The interactions outlined above are particularly relevant when considering self-renewal as a mechanism for growth control in stem cell populations. Under steady-state conditions, the small population of stem cells must maintain its own numbers while also allowing a proportion of its progeny to differentiate and amplify. This self-renewal probability, p, must by definition be set at or above 0.5 in order to conserve sufficient stem cell numbers for the lifespan of the organism. p values of less than and greater than 0.5 will respectively reduce and increase the stem cell population. Control of stem cell proliferation can therefore take place by one of two mechanisms. The first, which we have already considered, is cell cycle inhibition. The second is the ability to vary the number of daughter cells that remain stem cells, i.e., alteration of p; this is a mechanism that can govern the stem cell growth rate in, for example, hydra without the necessity of varying the length of the cycle.

Since observations suggest that the proliferation rate of hemopoietic cell populations is inversely related to its self-renewal capabilities, it is of interest to consider whether proliferation inhibition itself represents a mechanism to regulate self-renewal and differentiation. Proliferatively quiescent (G_0) stem cell populations are associated with the greatest self-renewal capacity. Induced proliferation and postcytotoxic therapy results in aging of the stem cell population and a reduction in marrow repopulating ability. Maintenance of a proliferatively quiescent stem cell population may therefore represent an evolutionary strategy to maintain the integrity of the genome. Under these circumstances the G_0 state may be viewed as a rest area, allowing cells to perform essential gene housekeeping functions and thereby reducing the risk of developing and propagating potentially damaging DNA mutations.

This possible relationship between proliferation inhibition and self-renewal suggests that feedback regulators may ultimately also control the switch between self-renewal and differentiation and not simply inhibit cell cycling. This hypothesis has recently been highlighted in a cytotoxic protection model using MIP-1α where hemopoietic recovery was enhanced due, at least in part, to improved self-renewal of the CFU-S population (Lord, 1995).

C. Assays

The low frequency of stem cells and early progenitors, together with their lack of distinguishing morphological features, precludes the use of direct methods of analysis such as autoradiography. Recent advances in immunophenotyping and fluorescence-activated cell sorter (FACS) analysis have improved our ability to purify stem cell populations; however, there remains

considerable overlap in phenotype for cell morphology and surface markers. As a result, assays for inhibitory regulators of stem cell proliferation have been based on the properties of colony formation and suicide techniques.

Hemopoietic colonies formed *in vivo* (CFU-S) or on semisolid culture media are clonal, i.e., they can be shown to be derived from a single cell. The number of colonies developing therefore becomes a measure of the progenitor cells in the sample being assayed. Theoretically, if an inhibitor completely suppressed proliferation, then no colony formation would occur. This could be interpreted as evidence for inhibitory regulation but it would also be indistinguishable from cytotoxicity.

A more specific means of analysis is available from suicide assays that exploit the property of specific S-phase-killing cytotoxics such as hydroxyurea (HU), cytosine arabinoside, and [^{3}H]thymidine. With sufficient dose, the incorporation of [^{3}H]thymidine into DNA of the test sample produces multiple double-strand breaks, thus rendering the cells incapable of further proliferation and colony formation. With appropriate controls using the same test sample and "cold" thymidine, the difference in colony numbers becomes a measure of the proliferative status. The addition of a further control group, using the same test cells, but in the absence of the putative inhibitor, allows quantification of the inhibitory effect and excludes any cytotoxicity.

Despite the common use of the suicide assay, a number of pitfalls have been recognized and not infrequently ignored (Maurer, 1981; Lord *et al.*, 1974a). Since the calculation of kill depends on the difference between two colony counts, each with its own error, the error on the kill can be very large. This is readily apparent when observing the variability in hemopoietic progenitor cycling reported in the literature. Several common S-phase cytotoxics may be used in the suicide assay but may not produce directly comparable results as [^{3}H]thymidine does (Lord *et al.*, 1974a). Caution is therefore required in extrapolating the data from one suicide technique to another. The large errors inherent in the assay are not always recognized and appropriate statistical analysis is essential. Contradictory results among laboratories are more likely to arise as a result of an inadequate database rather than methodological differences. An analysis that used hydroxyurea suicide on murine marrow colony-forming cells *in vitro,* for example, confirmed the large number of individual experiments necessary to detect a specific difference (Quesenberry and Stanley, 1990). To detect a specific difference of 25–30% with a probability of less than 0.05, 6–9 experiments are necessary. In order to be sure that a 25–30% difference is not present, 15–21 experiments would be required.

At present the suicide assay provides a useful tool for confirming the effect of potential inhibitors. Investigators familiar with the technique,

however, must also be aware of the possible pitfalls in order to maximize their chances of generating meaningful data.

IV. Inhibitors of Hemopoietic Stem Cell Proliferation

A. Macrophage Inflammatory Protein-1α

The active component of NBME-IV proved to be somewhat elusive and was not identified and characterized until 1990 (Graham *et al.*, 1990). Difficulties with biochemical purification were exacerbated by the lack of a suitable *in vitro* assay system for the target cell(s). The search was greatly simplified following the development of the colony-forming unit-A assay (Lorimore *et al.*, 1990). Preliminary experiments by Graham *et al.* (1990) confirmed the inhibitory effects of NBME-IV on colony growth in the CFU-A assay. Wright *et al.* had already defined a subpopulation of macrophages as the principal source of the inhibitory activity (Wright *et al.*, 1980; Simmons and Lord, 1985) and subsequent screening of the conditioned media from several murine macrophage cell lines identified J774.2 as an effective producer of the inhibitory activity. This activity included inhibition of CFU-A and CFU-S proliferation and retained the cellular specificity demonstrated by NBME-IV by having no effect on proliferation of the more mature GM-CFCs (Graham *et al.*, 1990). Chromatographic separation, sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS–PAGE) analysis, and high-performance liquid chromatography (HPLC) of J774.2-conditioned medium ultimately revealed the inhibitor to be identical to the previously characterized chemokine, MIP-1α (Sherry *et al.*, 1988). MIP-1 (doublet of MIP-1α and MIP-1β) and rMIP-1α, but not rMIP-1β, reversibly decreased the proliferative activity of CFU-A. The inhibitory activity of rMIP-1α was confirmed *in vivo* on CFU-S proliferation and using a neutralizing antibody, it was shown that MIP-1α is functionally and antigenically identical to the proliferation inhibitor in NBME-IV.

MIP-1 was originally recognized in 1988 by Wolpe *et al.*, who observed a third protein with interesting physical properties during the purification and characterization of cachectin/tumor necrosis factor (TNF). The protein, obtained as a doublet, was isolated from a murine macrophage tumor cell line (RAW.264.7) and although it had a molecular mass of 8 kDa on SDS–PAGE, it readily formed large multimeric aggregates of $>2 \times 10^6$ Da on gel filtration. The MIP-1 doublet was subsequently separated chromatographically, with MIP-1α representing the major component (109 amino acids) and MIP-1β the minor component (69 amino acids) (Sherry *et al.*, 1988).

The murine MIP-1α cytokine is a member of a large family of small, inducible and secreted cytokines (Wolpe and Cerami, 1989; Oppenheim *et al.*, 1991). The family members are all basic heparin-binding polypeptides that possess proinflammatory and reparative activity. They are defined by the presence of four conserved cysteine residues and have been subdivided into two groups. The first two cysteine residues are adjacent in the c-c group but are separated by a single amino acid in the c-x-c group (see Table I). Human MIP-1α (LD78) is approximately 75% homologous to murine MIP-1α at the amino acid level and comparison of the gene sequences has revealed approximately 77% homology within the first 350 bp of proximal promotor sequences, suggesting that the protein and its gene regulatory sequences have been conserved in evolution (Yamamura *et al.*, 1989; Blum *et al.*, 1990; Widmer *et al.*, 1991).

The macrophage origin of MIP-1α and its initial recognition as a product of endotoxin stimulation suggested a role as an inflammatory mediator. In addition to hemopoietic cell cycle regulation, MIP-1α is also known to be chemotactic for neutrophils (Wolpe *et al.*, 1988; Wolpe and Cerami, 1989), eosinophils (Rot *et al.*, 1992), monocytes (Wang *et al.*, 1993), and T-cell subsets (Schall *et al.*, 1993) in addition to its actions as a prostaglandin-E_2 (PGE_2)-independent pyrogen (Minano *et al.*, 1991; Davatelis *et al.*, 1989). A role in the immune response is suggested by the induction of MIP-1α gene expression in activated B and T cells (Zipfel *et al.*, 1989; Obaru *et al.*, 1986; Brown *et al.*, 1989). Despite a wealth of experimental data, the proinflammatory actions of MIP-1α have not been borne out in phase I clinical studies using a nonaggregating mutant of LD78 (BB10010; E. Marshall, unpublished observations). There is no clinical experience, however, with the wild-type, LD78 molecule.

1. Polymerization

A problem that rapidly came to light in early work with MIP-1α was one of molecular aggregation. MIP-1α is a rather "sticky" molecule and

TABLE I
The Chemokine Family

c-c structure		c-x-c structure	
Murine	Human	Murine	Human
MIP-1α	LD78	MIP-2	GRO
MIP-1β	ACT-2	Not known	NAP-1(IL-8)
Not known	RANTES	PF4(rat)	PF4
JE	MCAF	Not known	βTG(PBP)
TCA-3	I-309	CRC-2	*IP-10*

although its basic molecular weight is around 8 kDa, it has a strong tendency for noncovalent self-aggregation and displays a wide range of molecular sizes. This initially caused some problems in obtaining reproducible dose-response results and satisfactory interlaboratory comparisons. Aggregation appears to be greatly reduced in high ionic strength buffers and at least one commercial preparation is supplied in acetonitrile in order to maintain its monomeric form (Wolpe and Cerami, 1989).

The importance of this property is not clear. Mantel *et al.* (1993) reported that monomeric MIP-1α *in vitro* was 1000-fold more effective than polymerized material and, in spite of finding that polymerized MIP-1α does not interfere with suppression by monomeric MIP-1α, they came to the conclusion that polymerization of MIP-1α might be a control mechanism that limits the myelosuppresive effects of the monomeric molecule. One year later, the same group confirmed that a 1000-fold lower dose of monomeric MIP-1α, injected *in vivo,* rapidly reduced the cycling and numbers of progenitor cells in the bone marrow and spleen (Cooper *et al.,* 1994).

These findings were contradicted by Graham *et al.* (1994), who found that monomeric, dimeric, and tetrameric mutant MIP-1α molecules were equipotent in stem cell and monocyte shape change assays. They suggested that both aggregated MIP-1α and the aggregated mutants spontaneously disaggregate under assay conditions and function as monomers. They concluded that aggregation is a dynamic and reversible phenomenon that has little impact on bioactivity *in vivo.* Certainly, our own experience with the multimeric wild-type molecule is of good *in vivo* activity (Clements *et al.,* 1992). Subsequently, we tested an extensive range of LD78 mutants with widely varying degrees of molecular aggregation (prepared by British Biotech Pharmaceuticals, Ltd.) both *in vivo* and *in vitro* and found little variation in activity (B. I. Lord, C. M. Heyworth, and B. B. Personnel, unpublished results). From these studies, a nonaggregating tetrameric variant (BB10010) of LD78 with superior solution characteristics has been selected for extensive preclinical testing.

2. Hemopoietic Progenitor Cell Cycle Regulation

The inhibitory effects of MIP-1α appear to be specific for multipotential hemopoietic precursor cells that are intermediate to late in the stem cell hierarchy (see Fig. 3). Early day-12 CFU-Ss appear to be more sensitive to MIP1-α-induced inhibition than the later day-8 CFU-Ss (Graham *et al.,* 1990; Wright *et al.,* 1985; Lord *et al.,* 1992). *In vitro,* MIP-1α inhibits proliferation of primitive BFU-E (Broxmeyer *et al.,* 1991), human CFU-GEMM (Broxmeyer *et al.,* 1990, 1991), and colony formation in populations enriched for primitive hemopoietic progenitor cell subsets (Keller *et al.,* 1994; Broxmeyer *et al.,* 1990). Growth factor-stimulated proliferation of

the multipotent FDCP-mix A4 cell line is also inhibited (Clements *et al.*, 1992). The most primitive hemopoietic stem cells, or preCFU-Ss appear more resistant. MIP1-α did not inhibit the primitive high proliferative potential-CFC when combinations of interleukin-1 (IL-1), stem cell factor (SCF), and granulocyte macrophage colony-stimulating factor (GM-CSF) were used as growth promoters (Schneider and Moore, 1991). Cells that possess LTR potential normally reside in a quiescent state and are resistant to cycle-active drugs. They can, however, be triggered into proliferation by a single dose of 5-fluorouracil (5FU). Using a murine model, Quesniaux *et al.* showed that MIP-1α administered subcutaneously twice daily from day 0 to day 7 was unable to prevent the depletion of LTR stem cells by 5FU despite apparent inhibition of the more mature multipotential progenitor cells (Quesniaux *et al.*, 1993). The authors concluded that MIP1-α had no inhibitory effect on the LTR stem cells *in vivo*. However, one might have anticipated some effect, with CFU-S inhibition itself indirectly blocking the recruitment of preCFU-S populations.

There have been conflicting reports concerning *in vitro* effects of MIP1-α on the more mature, lineage-committed, colony-forming cells. Broxmeyer *et al.* reported that GM-CFCs induced to proliferate by a combination of growth factors are inhibited by MIP1-α (Cooper *et al.*, 1994; Broxmeyer *et al.*, 1990, 1991), an effect reproduced using a 1000-fold lower concentration of monomeric MIP1-α (Cooper *et al.*, 1994). Other groups, including our own, have been unable to show any consistent inhibitory effect of MIP1-α on lineage-committed cell populations (Graham *et al.*, 1990; Keller *et al.*, 1994; C.M. Heyworth, personal communication). In contrast, others have reported that MIP1-α may have a bidirectional effect on hemopoietic progenitor cell subsets (Broxmeyer *et al.*, 1989, 1990; Keller *et al.*, 1994). Broxmeyer *et al.* (1989, 1990) reported that the growth of the earlier progenitor cells, CFU-GEMM and subpopulations of BFU-E, was suppressed by MIP1-α. Paradoxically, the more mature GM-CFC and BFU-E were stimulated by MIP1-α in the presence of suboptimal concentrations of M-CSF and GM-CSF. Clements *et al.* (1992), however, observed stimulation in the presence of GM–CSF but no effect with M-CSF or IL-3. To add further confusion, Keller *et al.* (1994) showed that MIP1-α enhances IL-3, and GM–CSF induced colony formation of normal bone marrow progenitor cells and lineage-negative (Lin^-) progenitors but had no effect on G-CSF or M-CSF-induced colony growth. The significance of these apparent differences is unclear but they most likely reflect subtle variations in assay conditions and perhaps indirect effects of MIP1-α via contaminating accessory cells. It is important to note that the combination of cytokines and therefore the maturational stage of the CFCs, is central to the response observed with MIP-1α. No experiments have shown that MIP-1α has colony-stimulating activity pre se. It is noteworthy that this property of bidirectional hemopoi-

etic growth regulation has also been suggested for another putative inhibitor, TGF-β (see later discussion).

Owing to its effects on highly enriched progenitor cell populations, the inhibitory action of MIP-1α on its target cells is considered to be direct (Keller *et al.*, 1994; Broxmeyer *et al.*, 1990). To rule out any effects from contaminating accessory cells, Lu *et al.* (1993) investigated the effects of several members of the chemokine family on colony formation initiated by $CD34^+$ cells from single bone marrow and umbilical cord blood. Sorted into single wells in the presence of a combination of erythropoietin (Epo), stem cell factor, GM-CSF, and IL-3 in serum and serum-free conditions, proliferation of these cells was directly suppressed by MIP-1α. Inhibitory effects were also seen with the related chemokines MIP-2α, platelet factor 4 (PF4), IL-8, and monocyte chemotactic and activating factor (MCAF) (Table II). The significance of this functional overlap is unclear because some of these related members bind different receptors. They also span the structural families of the MIP-1 and MIP-2 groups (see Table I). Single-sorted cord blood $CD34^+$ cells were much less sensitive to inhibition by these cytokines, possibly as a result of their inherently lower cycling rates (Lu *et al.*, 1993).

The potential role of MIP-1α as a hemopoietic proliferation regulator has been particularly well illustrated in LTBMC, an *in vitro* model that reproduces the symbiotic relationship between the bone marrow stromal cells and the primitive hemopoietic progenitor cells (Dexter *et al.*, 1978). In this system, the primitive progenitor cells reside in the adherent layer and can be distinguished by their ability to generate very large colonies *in vitro* (more than 500 granulocytes and macrophages and more than eight clusters of erythroblasts) (Cashman *et al.*, 1985). The progenitors undergo cyclic oscillation in their proliferative status, each cycle triggered by a weekly change in culture medium (Fig. 4) (Cashman *et al.*, 1985; Eaves *et al.*, 1991; Toksoz *et al.*, 1980), which is a direct consequence of the opposing actions of endogenous inhibitors and stimulators in this system (Eaves *et al.*, 1991, 1993a; Cashman *et al.*, 1985, 1990, 1994; Toksoz *et al.*, 1980).

TABLE II

Members of the Chemokine Family That May Possess Proliferation Inhibitory Properties Against Hemopoietic Cells

MIP-1 Family(c-c)	MIP-2 Family(c-x-c)
MIP-1α	MIP-2α/β
MCAF	IL-8
PF4	

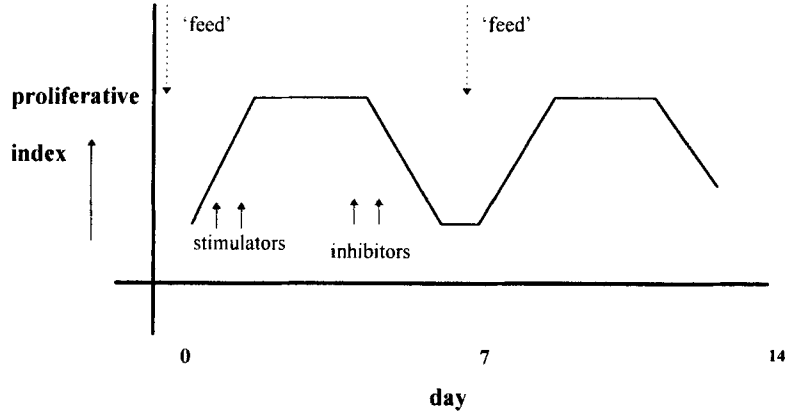

FIG. 4 Schematic representation of the cyclical changes in LTBMC progenitor cell proliferation associated with a weekly change in culture medium ('feed'). Stimulators and inhibitors are generated endogenously within the adherent stromal layer of the culture.

Cashman *et al.* (1990) reported that in the primitive progenitor population, DNA synthesis was triggered by a factor(s) present in horse serum and that the return to quiescence occurred under the influence of a proliferation inhibitor(s). MIP-1α mRNA had previously been identified in extracts of both primary and subcultured LTBMC adherent layers, confirming endogenous production and suggesting a possible role for the molecule in regulating proliferation in LTBMC (Otsuka *et al.*, 1991). Exploiting the antagonistic properties of MIP-1β on MIP-1α, Eaves *et al.* (1993a) reported that the addition of MIP-1β 2–3 days after feeding prevented the primitive hemopoietic progenitors in the adherent layer from returning to a quiescent state. Furthermore, the effect of exogenously added MIP-1β was itself overcome by the simultaneous addition of MIP-1α. The results using MIP-1β suggest that the return to quiescence of primitive progenitor cells occurs under the influence of endogenously produced MIP-1α. In agreement with the *in vitro* colony data, exogenously added MIP-1α did not block DNA synthesis in the more mature progenitors. Neither does it appear to be the only inhibitor present in the LTBMC system because both TGF-β (Cashman *et al.*, 1990) and AcSDKP (Cashman *et al.*, 1994) appear to act similarly. The addition of TFG-β antibody or MIP-1β to the LTBMC resulted in an increase in cycling of primitive progentior cells, suggesting that a combination of TGF-β and MIP-1α is required to maintain progenitor cell quiescence (Cashman *et al.*, 1990; Eaves *et al.*, 1993a), a feature that potentially has important clinical implications.

The potential importance of inhibitor cooperation is highlighted in CML. In contrast to normal progenitor cells, the primitive CML progenitor cells

are in a state of continuous turnover, irrespective of their location (marrow or blood) or differentiative potential (A. C. Eaves *et al.*, 1986; C. J. Eaves *et al.*, 1993b). This situation can be reproduced in LTBMC, thus permitting investigation of the underlying mechanisms. The results confirm the hypothesis that malignant transformation is associated with escape from the normal "braking" influence of inhibitory factors and not necessarily excess stimulation. The addition of MIP-1α (100ng/ml) around the time of feeding to CML–LTBMC did not produce any antiproliferative effect on the primitive CML granulopoietic and erythroid progenitors in the adherent layer (Eaves *et al.*, 1993a). An identical dose inhibited the normal counterpart in parallel cultures. The loss of MIP-1α sensitivity is in marked contrast to TGF-β, which specifically and reversibly arrests primitive (not mature) CML progenitor cells. This aberrant response of CML progenitor cells to MIP-1α suggests the presence of a signaling pathway that is presumably blocked or altered by the action of the BCR-ABL gene product. With the recognition that MIP-1α enhances self-renewal (Lord, 1995; Verfaille *et al.*, 1994), it is tempting to speculate that the dramatic reduction in the self-renewing capability of CML progenitor cells is perhaps related to MIP-1α resistance.

The use of the suicide technique as a means of evaluating potential proliferation inhibitory actions serendipitously provides a model of myeloprotection that has been both recognized and actively pursued. The practicality of this approach was first demonstrated *in vivo* by Lord *et al.* in an experimental model using repeated treatments with hydroxyurea (Lord and Wright, 1980). Hydroxyurea is a cytotoxic drug that kills cells in DNA synthesis and blocks further entry of non-S-phase cells into DNA synthesis for about 7 hr before a semisynchronized cohort of cells is released into the next period of DNA synthesis (Hodgson *et al.*, 1975). CFU-S normally proliferate slowly so that a single dose of HU kills relatively few cells. However, more mature cells that are proliferating rapidly are killed, their loss resulting in recruitment of the quiescent CFU-S population into DNA synthesis. A second dose of HU in mid-S phase (about 7 hr) kills a large proportion of proliferating CFU-S, with recovery to normal steady-state numbers occurring in about 7 days (Fig. 5) (Lord *et al.*, 1992). The addition of MIP-1α as a CFU-S-specific proliferation inhibitor theoretically should prolong the HU-induced block and therefore protect the CFU-S population against a further dose of the cytotoxic drug. In mice receiving 10 and 5 μg of MIP-1α at 3 and 6 hr, respectively, the femur contained 40% more CFU-S 3 days after the start of treatment than did those treated with HU alone (Lord *et al.*, 1992). Recovery to normal or supranormal levels was complete in 5 days. Increasing the doses to 15 + 5 μg or 15 + 15 μg offered full protection against the HU regimen, CFU-S numbers being normal or supranormal as early as 3 days. A similar study by Dunlop *et al.* (1992) using cytosine arabinoside, another S-phase cytotoxic agent, confirmed this pro-

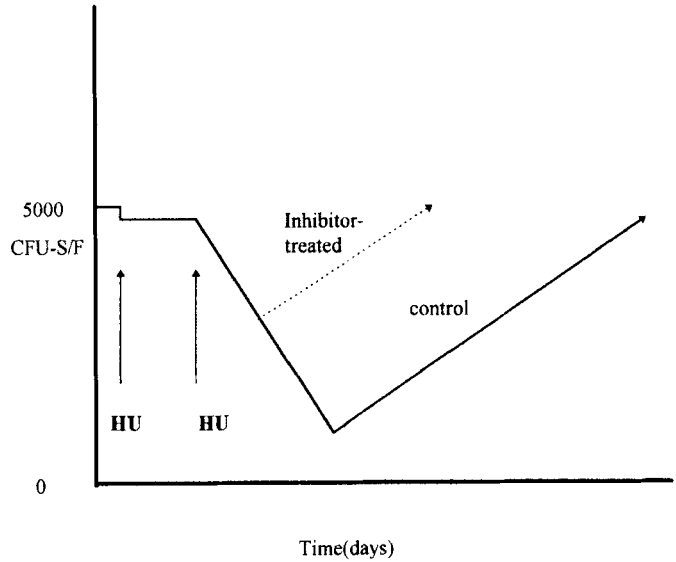

FIG. 5 Schematic representation of the CFU-S (stem cell) loss after repeated S-phase cytotoxic drug administration and the potential enhancement of recovery following drug use with a proliferation inhibitor.

tective role over the progenitor cells. In addition, there was an earlier neutrophil recovery, presumably led by the increased progenitor pool size, itself a consequence of protection and possibly enhanced self-renewal capacity.

The application of hemopoietic stem inhibitors to clinical practice offers exciting potential. The more rapid recovery of hemopoietic cells following cytotoxic insult should allow escalation of the intensity of delivered dose and ultimately may improve tumor response and possibly patient survival. This ability to allow a greater chemotherapeutic dose intensity is currently the subject of several phase II clinical studies evaluating the use of MIP-1α (BB10010) combined with chemotherapy.

The intimate relationship between proliferation inhibition and stem cell self-renewal has been outlined in an earlier section. Hints from the preliminary Ara-C/NBME-IV protection experiments *in vitro* suggested that during the recovery phase, inhibitor-treated cultures outperformed the controls (Lord *et al.*, 1987). Similarly, more rapid regeneration of the CFU-S population followed suboptimal protection treatment from hydroxyurea *in vivo* and it was speculated that secondary effects on the self-renewal and differentiation patterns of early stem cells might be involved (Lord *et al.*, 1992). Subsequent experiments measuring the generation of secondary CFU-S in

spleen colonies have corroborated the improved self-renewal quality of the post MIP-1α-treated CFU-S population (Lord, 1995), and MIP-1α treated LTBMC showed better maintenance of human LTC-ICs (Verfaille *et al.*, 1994). These results provide the rationale for using MIP-1α as a chemoprotectant and stem cell self-renewal enhancer during non-S-phase cytotoxic and radiation therapy. This approach has recently been confirmed experimentally: MIP-1α-protected marrow maintained its recovery potential much more completely over at least four cycles of sublethal γ irradiation (Lord *et al.*, 1996).

3. The MIP-1α Receptor(s)

The pleiotropic action of MIP-1α in inflammation, chemotaxis, and hemopoietic cell cycle regulation has understandably led to considerable difficulties in isolating the MIP-1α receptor(s). The diverse effects of the molecule and overlapping actions with several related chemokine members suggest that several receptors may exist, each specific for a given effect.

Oh *et al.* were the first to identify a receptor for MIP-1α on the T-cell line, CTLL-R8 and the LPS-stimulated macrophage cell line, RAW 267.7 (Oh *et al.*, 1991). Scatchard analysis indicated a single class of high-affinity receptor with approximately 1200 binding sites per Con A-stimulated CTLL-R8 cell and approximately 380 binding sites per RAW 264.7 cell. Additional receptors for MIP-1α have since been identified on human monocytes (Wang *et al.*, 1993), polymorphonuclear leukocytes (Gao *et al.*, 1993), basophils (Bischoff *et al.*, 1993), eosinophils (Van Riper *et al.*, 1994), and the HL60 cell line (Van Riper *et al.*, 1994). These receptors, however, do not appear to be unique for MIP-1α, having similar binding affinities with other members of the chemokine family, including RANTES and monocyte chemoattractant protein-1(MCP-1/MCAF) (Wang *et al.*, 1993; Gao *et al.*, 1993; Bischoff *et al.*, 1993; Van Riper *et al.*, 1994). MIP-1β, the second component of the MIP-1 doublet, also shares a common receptor with MIP-1α (Wang *et al.*, 1993) and it is this, together with similar binding affinities for both MIP-1α and MIP-1β, that presumably accounts for the reported antagonism of MIP-1β for MIP-1α's proliferation inhibitory properties.

The search for MIP-1α receptors on hemopoietic stem cells is limited by the relative infrequency and heterogeneous nature of this population. The murine multipotent FDCP-MIX cell line, however, possesses MIP-1α receptors that appear to be specific for MIP-1α and its related inhibitory chemokines (Graham *et al.*, 1993). In contrast, the MIP-1α receptor on the human myeloerythroleukemic cell line, K562, also binds noninhibitory chemokines e.g., RANTES, suggesting a separate receptor for inflammatory mediation.

The lack of a readily available MIP-1α receptor-bearing cell has hindered the flow of information on the signal transduction pathways that are linked to receptor binding. Furthermore, the elucidation of these pathways may be hampered by the variable responses elicited by MIP-1α on different cell populations. Evidence is available implicating both Rb and c-myc, in the cellular response to TGF-β (Pietenpol *et al.,* 1990) but there is currently no information concerning oncogene expression with MIP-1α. Preliminary work on anti CD3-stimulated T cells indicates that MIP-1α-induced inhibition is associated with decreased phosphorylation of MAP kinase, reduction of $p56^{lCK}$ autophosphorylation, and elevation of diacyl glycerol levels (Zhou *et al.,* 1993). The significance of these findings is still to be determined.

The sparsity of data on mechanisms of inhibition ultimately means the subject remains open to speculation. Growth factor antagonism, via receptor downregulation, or a reduction in the intracellular synthetic pathways for growth factors, may represent one possible mechanism. IL-2 is known to be a T-cell autocrine growth factor and downregulation of both IL-2 mRNA and receptors has been documented following MIP-1α binding to these cells (Zhou *et al.,* 1993). The molecular interactions of the other proliferation inhibitors are equally vague and little information is available to account for the apparent functional overlap. Receptor sharing between these molecules, however, appears unlikely as all are structurally dissimilar. TGF-β was shown to downregulate MIP-1α receptor numbers of FDCP-mix A4 cells without a change in the affinity of remaining receptors (Graham *et al.,* 1993), suggesting that MIP-1α may merely be a weak contributor to the overall physiological inhibition of stem cells. However, this is difficult to justify in light of LTBMC data that reveal a dramatic increase in progenitor cell proliferation with MIP-1β and anti MIP-1α antibodies despite the likely presence of physiological concentrations of TGF-β (Eaves *et al.,* 1993a).

The cellular response triggered by MIP-1α binding is associated with a rapid rise in cytosolic free Ca^{2+} (Gao *et al.,* 1993; Bischoff *et al.,* 1993; Van Riper *et al.,* 1994; Sozzani *et al.,* 1993) that is sensitive to pertussis toxin. This finding, indicating activation via a G-protein-coupled receptor(s) (Bischoff *et al.,* 1993; McColl *et al.,* 1993; Neote *et al.,* 1993), was confirmed by Gao *et al.* (1993) who cloned the cDNA for the human PMN receptor. The gene for the receptor was functionally expressed in *Xenopus* oocytes and mapped to human chromosome 3p21. The receptor was characterized as a seven-transmembrane spanning receptor belonging to the superfamily of G-protein-linked receptors that includes the related IL-8 receptor. Interestingly, the open reading frame US28 of the human cytomegalovirus (CMV) encodes a protein that is similar in sequence to the MIP-1α/RANTES receptor, hinting at a link with human CMV infection and viral replication (Gao *et al.,* 1993).

4. Effects on Nonhemopoietic Tissues

The spatial organization and growth regulatory mechanisms concerning the pluripotent stem cell are not unique to hemopoiesis, but are mirrored in other self-renewing tissues, including epidermis, gut mucosa, and seminiferous epithelium. Although the feedback systems are less well defined, there is some evidence that feedback regulators such as MIP-1α may function as pan-stem cell inhibitors without true tissue specificity. Several investigators have hinted at inhibitory effects of MIP-1α on clonic epithelial cells but this is largely unsubstantiated (Graham and Pragnell, 1992; Lord *et al.*, 1993). The recognition that MIP-1α transcripts are present in epidermal Langerhan's cells suggests a further potential role in the regulation of keratinocyte proliferation (Parkinson *et al.*, 1993). This hypothesis is strengthened by the knowledge that Langerhans' cells originate in hemopoietic tissue and have a special spatial relationship to the keratinocyte stem cell of the epidermal proliferative unit (Parkinson *et al.*, 1993; Breathnach, 1991). Parkinson *et al.* (1993) documented an inhibitory effect on MIP-1α on keratinocyte proliferation *in vitro* using partially purified recombinant murine MIP-1α, but it could not be reproduced using pure bacterially produced MIP-1α. Furthermore, the addition of antimouse MIP-1α neutralizing antibody did not stimulate the epidermal keratinocytes. A physiological role for MIP-1α in maintaining keratinocyte stem cell quiescence therefore seems unlikely.

The seminiferous epithelium provides an alternative model for studying the mechanisms of stem cell proliferation and differentiation and is the only mammalian tissue that contains both mitotic and meiotic cell cycles. Work by Hakovirta *et al.* (1994) suggests that MIP-1α may be a local regulator of both mitotic and meiotic DNA synthesis during spermatogenesis. Once again the molecule appeared to have a bidirectional effect, and as with hemopoiesis, the most sensitive phases—intermediate spermatogonia—were inhibited. Paradoxically, the most primitive type $A_{2\text{-}4}$ spermatogonia were stimulated.

At present, the role of MIP-1α in the regulation of nonhemopoietic stem cell proliferation is unclear. Further investigation is warranted, particularly because stem cell inhibition may represent a very real therapeutic option in ameliorating nonhemopoietic toxicity following cytotoxic therapy.

B. Transforming Growth Factor-β

Both MIP-1α and TGF-β show a remarkable degree of functional overlap despite the lack of structural similarity. Not only do these molecules cooperate in primitive hemopoietic cell cycle regulation, but they also share com-

mon properties as inflammatory mediators derived from monocytic sources. This functional overlap is all the more intriguing because both molecules have direct cellular actions and there is no current evidence suggesting a common inhibitory signaling pathway. The significance of this relationship remains uncertain but it may not be completely unexpected given the degree of functional overlap that is apparent with the hemopoietic growth factors.

TGF-β was the first cell-cycle inhibitory factor gene to be cloned and sequenced and its mRNA expression detected in a mammalian cell (Derynck *et al.*, 1985). The molecule, unlike other hemopoietic stem cell inhibitors, is both ubiquitous and pleiotropic in action, with diverse effects on a wide spectrum of cell phenotypes. Specific receptors for TGF-β have been found on almost all mammalian cells and it is this relative nonspecificity that brings its physiological role in unperturbed hemopoiesis into question.

TGF-β belongs to a superfamily of factors intimately involved in many aspects of cell growth, differentiation, and proliferation. Other closely related members include the inhibins, activins, müllerian-inhibiting substance, and decapentaplegic product. The effects of TGF-β on cell growth are specific to the cell type and environmental conditions, but generally TGF-β is stimulatory for cells of mesenchymal origin and inhibitory for cells of epithelial or neuroectodermal origin (Moses *et al.*, 1985; Roberts *et al.*, 1985). More recently, TGF-β has been postulated to be a bidirectional regulator of hemopoietic progenitor cell proliferation (Keller *et al.*, 1994).

TGF-β was originally purified to homogeneity from human platelets (Assoian *et al.*, 1983), placenta (Frolik *et al.*, 1983) and bovine kidney (Roberts *et al.*, 1983). The molecule is a disulfide-linked dimer consisting of two identical chains of 112 amino acids with an approximate molecular weight of 25 kDa on a nonreducing SDS–PAGE. TGF-β is in fact not a single entity but rather a term encompassing at least five isoforms, each sharing considerable sequence homology and defined as TGF-β1–TGF-β5. Sequencing of the cDNAs for the five isoforms indicates that each is initially synthesized as part of a larger precursor molecule containing the mature form of TGF-β at the C-terminal. Proteolytic cleavage of the precursor occurs just after a stretch of four basic amino acids and is mediated by a subtilisin-like protease (Barr, 1991). The two portions of the precursor remain together following cleavage and are secreted as a biologically inactive, noncovalently bound complex consisting of dimers of both the precursor remainder and mature TGF-β (Jalkowlew *et al.*, 1988).

In vitro, TGF-β activation can result from exposure to extreme pH (<4 or >9), chaotropic agents (sodium dodecyl sulfate, urea), or proteolytic cleavage by plasmin (Lawrence *et al.*, 1985; Lyons *et al.*, 1988). The *in vivo* mechanism of activation is unknown but clearly represents an important regulatory step for controlling and localizing the effects of the molecule. Cleavage of the precursor by cell-derived proteases appears to be the most

likely step. The production of an acidic environment in sites of would healing and bone resorption may also contribute. The importance of this regulatory step is illustrated by the differential activation of TGF-β by normal epithelial cells and their neoplastic counterparts (Keski-Oja *et al.*, 1987). Human A549 lung carcinoma cells are unable to activate the latent TGF-β precursor protein which they secrete, despite the fact that they generate TGF-β, bear receptors for TGF-β, and are susceptible to growth inhibition by exogenously added TGF-β. These cells have therefore lost the ability to activate latent TGF-β, a potential mechanism to escape growth regulation.

All the TGF-β isoforms share a high degree of sequence homology ranging from 64 (TGF-β1 vs. TGF-β4) to 82% (TGF-β2 vs TGF-β4) (Kondaiah *et al.*, 1990). Individual TGF-βs are extremely well conserved with >97% identity between the mature TGF-β1 sequences from various mammalian and avian species. Conservation is also maintained at the genomic level although various TGF-β genes are located on separate chromosomes in both man and mouse (Barton *et al.*, 1988). Despite the functional and structural similarities, the multiplicity of TGF-β forms and sequence conservation within each form through evolution suggest important specific roles for each of the TGF-βs.

1. Effects on Hemopoietic Progenitor Cells

TGF-β is produced by a variety of hemopoietic cells, including platelets, monocytes, and T lymphocytes. Immunohistochemical staining indicates that it is locally produced in ears of active hemopoiesis, including bone marrow and fetal liver (Ellingsworth *et al.*, 1986). *In situ* hybridization studies have shown that fetal liver produces large amounts of TGF-β mRNA (Wilcox and Derynck, 1988). These observations suggest that TGF-β may act has a regulator of hemopoietic cell growth. However, the production of a growth inhibitor by a rapidly proliferating tissue like fetal liver, which is known to produce stimulatory activity (Cork *et al.*, 1986), would appear to be somewhat paradoxical unless TGF-β were merely acting as a braking influence in this setting. It seems more likely, given the importance of TGF-β in cell differentiation and embryogenesis, that its intense expression in fetal tissue relates to its role as a coordinator of morphogenesis and remodeling rather than as a cell cycle inhibitor. Furthermore, the need for precursor activation means that the detection of TGF-β transcripts in tissues does not necessarily directly translate into activity.

TGF-β has been reported to produce both stimulatory and inhibitory effects on selected hemopoietic cell populations. In general, TGF-β inhibits a wide range of stem and progenitor cell subsets with similar potency and may have a growth stimulatory effect on certain lineage-committed cells.

Like MIP-1α, however, the effect of TGF-β on hemopoietic progenitor colony growth *in vitro* is dictated by the growth factors present, and therefore, the maturational age of the colony-forming cell. *In vitro,* TGF-β is a potent inhibitor of erythroid an granulopoietic precursor cells. Colony inhibition has been reported for multipotential CFU-GEMM (Keller *et al.,* 1988, 1994; Sing *et al.,* 1988; Ruscetti *et al.,* 1991) and the most primitive progenitor cell assayed *in vitro,* HPP-CFC (Keller *et al.,* 1990, 1994; Ruscetti *et al.,* 1991). *In vivo,* TGF-β inhibits CFU-S proliferation (Ruscetti *et al.,* 1991; Keller *et al.,* 1990; Goey *et al.,* 1989; Migdalska *et al.,* 1991) and IL-3-responsive bone marrow progenitor cells (Keller *et al.,* 1990; Goey *et al.,* 1989; Migdalska *et al.,* 1991).

Keller *et al.* (1994) investigated the spectrum of activity of TGF-β *in vitro* utilizing normal murine bone marrow progenitor cells and progenitor cell subsets enriched for long-term repopulating cells. It inhibited IL-3- and CSF-1-induced colony formation from cells lacking lineage-specific antigens (Lin^-) and directly inhibited the more primitive Thy-1^{LO} lin^- cells regardless of the cytokine used to stimulate growth (IL-3, GM–CSF, or CSF-1). TGF-β also inhibited additional primitive stem cell subsets, including rhodamine-dull and Lin^- Sca-1^+ cells, in contrast to MIP-1α, which appeared to have no effect on these populations.

In the presence of combinations of growth-stimulating cytokines (Eop, SCF, GM–CSF and IL-3), the growth of single-sorted human bone marrow $CD34^{3+}$ cells is directly inhibited by TGF-β. Proliferation of individual cells in inhibited by TGF-β when stimulated to proliferate (Lu *et al.,* 1993). The CD34 antigen, however, is expressed on a wide range of hemopoietic progenitor cells, including multipotential and more lineage-restricted progenitor cells. Work by Lardon *et al.* (1994) suggests that the effect of TGF-β on all $CD34^+$ cells may not be identical. Using highly purified human bone marrow progenitor cells ($CD34^+$ sorted fraction), they have evaluated the immediate kinetic response to the inhibitory effects of TGF-β. Very primitive $CD34^{3+}$ progenitor cells that are recruited into cell cycle by the early-acting factors IL-1 and SCF are arrested by TGF-β specifically in the 6_1 phase of the second cycle. Addition of TGF-β to $CD34^+$ progenitor cells responding to IL-3 alone resulted in a general growth retardation but without apparent specific accumulation at any point of the cell cycle. Within the $CD34^+$ compartment there also appeared to be a subset of IL-3-responsive cells that were not inhibited by the addition of TGF-β.

In human LTBMCs TGF-β inhibited primitive hemopoietic progenitor cell proliferation (Cashman *et al.,* 1990; Eaves *et al.,* 1991) and a physiological role in steady-state hemopoiesis was suggested by the detection of TGF-β mRNA expression in the stromal cells of the culture (Eaves *et al.,* 1991). Conversely, TGF-β antibody stimulated or quiescent cultures, respectively (Eaves *et al.,* 1991). Thus, if LTBMC is a true reflection of *in vivo* regulation,

these results suggest that TGF-β plays a role in regulating the proliferation of primitive hemopoietic stem cells. As previously noted, similar findings were reported for MIP-1α in the LTBMC, suggesting that inhibitors may act in concert to maintain stem cell quiescence.

The more mature, lineage-restricted progenitor cells are resistant to the inhibitory effects of TGF-β and in combination with GM-CSF, at least, their growth may be promoted, the size of the GM colonies increasing three- to fivefold (Ruscetti *et al.*, 1991). The size increase is primarily due to extra amplification in the production of mature granulocytes. In suspension cultures, the same combination resulted in markedly enhanced neutrophilic differentiation (Ruscetti *et al.*, 1991). TGF-β has also been reported to stimulate human myeloid progenitor cells. In the presence of different colony-stimulating factors, TGF-β enhanced human day-7 but not day-14 GM–CFC colony formation (Ottman and Pelus, 1988).

The pharmacodynamics of exogenously administered TGF-β are reported to be unfavorable due to binding to serum components, including α_2-macroglobulin (O'Connor and Wakefield, 1987) and first-pass hepatic extraction (Coffey *et al.*, 1987). As a consequence, drug evaluation *in vivo* may be compromised by the route of administration. Goey *et al.* (1989) devised a model to analyze the effects on basement membrane (BM) progenitor cells by administering TGF-β locoregionally. Direct injection into the femoral artery circumvented first-pass hepatic clearance of the molecule and maintained optimal local biodistribution to the bone marrow. An intrafemoral bolus of TGF-β significantly reduced tritiated thymidine incorporation by BM cells, particularly by the earlier CFU-GEMM population, although the number of colonies formed was very low in both treated and control mice. A dose-response experiment confirmed 1–5 μg/mouse (40–200 μg/kg) to be an effective dose range and inhibitory effects occurred 3 to 24 hr following TGF-β treatment (Goey *et al.*, 1989). Migdalska *et al.* (1991) extended these findings and confirmed activity via the intraperitoneal route. Mice were administered TGF-β twice daily for 5 consecutive days with doses ranging from 1 to 250 μg/kg/day. doses of 50 and 100 μg/kg/day resulted in a decrease in bone marrow cellularity that reaches a nadir by 6.5 days (24 hr after the final TGF-β injection). A corresponding reduction was seen in the numbers of day-8 and day-11 CFU-S and IL-3 responsive progenitor cells. In contrast to Goey's findings, cell cycle inhibition occurred gradually so that only by day 5 were the day-8 and day-11 CFU-Ss quiescent. Examination of the small intestine revealed a significant reduction in crypt and villus size, hinting at a similar antiproliferative effect on gut mucosa. This "slow brake" effect of TGF-β on proliferation is dissimilar to the inhibition induced by MIP-1α and points to a different mode of action. Hampson *et al.* (1991) showed that NBME-IV inhibited thymidine incorporation in FDCP-mix A4 cell lines with maximal effects within 16 hr,

while TGF-β was still ineffective. Indeed, other investigators also reported inhibitor effects on similar factor-dependent cell lines only following at least 48 hr of exposure (Keller *et al.*, 1988; Ohta *et al.*, 1987).

The study performed by Migdalska was associated with significant TGF-β-induced toxicity and morbidity. Mice receiving repeated TGF-β, 250 μg/kg/day, developed dramatic weight loss and ultimately died. The potential adverse effects of TGF-β, following chronic administration, have also been evaluated by several other groups. Carlino *et al.* (1990) injected TGF-β1, 25 μg/mouse (1.25 mg/kg/day) subcutaneously for 14 days. Red blood cells and platelets were reduced but there was an increase in total white blood cells (WBCs) which correlated with increased granulopoiesis in the spleen and bone marrow. The effects were consistent with *in vitro* data but the increased dose (four times the Migdalska dose) and limited toxicity suggest that subcutaneous injection may not be an effective route of administration.

Chuncharunee *et al.* (1993) confirmed the suppressive effect on erythropoiesis using a lower dose of 7.5 μg/mouse (375 μg/kg/day) given intraperitoneally daily for 6 days. The suppression was manifested by a decline in reticulocyte count, marrow erythroblasts, and marrow and spleen CFU-E. A reduction in platelets was also observed, presumably reflecting the inhibitory effects of TGF-β on megakaryopoiesis (Greenberg *et al.*, 1990). The decline in erythropoiesis was associated with falling erythropoietin levels (which were undetectable by 5 days) and an up to 200-fold increase in tumor necrosis factor-α in the blood that was linearly related to the dose of TGF-β. Mice injected with TGF-β exhibited progressive and profound weight loss comparable to the study by Migdalska with a 25% mortality rate by the seventh injection. The authors concluded that the combined findings of a cachexia syndrome and dyserythropoiesis could be explained by the indirect effects of TGF-β, via TNF. This finding may also explain the delayed onset of action of TGF-β seen in other studies. Whatever the mechanism of action, the toxicity associated with TGF-β is likely to prove a major obstacle in the design and development of clinical trials with this agent.

The ability to inhibit primitive progenitor cell cycling has been shown to be of potential clinical importance in protecting these cell populations from S-phase-specific cytotoxic agents. TGF-β may offer protection similar to that demonstrated with MIP-1α in the HU model (see earlier discussion) although the timing of administration may be quite different. However, TGF-β-induced toxicity remains a significant drawback. using a repeated cytotoxic treatment model in mice, Molineux *et al.* (1994) were able to show that pretreatment with stem cell factor sensitizes both hemopoietic progenitor cells and gut epithelium to an injection of 5-fluorouracil (5FU). In groups pretreated with SCE, all the mice died after two cycles of 5FU. The timing of death (14–20 days) and the ability to rescue all mice with bone

marrow transplantation confirmed bone marrow failure as the principal cytotoxic effect. The simultaneous addition of TGF-β abrogated the toxicity of SCF pretreatment with no significant difference in survival compared with the group treated with 5FU alone (70–80%). The investigators concluded that the mechanism of this protection was possibly related to down-regulation of SCF receptors by TGF-β and a resultant proliferation block of hemopoietic progenitors. However, the protective effect is intriguing because the survival advantage was significantly greater compared with the modest protection in CFU-S numbers seen after two cycles of 5FU. These experiments appeared to give a modest protective effect to gut mucosa with an increased villus height and crypt mitotic index. This was possibly related to the use of TGF-β3, which has similar effects, but is potentially less toxic than the related isoform-β1. Despite the short-term administration of TGF-β3, however, a cachexia syndrome was once again noted.

2. Receptors

TGF-β receptors were initially characterized by cell-surface cross-linking experiments using ^{125}I-TGF-β. Two glycoproteins (receptors I and II) of 50 and 70–100 kDa, respectively, bind TGF-β with high affinity and are ubiquitously present at low levels in mammalian and avian cells (Cheifetz *et al.*, 1986; Massagué and Like, 1985). Many cells also possess a larger type III receptor (betaglycan) that binds TGF-β with less affinity and is not detectable on various cell types that respond to TGF-β, including hemopoietic progenitor cells (Massagué and Like, 1985). Cross-linking experiments have identified additional cell-surface proteins that bind TGF-β (type IV to type IX receptors)(Massagué, 1992), but most attention remains focused on the type I and II receptors which have now been cloned and identified as transmembrane Ser/Thr kinases (Matthews and Vale, 1991; Attisano, 1994). Although the receptor interactions are not yet fully elucidated, current evidence suggests that the functional TGF-β receptor is in fact a heteromeric complex of type II and type I receptors.

Differential expression of the TGF-β receptors on hemopoietic cells may account for the diverse, bidirectional effects on these populations. Acquisition or the presence of a functional type II receptor may be necessary for the antiproliferative effect of TGF-β. Transfection of a truncated type II receptor lacking a cytoplasmic domain into Mv1Lu cells blocks the antiproliferative effect of TGF-β without affecting several other responses (Chen *et al.*, 1993). This uncoupling can also be seen in several cell lines that lack a functional type II receptor and are resistant to the inhibitory effects of TGF-β, yet retain the ability to respond by increased synthesis of extracellular matrix molecules (Fafeur *et al.*, 1993). Interestingly, the latter property has also been observed in several tumor cell lines that are

not inhibited by TGF-β, yet respond by increasing extracellular matrix synthesis (Laiho *et al.*, 1991).

Murine and human hemopoietic progenitor cells treated with IL-3 or GM–CSF acquire TGF-β receptors in a dose- and time-dependent manner (Falk *et al.*, 1991). Initially, predominantly type I receptors accumulate, then all three binding proteins. This suggests that TGF-β is unlikely to be involved in maintaining the G_0 status of quiescent stem cells but rather to act as a decelerating force on proliferating progenitors (Ruscetti *et al.*, 1991). This is supported by *in vitro* studies designed to evaluate the growth inhibitory properties of TGF-β on hemopoietic progenitor cells. While TGF-β consistently inhibited growth factor-induced colony formation by human bone marrow progenitor cells, it exerted no inhibitory effect on unstimulated cells (Sing *et al.*, 1988).

3. Mechanisms of Inhibition

The intracellular signaling pathways effecting the antiproliferative actions of TGF-β remain poorly understood although what is known is considerably more extensive than that for other cell cycle inhibitors. Maintenance of a proliferatively quiescent hemopoietic progenitor populations is intimately related to microenvironmental–progenitor cell interactions. Although TGF-β has a direct inhibitory effect on single-sorted progenitor cells *in vitro* (Lu *et al.*, 1993), the molecule also has a polyfunctional role in regulating extracellular matrix deposition and cell adhesion molecules (Massagué, 1990; Ignotz and Massagué, 1987b). Integrin expression may be involved and its upregulation may represent one of many mechanisms by which TGF-β promotes local cellular interactions that terminate in cell cycle inhibition (Ignotz and Massagué, 1987a,b; Ignotz *et al.*, 1989; Massagué, 1990).

In addition to its microenvironmental effects, TGF-β is also known to regulate cytokine receptor numbers with receptor downmodulation on murine hemopoietic cell lines and murine factor-dependent myeloid progenitor cell lines reported for IL-1 (Dubois *et al.*, 1990), GM-CSF, IL-3, and G-CSF (Ruscetti *et al.*, 1991). The rate of receptor downmodulation is variable with several cytokine receptors, including GM-CSF, Il-3 and G-CSF, reduced to a minimum by 72–96 hr (Ruscetti *et al.*, 1991), which is in agreement with *in vivo* data showing delayed inhibition (Migdalska *et al.*, 1991). More recently, TGF-β1 was shown to downregulate SCF receptors on acute myeloid leukaemia (AML) blasts (De Vos *et al.*, 1993), providing a possible explanation for the TGF-β protective effect in the 5FU–SCF model described by Molineux *et al.* (1994).

Considerable advances have been made concerning the intracellular signaling pathways that follow TGF-β–receptor binding. TGF-β is known to play a role in determining the phosphorylation state of Rb (Lahio *et al.*,

1990). It maintains Rb in its underphosphorylated form, which leads to a block on growth, preventing the G_1-S transition from taking place. More recently, investigators have focused on the G_1 cyclins and associated cyclin-dependent kinases (cdk's) that act upstream of Rb. TGF-β appears to have complex actions on cdk4, a major partner of cyclin D_1 and D_2 and an essential element in G_1 progression (Geng and Weinberg, 1993). More recently, the intracellular free level of one of the growing family of cdk inhibitors, $p27^{kip1}$ (binds to and inhibits cdk2 and cdk4), was shown to be elevated following TGF-β exposure (Polyak *et al.,* 1994).

Of further interest and complexity is the recent recognition that TGF-β may interact with P53 inhibitory signaling. The nuclear phosphoprotein P53 is known to play a significant role in preventing malignant transformation. Its inactivation, either by point mutation or indirect mechanisms, appears to be essential for the progression of most tumor types. A loss of response to the growth inhibitory action of TGF-β, well recognized in many human cancer cells in cultures, has been correlated with P53 mutation. Further support for this hypothesis comes from TGF-β resistant epithelial cell lines induced by the introduction of vectors expressing mutant P53 (Reiss *et al.,* 1993). The mode of P53-induced TGF-β resistance is unknown but presumably lies at the level of cdk activation. Recent work by Blaydes *et al.* (1995) suggests that P53 is not a mediator of TGF-β action but more likely P53 and TGF-β activate independent inhibitory signal pathways that converge at the level of cdks controlling the G–S transition.

4. Nonhemopoietic Effects

TGF-β has far-reaching effects on many tissues, the nature of the response depending on cell type, growth conditions, state of cell differentiation, and the presence of other growth factors. The TGFs-β are the most potent growth inhibitors known and all forms tested display reversible growth inhibitory activity on normal as well as transformed epithelial, endothelial, fibroblast, neuronal, lymphoid, and hemopoietic cells. Its multifunctional role also includes diverse actions on immunomodulation, wound healing, and bone remodeling. These actions have been well described in several comprehensive reviews on TGF-β (see Massagué, 1991).

C. Hemoregulatory Pentapeptide (pEEDCK)

In 1968, Rytomaa and Kiviniemi (1968a) identified an inhibitor of myelocyte proliferation that was present in medium conditioned by mature granulocytes. The "granulocyte chalone" was active; *in vivo* injection into mice resulted in reduced tritiated thymidine uptake and autoradiographic label-

ing indices in myelocytes (Lord, 1975). Other workers confirmed the inhibitory activity of the crude extract but also reported inhibition of GM–CFC growth (Paukovits, 1971; Laerum and Maurer, 1973; Lord *et al.*, 1974b; Maurer *et al.*, 1978). The active component of this "chalone" was ultimately characterized as an acidic pentapeptide with the amino acid sequence pyro-Glu-Glu-Asp-Cys-Lys(pEEDCK) (Paukovits and Laerum, 1982). Despite the earlier reports showing relative specificity for the maturing myeloid precursor cells, it now appears that the pentapeptide has a much wider spectrum of activity, including CFU-S and pre-CFU-S inhibition (Laerum and Paukovits, 1984; Paukovits *et al.*, 1993). The physiological significance of this nonspecificity is not entirely clear and may represent a dose-dependent phenomenon. For example, using a dose that inhibited myelocyte proliferation, Lord *et al.* (1977b) showed that the crude extract had no effect on either GM–CFC or CFU-S proliferation. Teleologically, an inhibitory signal originating from mature end cells such as granulocytes might be thought a rather crude feedback mechanism for a multipotent stem cell population that ultimately gives rise to several distinct cell lineages.

A synthetic analog of the peptide, designated hemoregulatory peptide 5b (b distinguishes the active molecule from other inactive pentapeptide analogs), has reversible inhibitory effects on both murine and human hemopoietic colony formation. Interestingly, the molecule is inhibitory only in its monomeric form. (In its dimeric form it is a stimulator of hemopoietic progenitor cells; see later discussion). *In vitro*, pEEDCK inhibits GM–CFC at concentrations ranging from 10^{-9} to 10^{-5} *M* (Laerum and Paukovits, 1984). The inhibitory activity is relatively specific for the myeloid lineage. Moderate to slight inhibition of human T-, B-lymphocyte, and erythroid colony formation has also been reported using 1000 times the optimal doses for myelopoiesis (Kreja *et al.*, 1986; Laerum *et al.*, 1990a). When injected into mice, the molecule has a dose-dependent inhibitory effect on CFCs (Laerum and Paukovits, 1984; Laerum *et al.*, 1990a) with maximal effects documented following continuous infusion (1.4 μg/hr) (Laerum and Paukovits, 1984; Laerum *et al.*, 1990a). When the peptide was administered over 6 days, CFC numbers were reduced by 80%; the reduction was less pronounced for CFU-S. Subsequently, both CFC and CFU-S recovered rapidly, overshooting normal levels. No direct toxic effects have been seen in mice receiving up to 9 mg of the peptide (Laerum and Paukovits, 1984).

The ability of pEEDCK to inhibit CFU-S proliferation *in vivo* has been exploited in a chemoprotection model using repeated doses of cytosine arabinoside (Paukovits *et al.*, 1991a). Mice received two injections of ARA-C (300 mg/kg), 12 hr apart. pEEDCK, 30 μg/kg, administered at various times beginning 2 hr before the second ARA-C injection, reduced CFU-S cycling by approximately 50%, compared with the Ara-c-treated control This was reflected by total abrogation of the cytotoxic-induced CFU-S

loss (100% CFU-S survival in the pEEDCK-treated cohort vs. 27% in the control). Subsequently, in the peptide-treated group, the leukocyte nadir was delayed by approximately 2 days, and its ultimate recovery was delayed in the peptide-treated mice by 1 day. The explanation for these findings is not entirely clear. The authors suggest that the delayed fall in leukocyte numbers is related to the inhibitory effects of pEEDCK on the more mature precursor cells, including GM-CFC and myelocytes. However, the treatment schedule delayed the peptide administration until 10 hr after the initial ARA-C dose. Under normal circumstances, the lineage-committed progenitor cells are proliferating and would be targeted by that first ARA-C injection, unlike the proliferatively quiescent CFU-S population. One would therefore anticipate an equivalent acute progenitor cell kill in both groups and an identical fall in neutrophil numbers. It is equally unlikely that the peptide is behaving as the stimulatory dimer in ths situation because this would potentially exacerbate the cytotoxic-induced damage.

The protective effect of the peptide on pre-CFU-S was also analyzed using a model with repeated ARA-C injections (Paukovits *et al.*, 1993). Nine hours after ARA-C treatment, the members of CFU-S and pre-CFU-S were reduced to 10 and 30%, respectively. Although pre-CFU-S are known to be extremely proliferatively quiescent and may take 3–5 days to enter the cell cycle (Quesniaux *et al.*, 1993; Lord and Woolford, 1993). The investigators confirmed that the pre-CFU-S were in fact proliferatively quiescent when assayed immediately following the ARA-C scheduling; the dramatic loss in pre-CFU-S numbers appears to be result of differentiation into the depleted CFU-S pool (Quesniaux *et al.*, 1993; Lord and Woolford, 1993). However, this depletion, surprisingly, was also long lasting, with no recovery up to a year later, suggesting a significant loss of self-renewal capability for the MRA cells that has not previously been recognized with short-term repeated administration of an S-phase cytotoxic agent. The addition of the pEEDCK peptide around the time of chemotherapy appeared to attenuate the pre-CFU-S loss. Thirty micrograms per kilogram administered at 2, 4, and 6 hr after the second ARA-C injection was thus considered to have blocked CFU-S recruitment and prevented the excessive loss of the pre-CFU-S pool. Fifty percent of these cells were present 2 months after cytotoxic drug treatment compared with <10% in the control group treated only with ARA-C.

A general problem encountered with the pEEDCK molecule has been its instability; short exposure to air, as well as repeated freezing and thawing destroys its inhibitory properties, sometimes rendering it stimulatory (Paukovits *et al.*, 1991a; Laerum *et al.*, 1986, 1987) due to dimerization of the molecule (Paukovits *et al.*, 1991a; Laerum *et al.*, 1988). The molecular instability and paradoxical stimulatory actions of the peptide have caused some confusion over its often diverse effects *in vitro* and *in vivo*. Replace-

ment of the -SH group of cysteine with an isoteric methylene group, however, creates a stable monomer (SK&F108636) that retains potent inhibitory actions. *In vivo,* this molecule produces growth inhibitory effects in a wide range of progenitor cells, including the most primitive HPP-CFC and pre-CFU-S (Veiby *et al.,* 1994).

pEEDCK dimerization results from monomeric oxidation with the formation of disulfide bridges between the two cysteine residues (Paukovits *et al.,* 1991a; Laerum *et al.,* 1988). This dimeric peptide has growth-enhancing properties on both murine and human colony formation, which appear to be indirect and probably mediated by stromal cell activation (King *et al.,* 1992; Pelus *et al.,* 1994). Somewhat surprisingly, the effects *in vitro* are reported to occur at doses as low as 10^{-16} *M* (Paukovits *et al.,* 1991b; Laerum *et al.,* 1988). In addition, the molecule remains inherently unstable and is easily reduced to the inhibitory, monomeric form which itself, as we have seen, is unstable, readily forming the dimer. Removal of the disulfide bridge and replacement with a nonreducible carbon bridge results in a stable dimer, SK&F 107647, that possesses more potent stimulatory activity. When injected into mice, a two- to sixfold increase in colony-stimulating activity occurs with a peak at about 6 hr (Pelus *et al.,* 1994). *In vivo,* the analog is active at significantly lower doses than those required for other directly acting stimulators such as G-CSF (optimum dose in mice, 1 ng/kg vs. 100 μg/kg, respectively). Intriguingly, the molecule may be orally bioactive although significantly higher doses are required (Pelus *et al.,* 1994). This molecule is being evaluated in phase I clinical studies.

1. Structure

In addition to the relative ease of monomeric–dimeric interchange and dramatic reversal of activity, significant quantitative differences in activity also occur with relatively minor structural changes in the monomer. Analysis of a series of synthetic peptide variants showed that subtle amino acid rearrangements resulted in a reduction in potency or different tissue specificity (Laerum *et al.,* 1990b; Jenson *et al.,* 1990)(see Table III). It is of interest that several other small peptides have also been described that have proliferation-modulating effects on both hemopoietic and epithelial cells (see the later discussion on oligopeptide inhibitors).

pEEDCK-like sequences have been identified in the Giα chain of GTP-binding proteins (Laerum *et al.,* 1990b). These sequences occur at positions 63 through 67, a potential site for effector interactions. The greatest homology occurs with a rat Giα and not the human Giα. Similarities in structure suggest that the peptide may interfere with G-protein–effector binding, although this is purely speculative.

TABLE III
Hemoregulatory Peptide(pEEDCK) Analogs[a]

	Analogs					Haemopoietic activity
HP5a	pGlu	Asp	Asp	Cys	Lys	None
HP5b	pGlu	Glu	Asp	Cys	Lys	Yes (pEEDCK)
HP5c	Glu	Glu	Asp	Cys	Lys	None
HP5d	pGlu	Glu	Glu	Cys	Lys	Reduced
HP5e	pGlu	Asp	Glu	Cys	Lys	Reduced
HP5f	pGlu	Glu	Asp	Cys	Arg	Reduced
HP5g	pGlu	Glu	Glu	Ser	Lys	None
HP5h	pGlu	Glu	Glu	Cys	Arg	Reduced
epid. peptide	pGlu	Glu	Asp	Ser	GlyO	Keratinocytes
epid. analogue	pGlu	Glu	Asp	Ser	Lys	Keratinocytes

[a] From Laerum *et al.*, (1990b) and Jenson *et al.* (1990). Amino acid Substitution's highlighted.

It is currently not known whether the pEEDCK molecule exists as such *in vivo* or whether it exists bound to a larger precursor protein (or Giα protein). A physiological role for proliferation regulation is suggested by immunization studies that show increased GM–CFC numbers following removal of natural pEEDCK from the organism (Paukovits *et al.*, 1991b). At present, there are no data on the pEEDCK receptor or intracellular signaling mechanisms for either the monomer or dimer.

2. Effects on Other Tissues

The synthetic pentapeptide monomer is specific for hemopoietic cell subsets, as outlined above. Unlike TGF-β, and possibly MIP-1α, its inhibitory activity does not appear to extend to other self-renewing tissues. However, several small peptides with considerable structural similarity to pEEDCK have been shown to have inhibitory effects on nonhemopoietic tissues. Jensen *et al.* (1990) documented reversible inhibitory effects of an epidermal pentapeptide on keratinocyte proliferation which differs from HP5b at positions 4 and 5 (pyroGlu-Glu-Asp-Ser Gly-OH) with a serine and glycine substituted for cysteine and lysine, respectively. A dipeptide structure, pyroGlu-Gly-OH, has similar inhibitory effects on keratinocytes while HP5b has no effect. A derivative of HP5b, in which cysteine is exchanged for serine, does, however, show inhibitory effects on keratinocyte proliferation.

D. Hemoregulatory Tetrapeptide (AcSDKP)

An inhibitory oligopeptide was isolated from fetal calf bone marrow (Frindel and Guigon, 1977). Structurally unrelated to pEEDCK, it has the amino acid sequence, *N*-acetyl-seryl aspartyl-lysil-proline (AcSDKP) (Lenfant *et al.*, 1989). Originally trademarked as Seraspenide, it is now known as Gorolotide. The crude extract, first isolated in 1977 by Frindel and Guigon, was shown to inhibit CFU-S recruitment (Frindel and Guigon, 1977) and in a radiation model, addition of the bone marrow extract reduced the proportion of CFU-S in DNA synthesis from 44% to a level as low as 3%. This crude extract was also shown to have a similar action in preventing CFU-S recruitment induced by ARA-C treatment (Guigon *et al.*, 1980).

The early experiments suggested that the synthetic peptide was specific for the CFU-S population and was equipotent to the naturally occurring peptide (Lenfant *et al.*, 1989). No effects were seen on murine GM-CFC, suggesting that the maturing progenitors were resistant to the inhibitory effects of the molecule. More recently, Guigon *et al.* reported that prolonged incubation of human mononuclear cells with AcSDKP resulted in significant growth inhibition of the lineage-committed GM-CFC, BFU-E, and in some cases the unipotent CFU-E (Guigon *et al.*, 1990). A dose-response effect was noted, with the peak inhibition at concentrations of 10^{-9} to 10^{-10} *M*. The inhibitory effects seen with unfractionated bone marrow were confirmed using enriched progenitor cell populations, including cells with marrow-repopulating potential.

Bonnet *et al.* (1993) evaluated the effects of AcSDKP on highly purified human progenitors. FAC-sorted $CD34^{2+}$/HLA-DR^{high} and $CD34^{2+}$/HLA-DR^{low} populations were incubated with AcSDKP for 20 hr in the presence of seven growth factors (IL-3, IL-6, IL-1β, GM–CSF, G-CSF, Epo, and SCF), a combination that had previously been found to enhance both the proliferation of $CD34^{2+}$ cell subsets and colony generation (Lemoine *et al.*, 1992). AcSDKP (10^{-10} mol/liter) reduced the number of BFU-Es and GM-CFCs generated from both $CD34^{2+}$ fractions by approximately 30%. Added daily for 6 days to $CD34^{2+}$ subsets grown in liquid culture under optimal conditions, AcSDKP reduced the thymidine incorporation of HLA-DR^{high} and HLA-DR^{low} $CD34^{2+}$ cell subsets by 48 and 65%, respectively. There was a bell-shaped dose-response curve with inhibition observed only between 10^{-7} and 10^{-10} mol/liter. Single $CD34^{2+}$ cell analysis strongly suggested that the inhibitory effects were direct.

In the LTBMC, Cashman *et al.* (1994) found that the addition of 100 ng/ml of AcSDKP was sufficient to prevent the onset of DNA synthesis in primitive erythroid and granulopoietic progenitor cells in the adherent layer of the culture. In contrast to Bonnet *et al.'s* (1992) findings, this effect was specific for the more primitive populations. No concentration of AcSDKP

blocked proliferation of the more mature progenitors cells while the addition of a closely similar control peptide (AcSDKE) or the unacetylated SDKP had no inhibitory effects on the proliferation of the primitive progenitor cells in the LTBMC adherent layer. Furthermore, AcSDKP incorporated into methylcellulose assays of freshly isolated normal bone marrow cells did not produce inhibitory effects if the adherent cells had been rigorously removed before plating. This finding, again in contrast to Bonnet *et al.'s* observations, suggests an indirect inhibitory effect. Interestingly, the inhibitory effects of the tetrapeptide could be blocked by the addition of the chemokine, MIP-1β, suggesting that AcSDKP may in fact act indirectly through MIP-1α or another member of the cytokine family that is also blocked by MIP-1β. This could also explain why primitive CML progenitor cells are resistant to the inhibitory actions of AcSDKP (Cashman *et al.*, 1994). An indirect inhibitory action has also been highlighted by the conflicting effects of AcSDKP on hepatocyte proliferation, *in vitro* and *in vivo. In vivo,* AcSDKP blocks hepatocyte proliferation induced by partial hepatectomy; *in vitro,* it has no effect on primary hepatocyte cultures (Lauret *et al.*, 1989a). While these findings consolidate the hypothesis that AcSDKP acts indirectly, the effects on bone marrow and liver may occur via different mechanisms.

In vitro evaluation of the effects of AcSDKP have proved somewhat difficult, which is probably due at least partly to its rapid degradation by proteases in the added sera. Grillon *et al.* (1993) showed that the *in vitro* metabolism of AcSDKP can be delayed and its half-life extended by adding the metalloproteinase inhibitor Captopril to the assay conditions, findings that were subsequently confirmed by Reiger *et al.* (1993) using radiolabeled AcSD[4-3H]KP. In human plasma, AcSDKP was completely metabolized, with a half-life of 80 min, leading exclusively to the formation of lysine. The peptide cleavage was insensitive to classic proteinase inhibitors including lekpeptin, but was completely blocked by specific inhibitors of angiotensin-I-converting enzyme.

AcSDKP is active *in vivo* and can prevent the CFU-S recruitment induced by cytotoxic treatment (Frindel and Guignol, 1977; Frindel *et al.*, 1992; Guigon *et al.*, 1990; Bogden *et al.*, 1991; Monpezat and Frindel, 1989). However, the timing of AcSDKP administration in relation to the cytotoxic agent is critical. Mice that received AcSDKP following ARA-C had the highest survival when the peptide was administered simultaneously and 2 hr after each treatment of ARA-C treatment (Bogden *et al.*, 1991). The ability to block CFU-S recruitment is lost if AcSDKP is administered beyond 6–8 hr following Ara-c treatment (Monpezat and Frindel, 1989). This appears to reflect the position of the target cells in G_1, with decreasing sensitivity to the tetrapeptide as the cells approach the G_1–S boundary. The protective effect of AcSDKP has also been confirmed against the S-phase agent 3′-azido-3′-deoxythymidine (AZT) (Grillon *et al.*, 1994) and

the non-S-phase-specific agents cyclophosphamide (Bogden *et al.,* 1991) and adriamycin (Ramirez *et al.,* 1994). The mode of toxicity of these latter compounds is qualitatively different than conventional S-phase drugs, suggesting, in this case, that cell cycle inhibition may not be a primary cytoprotective mechanism. Bogden *et al.* (1991) showed a survival advantage in mice when AcSDKP was administered (optimally 8 hr) following a lethal dose of cyclophosphamide. The protective effect extended the median survival time from 1.4 to 7.3 days compared with the mice receiving cyclophosphamide alone.

Unfortunately, survival experiments do not offer any insight into the mechanisms of protection and the cyclophosphamide model raises several questions. Given that the CFU-S population is proliferatively quiescent under normal conditions, why should the addition of a CFU-S proliferation inhibitor offer any protection following a single administration of a cytotoxic agent? Furthermore, the acute lethality at day 1 is consistent with a nonhemopoietic etiology as a cause of death in the animals. The specificity of the AcSDKP dosing time in this model is also unclear unless AcSDKP in some way interrupts the normal activation of cyclophosphamide by hepatic microsomal enzymes. Similar survival experiments using repeated adriamycin with AcSDKP gave a small but statistically significant ($p < 0.05$) cumulative probability of survival of 53.3 vs. 38.8% in the control (Ramirez *et al.,* 1994).

A physiological role for AcSDKP was highlighted by the recognition of its endogenous production both *in vitro* and *in vivo* and by neutralizing antibody experiments (Coffey *et al.,* 1988; Wdieczak-Bakala *et al.,* 1990; Frindel and Monpezat, 1989). *In vitro,* synthesis of AcSDKP was documented in LTBMC (Grillon *et al.,* 1994). Endogenous production *in vivo* was confirmed using radioactive precursors (Grillon *et al.,* 1994). Serum levels of the tetrapeptide were measured in 34 healthy human volunteers at between 0.7 and 2.5 pm/ml (Liozon *et al.,* 1993). The addition of polyclonal antisera to mice resulted in enhanced proliferation of CFU-S (Frindel and Monpezat, 1989; Lauret *et al.,* 1989b), and comparable increases in the proliferative activity of primitive hemopoietic progenitor cells was observed when antisera were added to LTBMC (Grillon *et al.,* 1994).

While the bone marrow is a recognized source of AcSDKP, competitive immunoassays have demonstrated its presence in numerous murine tissue extracts, suggesting that the molecule is more ubiquitious than previously recognized (Pradelles *et al.,* 1991). Within these tissues, AcSDKP is probably synthesized from thymosin $\beta 4$, through the action of an endoproteinase that cleaves the Pro-4, Asp-5 peptidic bond (Grillon *et al.,* 1990). The SDKP sequence is also found in tumor necrosis factor, another cytokine that possesses antiproliferative activity on hemopoietic colony formation (see

later). However, addition of TNF-α to LTBMC does not reproduce the inhibitory effects of AcSDKP (Cashman *et al.*, 1994).

The mechanism of action of AcSDKP is currently unknown, with no information on the receptor or signal transduction pathways. As outlined earlier, there are conflicting reports on the possible direct or indirect effects on hemopoietic progenitors. Using the *in vitro* assay for a murine HPP-CFC, Robinson *et al.* (1992) showed that AcSDKP had no direct inhibitory effect but rather blocked the action of a stimulator of hemopoietic stem cell formation. Several structurally modified forms (AcSDDKP, AcSDβKP) were unable to stimulate the blocking effect of AcSDKP. However, the tripeptide Ser-Asp-Lys was effective, suggesting that the SDK sequence may represent the active component of the molecule. Lenfant *et al.* (1990) showed that AcSDKP enhanced the adherence of CFU-S to the bone marrow-derived hemopoietic stromal cell line MS1-T. It also suppressed MS1-T proliferation, but without modifying the granulocyte to macrophage colony-stimulating activity of these cells. The importance of microenvironmental influences on cell proliferation has already been alluded to and it is possible that alteration in progenitor–stromal cell interactions may well represent a common factor in the actions of all the stem cell inhibitors so far discussed.

E. Other Inhibitors

It is somewhat ironic, considering the initial scepticism surrounding the chalone hypothesis, that the list of potential hemopoietic proliferation inhibitors has now expanded to rival that of the hemopoietic growth factors (see Table IV). A number of these molecules, e.g., MIP-1α and AcSDKP, appear to be relatively specific for hemopoietic progenitor cells subsets, but the physiological role of other factors is less clear, and TGF-β, TNF-α, and interferon (IFN) have diverse effects on many tissues. Their growth-modulating effects on hemopoiesis may, therefore, merely represent a non-specific or secondary effect. Studies evaluating these factors have frequently produced conflicting results. Growth inhibition and growth augmentation have been reported and the response appears to be determined by the assay conditions. Presumably this relates to the presence of endogenous factors that may potentiate or antagonize the actions of the molecule under investigation. In addition, the presence of contaminating accessory cells also appears to have an important influence on the response observed. Single-cell sorting and serum-free conditions may allow a more precise evaluation of specific factors, but it is important to understand that such models are an artificial scenario and take no account of the complex biochemical and cellular interactions that take place *in vivo*.

TABLE IV
Proposed Physiological Inhibitors of Hemopoiesis

Macrophage inflammatory protein-1α (BB10010)
Transforming growth factor-β
Hemoregulatory pentapeptide (HP5b, pEEDCK)
Hemoregulatory tetrapeptide (AcSDKP, Seraspenide,Gorolotide)
Negative regulatory protein (superoxide dismutase)
Tumor necrosis factor
Interferon (α,β,γ)
Glutathione
Lactoferrin
Isoferritins

Despite the cautionary note, it is likely, given the complexity of the hemopoietic system, that multiple inhibitory molecules are involved in feedback regulation at all levels of progenitor cell development. Considerable knowledge has been gained concerning regulation at the stem cell level, but less is known about the control exerted on maturing populations. Some of the hemopoietic stem cell inhibitors, e.g., pEEDCK, show activity on a wide spectrum of developing hemopoietic progenitor cells. However, the observation that maturing progenitor cells such as GM–CFC are actively proliferating under steady-state conditions suggests that inhibitors may play less of a role at this stage. On a mechanistic level, the effect on committed cells is more likely to represent a decelerating force, as opposed to the on/off switch that is necessary to maintain stem cell quiescence.

1. Negative Regulatory Protein

Negative regulatory protein (NRP) was isolated from bone marrow supernatants of C57B1/6(B6) mice and appears to be specific for the early erythropoietic progenitor cell, BFU-E (Axelrad *et al.*, 1981). The protein is nontoxic and its inhibitory action is readily reversed by washing the cells. *In vitro,* the molecule acts extremely rapidly (within 20 min), suggesting that NRP acts at the G_1/S boundary or possibly on DNA synthesis itself (Axelrad *et al.*, 1981; Del Rizzo *et al.*, 1990). The active component of B6 marrow supernatant has now been characterized as the Cu, Zn-containing form of the antioxidant enzyme superoxide dismutase (SOD) (Pluthero *et al.*, 1990). This enzyme is normally present in large amounts in erythrocytes, suggesting a feedback loop from this population to its precursor BFU-E. [It is of note

that several reports of an inhibitor of erythropoiesis that is derived from erythrocytes have appeared in the literature dating from as early as 1971 (Kivilaasko and Rytomaa, 1971).] Interestingly, marrow supernatant from congenic B6S mice does not inhibit DNA synthesis in BFU-E despite similar levels of SOD in both B6S and B6 mice (Pluthero and Axelrad, 1991). The inhibitory effects of SOD are opposed by IL-3 in a dose-dependent manner (Pluthero *et al.*, 1990; Pluthero and Axelrad, 1991). Pluthero and Axelrad (1991) suggest that the differential rate of DNA synthesis observed in B6 and B6S mice is related to the altered balance between stimulatory and inhibitory signals found in these two strains.

2. Oligopeptide Inhibitors

Several inhibitory oligopeptides sharing remarkably similar amino acid structures have been characterized (Table V). Two of these, AcSDKP and pEEDCK, have already been discussed. More recently, an inhibitory peptide has been purified from calf spleen (Fetsch and Maurer, 1987) and identified as glutathione, Gly-Cys-Glu (GSH). It is active in the nanomolar range and appears to be specific for colony formation by GM-CFC. This finding is of particular interest because oncologists have recognized for some time that glutathione metabolism may have an important impact on the cytotoxicity of various chemotherapeutic agents (Arrick and Nathan, 1984). The elevation of intracellular concentrations of glutathione produces a protective effect on bone marrow against cyclophosphamide treatment (Carmichael *et al.*, 1986), which is thought to involve a metabolic interaction between GSH and an alkylating moiety (Chasseaud, 1979). It is tempting to speculate that a component of this cytotoxic resistance may be related to inhibition of the cell cycle. Several oligopeptides have been described that have growth-modulating effects on nonhemopoietic tissues, and it appears that subtle amino acid substitutions confer different lineage and

TABLE V
Oligopeptide Inhibitors

Peptide	Structure
AcSDKP	*N*-acetyl-Ser-Asp-Lys-Pro(Lenfant *et al.*, 1989)
pEEDCK	pyroGlu-Glu-Asp-Cys-Lys(Paukovits and Laerum, 1982)
Glutathione	Gly-Cys-γGlu(Fetsch and Maurer, 1987)
Epidermal pentapeptide	pyroGlu-Glu-Asp-Ser-Lys(Jenson *et al.*, 1990)
Colonic epithelium inhibitor	pyroGlu-His-Glu(Skraastad *et al.*, 1987)

tissue specificity. This is particularly well illustrated by the epidermal pentapeptide (Jenson *et al.*, 1990) and the tripeptide pGlu-His-Gly (Skraastad *et al.*, 1987), which inhibit keratinocyte and colonic epithelial proliferation, respectively. Despite increased awareness of them, it remains uncertain whether these molecules exist as oligopeptide structures *in vivo* or as part of larger precursor proteins that require enzymatic cleavage. Similarly, there is no current information on receptor binding or signal transduction mechanisms.

3. Iron Binding Factors

a. Isoferritins Ferritin is a ubiquitous protein whose major function is iron storage. It has an outer protein shell consisting of 24 subunits and an inner core consisting of a variable amount of iron deposited as ferric hydroxyphosphate complex. Isoferritins consisting of different subunits are found in almost all tissues. Three subtypes are recognized, L(light), H(heavy), and G(glycosylated). Variation in the proportion of H and L dictates the pH of the molecule, with a greater proportion of H subunits resulting in an acidic subtype.

Broxmeyer *et al.* (1981, 1984a,b) reported that extracts and conditioned media of marrow and blood cells from patients with leukemia inhibited colony formation of normal GM-CFC. Leukemic GM-CFCs were not affected by this leukemia inhibitory activity (LIA), which was subsequently identified as acidic isoferritin and proposed as a regulator of granulocyte–macrophage production (Broxmeyer *et al.*, 1981). Further studies have shown that the inhibitory effects are specific to the acidic isoferritins: L-subunits or basic ferritins have no effect on hemopoietic cell proliferation (Broxmeyer *et al.*, 1986a). The initial experiments using purified tissue H-subunit-rich isoferritins and inhibiting of both human CFU-GEMM and BFU-E were subsequently reproduced using recombinant H subunit ferritin (Broxmeyer *et al.*, 1986a). Using human bone marrow mononuclear cells, Dezza *et al.* (1987) showed that this recombinant H-subunit ferritin inhibited 7-day GM colony formation in the presence of fetal calf serum and conditioned medium (5637 bladder carcinoma cell line). The inhibition was blocked by preincubation with the monoclonal antibody 2A4, directed against the H subunit. As with the native material, recombinant L-subunit ferritin was not inhibitory.

Despite these findings, the physiological role of isoferritins in hemopoietic cell cycle regulation is subject to considerable debate and may simply represent experimental phenomena; for example, there is the apparent ability to inhibit at concentrations that are several orders of magnitude below the normal physiological background (Cazzola *et al.*, 1990). Inhibitory effects have not been confirmed by all investigators (Sala *et al.*, 1986) and

the experimental findings do not correspond with any known clinical data. Ferritin is an acute-phase reactant and is elevated during periods of acute stress, such as inflammation or necrosis. It would therefore appear inappropriate, in physiological terms, to inhibit hemopoietic subsets that are required to aid in the hosts defense mechanisms. In addition, bone marrow suppression is not associated with pathological states of iron overload such as primary hemochromatosis and transfusional hemosiderosis.

b. Lactoferrin The iron-binding glycoprotein lactoferrin has also been implicated in negative feedback regulation of hemopoietic progenitor cells. Its effects were illustrated using both murine and human bone marrow and included inhibition of erythroid and myeloid progenitor cells (BFU-E and GM-CFC) (Broxmeyer *et al.*, 1978, 1980, 1984b,c). The suppressive effects are thought to be indirect and dependent on the presence of monocytes or macrophages, acting via other known macrophage-derived inhibitors, or alternatively, blocking their production of growth stimulatory factors. Several investigators have reported reduced GM-CSF and IL-1β activity from monocytes following incubation with lactoferrin (Broxmeyer *et al.*, 1978, 1984c; Zucali *et al.*, 1989). Gentile and Broxmeyer (1991) were unable to override the inhibitory effects of lactoferrin on GM-CFC development by adding excess rhuGM-CSF or rhIL-1 to the culture medium. However, the myelosuppression could be ablated by rhu-IL6.

The inhibitory effects of lactoferrin have not been accepted by all groups (Winton *et al.*, 1981; Gilraith, 1986) and the postulated role, like that for ferritin, does not correlate with clinical findings. Lactoferrin is present in secondary granules of neutrophils and is released when a cell dies or amounts are increased during a stress response. Under these circumstances it appears more likely that the molecule would act as a stimulator to maintain or increase neutrophil numbers. Rich and Sawatzki (1987) reported that *in vivo* administration of purified species-specific lactoferrin results in an increase in plasma GM-CSF levels within 12 hr, leading to a signficant GM-CFC increase after 48 hr. They concluded that lactoferrin release is a signal that enhances macrophage GM-CSF synthesis and is not in fact a negative feedback regulator.

4. Tumor Necrosis Factor

Tumor necrosis factor was originally described as an endotoxin-induced serum factor that produced hemorrhagic necrosis in subcutaneous Meth.A. sarcoma transplanted tumors (Carswell, 1975). A closely related molecule, lymphotoxin (LT,TNF-β), was purified and characterized in the early 1980s and shows activities identical to TNF-α (Aggarwal and Eessalu, 1987). It is 32% homologous to TNF-α and appears to act via the same receptor.

TNF-α is released from monocytes, macrophages, and lymphocytes, and possesses a wide range of cell regulatory, immune, and inflammatory properties that overlap with other members of the cytokine network, including IL-1 and interferon-γ (IFN-γ) (Balkwill *et al.*, 1990). A role in hemopoietic proliferation control is suggested by its ability to activate stromal cells and augment colony-stimulating factor release (G-CSF, GM, CSF, M-CSF) (Koeffler *et al.*, 1987; Munker *et al.*, 1986; Oster *et al.*, 1987).

Receptors for TNF-α are present on nearly all cell types, with a few exceptions such as erythrocytes and unstimulated T cells. Two types of receptor have now been recognized (Hohmann *et al.*, 1989; Brockhaus *et al.*, 1990). Type I receptors (TNFR I) are ubiquitous while the type II (TNFR II) receptor appears more specifically on cells of hemopoietic origin.

There are conflicting reports concerning the role of TNF-α on the growth modulation of hemopoietic progenitor cells. Paradoxical stimulatory and inhibitory effects have been obtained using *in vitro* colony assays. The inconsistencies and sometimes confusing results emphasize the influence of other cellular and soluble factors on the effects seen with this molecule.

Several groups have reported that both TNF-α and lymphotoxin (recombinant and purified preparations) can inhibit colony formation by granulocyte, monocyte, erythrocyte, and multipotential precursor cells (Broxmeyer *et al.*, 1986b; Wisniewski *et al.*, 1987; Murphy *et al.*, 1988). In general, the earlier multipotential progenitor cells appear more sensitive to their inhibitory effects. However, the experimental conditions have not always differentiated between direct and indirect effects via accessory cells and other growth-modulating factors. Enriched progenitor cell populations and differing sources of colony-stimulating activity have helped. After most of the lymphocytes and monocytes were removed from human bone marrow mononuclear cells by a combination of plastic adherence and indirect rosetting, rTNF and rLT consistently inhibited the more immature, day-14 GM-CFC, suggesting that these factors have direct effects (Murphy *et al.*, 1988). Inhibition was independent of the source of CSA. Conversely, the growth-inhibitory effects of TNFα on the more mature 7-day GM–CFCs was extremely dependent on some factor present only in conditioned medium from the human bladder carcinoma cell line (5637CM). No inhibitory effects were obtained using rG-CSF or the conditioned medium from the giant cell tumor cell line (GCT-CM).

Bonnet *et al.* (1995) confirmed the inhibitory nature of rTNFα for human progenitor cell subsets enriched for $CD34^+$/HLA-$DR^{high/low}$. Short-term (20 hr), and long-term incubation (daily addition for 6 days) resulted in consistent inhibition in both subsets of $CD34^+$ populations. Following the short-term incubation, the more mature GM–CFCs obtained in the $CD34^+$/HLA-DR^{high} cell population were suppressed to a greater extent than the more immature GM-CFCs in the $CD34^+$/HLA-DR^{low} fraction.

This differential effect was lost following the 6-day incubation. In liquid culture, the same cell subsets were induced to proliferate over 6 days in the presence of a prescribed cocktail of seven recombinant growth factors (IL-3, IL-1β, IL-6, G-CSF, GM-CSF, SCF plus Epo). A single addition of TNF-α at day 0 was sufficient to produce a 72–76% inhibition in both the HLA-DR^{high} and HLA-DR^{low} subpopulations. The inhibitory effects thus span the whole spectrum of the CD34$^+$ progenitor cell populations but comparable inhibition with MIP-1α, TGF-β, and AcSDKP suggested a high degree of functional overlap.

In complete contrast to these inhibitory properties, several groups have now reported that TNF-α augments proliferation of the GM-CFC population. Using highly purified CD34$^+$ human progenitor cells, Caux *et al.* (1990) showed that rTNF-α strongly potentiates IL-3 and GM-CSF-induced growth of GM-CFC. These effects were observed using the same dose of rTNF-α (25 ng/ml) as Bonnet *et al.* used and must presumably reflect the difference in cytokine combinations. in contrast to the findings of Murphy *et al.* (1988), committed G-CSF-sensitive subpopulations were consistently inhibited in the presence of TNF-α. These findings were also confirmed by Backx *et al.* (1991), who showed that rTNF-α produced a dose-dependent suppression of G-CSF-induced granulocytic colony formation and Epo-induced burst formation, but augmented the growth of the more primitive precursor cells that respond to IL-3 and GM-CSF.

The paradoxical effects of TNF-α on *in vitro* colony formation are probably explained by the complexity of its molecular interactions with other growth-modulating factors, including the interferons, interleukins, and the colony-stimulating factors. Furthermore, the interaction with accessory cells, in particular, macrophages, may result in indirect effects that are dictated by the secondary production of stimulators and/or inhibitors. It may be possible to obtain some insight into the physiological role of TNF-α from preliminary clinical studies that have been designed to evaluate the antineoplastic effects of the molecule. In Phase I studies, TNF-α produced fever, myalgia, and alterations in hemodynamic parameters (Hieber and Heim, 1994). These effects are almost certainly related to the release of secondary mediators following TNF-α-induced macrophage and natural killer cell activation. Hemopoietic progenitor cell suppression did not appear to represent a major sequelae of TNF-α administration (Aulitzky *et al.*, 1991). Transient reductions in neutrophils, monocytes, and lymphocytes were documented following 5-day schedules (Hieber and Heim, 1994; Aulitzky *et al.*, 1991). Anemia and thrombocytopenia were reported, but were generally modest.

5. Interferons

The IFNs are a family of glycoproteins that exhibit antiproliferative effects on normal and malignant cells, *in vitro* and *in vivo*. They can be classified

into three major groups based on immunological criteria. The current nomenclature defines the three classes as IFN-α (lekcocyte), IFN-β (fibroblast), and IFN-γ (immune). The IFNs are produced by a variety of cell types in response to several classes of inducers, in particular, virus infections. They were the first well-defined, highly potent group of polypeptides with antimitotic effects (Taylor-Papadimitrou, 1980). The inhibition of cell proliferation appears to be cell-cycle specific since IFN-α is active on cells in the G_1 phase but not on cells that have already progressed into DNA synthesis (Tominaga and Lengyel, 1985). The IFN family appears to act by inhibiting the expression of growth-inducing genes. When given in combination with platelet-derived growth factor, for example, IFN-β blocks the induction of several proteins that are typical of PDGF-stimulated cells (Tominaga and Lengyel, 1985). PDGF-stimulated cells also produce IFN (Zullo *et al.*, 1985), a response that presumably represents an autocrine feedback inhibitory loop, to regulate cell growth.

Many of the conflicting reports concerning TNF-α and hemopoietic colony formation can be reiterated for the IFNs, particularly immune IFN (IFN-γ). This may not be totally unexpected because the two families interact considerably, both at the receptor and gene level. IFN-α and IFN-γ have been shown to enhance TNF-α binding to cells (Tsujimoto and Vilcek, 1986; Aggarwal *et al.*, 1985). In addition, some of the growth-inhibitory properties of TNF-α appear to be mediated via IFN-β_2 induction (Kohase *et al.*, 1986).

Broxmeyer *et al.* (1983, 1985) showed that both natural and recombinant IFNs from all three classes (α, β, and γ) can suppress colony formation by human GM-CFC, BFU-E, and CFU-GEMM. When evaluated individually, the IFNs inhibited both mature, 7-day colony growth and the more immature, 14-day colony growth with well-defined dose responses. When used in combination, the IFNs synergized to produce colony inhibition at concentrations 2 log units lower than those required for any individual IFN. Synergism occurred between IFN-γ and IFN-α and to a lesser extent between IFN-γ and IFN-β, but not between IFN-α and IFN-β. Raefsky *et al.* (1985) have confirmed these results using IFN-γ and rIFN-α. However, in contrast to the much lower dose of 10 U/ml described by Broxmeyer's group, they observed that a 50% inhibition required approximately 200 U/ml. The inhibition observed by Raefsky *et al.* was highly dependent on the culture conditions. Reduction of the fetal calf serum concentration from 30 to 20% significantly enhanced the action of IFN-γ. Similarly, IFN-induced inhibition varied with the concentration of human placenta-conditioned medium used as a source of CSA (5637-CM and 10% FCS were used in Broxmeyer's investigations).

Contaminating accessory cells can introduce further variability. Cannistra *et al.* (1988) examined the effects of autologus monocytes and T lymphocytes on IFN-γ-induced inhibition of GM-CFC. T-cell depletion from the mono-

nuclear fraction of normal BM had no effect, but removal of the adherent cells significantly reduced the inhibitory effects of IFN-γ. Replacement of the autologous monocytes introduced a concentration-dependent restoration of inhibition. Interestingly, the effects were completely abolished by adding neutralizing antibody to TNF-α, suggesting that monocyte-derived TNF-α was the active mediator of GM-CFC inhibition. Again, these findings conflicted with the observations made by Broxmeyer *et al.* (1983), who noted that depletion of bone marrow-adherent cells did not influence the inhibitory effectsof IFN-γ on day-7 GM-CFC growth. In summary, it appears that the IFNs possess growth-modulating effects on BM progenitors but that these are dependent on the balance of growth factors (and inhibitors) and the presence of accessory cells.

As discussed earlier, the antiproliferative effects of the IFNs also extend to include malignant tissues. IFN-α, in particular, has significant activity in a number of hemopoietic malignancies, including CML (Talpaz *et al.*, 1987), hairy cell leukemia (Quesada *et al.*, 1986a), and multiple myeloma (Quesada *et al.*, 1986b). The exact mechanism underlying the efficacy of IFN in these conditions is unknown but there have been some intriguing findings in CML. Accumulating clinical trial data show that IFN-α produces hematological remissions in 70–80% of CML patients (Talpaz *et al.*, 1987) while Bhatia *et al.* (1994) demonstrated that IFN acts directly on CML progenitors to restore their adhesion to normal bone marrow stroma by modulating the β_1 integrin receptor function. Surprisingly, this effect could be blocked by the addition of neutralizing antibodies to TGF-β or MIP-1α and augmented by the addition of MIP-1α itself. Thus, IFN therapy may regulate CML adhesion, and possibly proliferation, via mechanisms involving the stem cell inhibitors TGF-β and MIP-1α.

V. Feedback Regulators and Tumor Tissues

The concepts of feedback inhibition and tissue hemostasis are especially pertinent when one considers malignancy and the dysregulated growth that accompanies neoplastic transformation. The escape from proliferation control that typifies the malignant phenotype has, historically, been associated with an excessive proliferative stimulus. Numerous proto-oncogenes have now been described that have the latent potential to transform cells and therefore initiate or promote neoplastic change. All of the proto-oncogenes recognized to date are linked to growth regulatory pathways and, under normal conditions, participate in proliferation and differentiation decisions under the influence of exogenous signals. Altered expression of these genes, e.g., by mutation or deletion, can be detected in many cancers

and generally leads to a stimulatory autocrine loop or autonomous function of growth factor receptors. Examples include the *c-erbB* oncogene that codes for a protein that is a truncated form of the epidermal growth factor (EGF) receptor (Downward *et al.*, 1984). This truncated receptor can stimulate cellular growth in the absence of ligand–receptor binding. Similarly, overexpression of the *c-sis* oncogene results in an excess production of the B-chain of platelet-derived growth factor and provides the cell with an autocrine growth stimulatory pathway (Dolittle *et al.*, 1983).

While these findings are important, they fail to take into account proliferation inhibition and its role in carcinogenesis. More recently, it has become increasingly evident that malignant transformation may occur through loss or attenuation of sensitivity to an inhibitory signal. Studies with hybrid cells have shown that the neoplastic phenotype of several tumor cells can be attenuated after fusion with normal cells (Sagar, 1989). These results imply that complete transformation, with acquisition of tumor-forming ability, requires the loss of a suppressive function. The hypothesis that tumor cells are controlled by recessive genes has now been substantiated with the recognition of the tumor suppressor genes. Both P53 and the retinoblastoma (RB) genes function as key intracellular inhibitory regulators of cell proliferation and act to block cell cycle progression beyond the G_1–S interface (Pietenpol *et al.*, 1990; Chen *et al.*, 1989; Blaydes *et al.*, 1995; Lane and Benchimol, 1990). Furthermore, there is accumulating experimental evidence linking at least one of the hemopoietic progenitor cell proliferation inhibitors, TGF-β, to the RB pathway (Pietenpol *et al.*, 1990; Lahio *et al.*, 1990).

Many of the putative inhibitory regulators of hemopoiesis have now been characterized and reproduced by chemical synthesis or recombinant DNA technology. Their availability will allow further evaluation of their function, using appropriately designed clinical trials. However, their potential role in malignant transformation poses a number of questions.

- Following malignant transformation, do cells continue to synthesize and secrete proliferation inhibitors, and if so, do the malignant cells retain any sensitivity to feedback regulation?
- If tumor tissues retain any sensitivity to proliferation inhibitors, can these molecules be utilized as novel antineoplastic agents?
- If there is a differential sensitivity between normal and neoplastic tissues, can proliferation inhibitors be used to expand the therapeutic window for cytotoxic agents?
- What effect do the known proliferation regulators have on tumor tissues?

Some of these points have been addressed by limited experimental studies and are discussed briefly in the following sections. Although these studies

provide some insight, further clarification is required if the full clinical potential of the inhibitors is to be achieved.

A. Growth Inhibitory Factors

It has been recognized for some time that the degree of pancytopenia in leukemia patients is frequently out of proportion to the extent of marrow infiltration by the malignant clone. In some cases, bone marrow failure is evident despite minimal morphological evidence of leukemia or is merely associated with focal infiltration by leukemic cells. Furthermore, following remission induction therapy, the absence of recovery from chemotherapy-induced bone marrow aplasia is not infrequently followed by florid leukemic relapse. These clinical observations, supported by a number of experimental studies, suggest that leukemia-associated bone marrow failure may be related, at least in part, to the inappropriate production of hemopoietic inhibitors.

Suspensions of the normal human bone marrow cells, cocultured with the leukemia cell lines HL60 or K562, are inhibited in a dose-dependent manner with reduction in the erythroid progenitors, CFU-E and BFU-E (Steinberg, 1987). The inhibitory effect of the leukemia cell lines is lost when they are first induced to differentiate by cis-retinoic acid or sodium butyrate. Similar findings have been reproduced *in vivo* using plasma clot diffusion chambers seeded with normal marrow and implanted in rats with Shay chloroleukemia. As the number of leukemia cells increased in the animal's bone marrow, a decrease in the number of normal progenitors was observed in the chamber (Steinberg, 1987). Since the decrease in hemopoiesis occurred within a compartmentalized area, the inhibition observed must have been mediated by a diffusible, leukemia-associated inhibitory factor (LAI) (Steinberg, 1987; Olsson and Olofsson, 1980). These experimental observations are probably a realistic reflection of the situation in leukemia patients. Sera from patients with hairy cell leukemia can inhibit *in vitro* colony formation by normal bone marrow progenitor cells (Lauria *et al.*, 1989). The inhibitory effect is proportional to the tumorburden and is abrogated with the induction of successful remission following interferon-α therapy.

Studies by Olsson and Olofsson (1980) have shown that LAI is a nontoxic factor that specifically inhibits proliferation in normal marrow progenitor cells. Using a modified Marbrook chamber, normal marrow cells were incubated in the inner chamber, which was separated from the outer chamber by a nucleopore filter. The outer chamber contained marrow cells from patients with AML or CML or tissue culture medium only (control). Following a 70-min incubation, the normal cells were recovered, subjected

to a thymidine suicide technique, and plated for *in vitro* colony formation. Compared with controls, there was a significant reduction in the percentage of GM-CFCs in DNA synthesis in those chambers adjacent to AML or CML cells but there was no indication of cytotoxicity. Somewhat surprisingly, LAI production was associated with a nonleukemic cell that was present in the blood mononuclear cell fraction. This paraneoplastic effect has also been noted in patients with chronic lymphocytic leukemia (CLL) in which bone marrow stromal cells have been shown to produce excess amounts of TGF-β (Lagneaux *et al.*, 1993).

At present, the active component of LAI has not been characterized. It does not appear to be identical to the leukemia inhibitory activity described by Broxmeyer *et al.* (1981) and subsequently reported to be acidic isoferritin. It appears more likely that leukemia-associated inhibitory factors(s) is in fact a heterogeneous group. The expression of other inhibitory factors [TNF-α (Brockhaus *et al.*, 1990; Lindeman *et al.*, 1989), TGF-β (Lagneaux *et al.*, 1993; Kremer *et al.*, 1992), and MIP-1α (Yamamura *et al.*, 1989)] has also been demonstrated in cells from leukemia patients, as was the original granulocyte chalone, the forerunner of the hemopoietic regulatory pentapeptide found to be produced by Shay chloroleukemia cells (Rytomaa and Kiviniemi, 1968b).

B. Tumor Growth Modulation by Proliferation Inhibitors

If neoplastic cells produce one or more feedback inhibitor, they may retain some degree of sensitivity to these molecules. Alternatively, the neoplastic clone may have developed an inherent resistance to these inhibitory signals, thus ensuring a continued growth advantage. More than 20 years ago Bichel showed that the hypotetraploid JB-1 and Erlichs ascites tumors each grow to a maximum size of 10^9 cells (Bichel, 1972). Nevertheless, the growth of each tumor was limited by a factor in its own ascitic fluid. Cell-depleted ascitic fluid from a tumor-bearing mouse had a specific antiproliferative effect and was shown to block the entry of cells into mitosis. It therefore appears that some tumors continue to regulate their own growth rate. However, it is also possible that the inhibitory factor present in the ascites was of host origin and not tumor derived.

Despite Bichel's findings, it is likely that tumor cells are intrinsically more resistant to feedback inhibitory signals than their normal counterparts and that this, at least in part, accounts for the observed hyperproliferative state in malignancy. Rytomaa and Kiviniemi (1968b) found that Shay chloroleukemia cells generate large quantities of the granulocyte chalone but are themselves less sensivity to its effects. It remains to be seen whether

tumors have a differential sensitivity to inhibitors that is dependent on the tissue of origin.

A number of limited studies have evaluated the antiproliferative effects of the hemopoietic stem cell inhibitors on neoplastic cells. The majority have not shown any growth modulation. However, the conclusions are based largely on *in vitro* effects of inhibitors on a spectrum of hemopoietic and nonhemopoietic cell lines. The pEEDCK monomer had no effect on the MCF7 human breast carcinoma cell line or the GaMg human glioblastoma cell line (Laerum *et al.,* 1990a). A modest inhibition was seen with the Erhlichs ascites tumor using higher doses (10^{-7} *M*) than those required for hemopoietic progenitor inhibition. The pentapeptide also inhibited the HL60 leukemia cell line but, surprisingly, this did not modify the leukemia'a response to cytosine arabinoside (Paukovits *et al.,* 1990). The stimulatory dimer had no effect on MCF7 cells *in vitro* though it has been reported to produce a slight but variable stimulatory effect on murine SC1 lymphoma cells and the human GaMg glioblastoma cell line (Frostad *et al.,* 1993). The tetrapeptide AcSDKP does not appear to inhibit tumor cell growth *in vitro,* but once again the experimental evidence is extremely limited. Guigon *et al.* (1991) found that AcSDKP had no effect on the leukemia cell lines HL60 or K562. Similarly, no inhibitory effect has been noted on leukemic cells from CML patients (Cashman *et al.,* 1994).

The effect of MIP-1α has been evaluated using solid tumor cell lines and leukemia cells, with variable results. Lord *et al.* (1987) first reported that a potentially leukemic cell line was highly resistant to the inhibitory effects of NBME-IV. This was in contrast to a clear inhibition of the IL-3-dependent hemopoietic cell line from which the independent line was derived. In a further model, L1210 lymphoid leukemia cells were cocultured with normal bone marrow (Tsyrlova and Lord, 1989). Under these circumstances the L1210 cells dominated the culture, suppressing normal hemopoiesis. Concurrent treatment with NBME-IV and cytosine arabinoside resulted in specific cytotoxicity on the leukemia cells and the remergence of a normal marrow-like culture. More recently, leukemia cells from patients with CML have been shown to be MIP-1α resistant (Eaves *et al.,* 1993a,b; Holyoake *et al.,* 1993).

The differential toxicities of normal and leukemic cells in the L1210 model and CML thus suggest that combined chemotherapy and inhibitor protocols may be beneficial in clinical practice, MIP-1α providing protection for the normal stem cells while the chemotherapy attacks the malignancy. Despite the potential benefit in CML, a degree of caution is still required; MIP-1α resistance may not extend to all leukemia subtypes. Recently Ferrajoli *et al.* (1994) reported that rMIP-1α prevented AML progenitors (variable FAB subtypes) from entering DNA synthesis in a significant number of patients. While conflicting, these findings are perhaps not surprising

given the marked heterogeneity in the response of leukemic cells to growth factors and cytotoxic agents. Clearly, further experimental studies are necessary to fully elucidate the effects of MIP-1α on hemopoietic-derived malignancies.

Nonhemopoietic tumor cell lines do not appear to be sensitive to MIP-1α, at least *in vitro*. The clonal growth of a wide spectrum of solid tumor cell lines remained unaffected by continuous exposure to rMIP-1α (Korfel *et al.*, 1994). However, the report failed to confirm the activity of the MIP-1α on normal cells.

The inhibitory effects of AcSDKP, pEEDCK, and MIP-1α are relatively specific for normal hemopoietic progenitor cell subsets. Other inhibitory molecules, including TGF-β, TNF-α, and interferon, have more complex interactions with normal and malignant tissues. These effects may be growth inhibitory or cytotoxic. TGF-β is a potent inhibitory glycopeptide that has been shown to have growth-modulating effects on both normal and tumor tissues (Roberts *et al.*, 1985; Sing *et al.*, 1988). In addition to the inhibitory actions on hemopoietic progenitors, TGF-β is known to inhibit the anchorage-dependent growth of human tumor cell lines (Roberts *et al.*, 1985) and inhibits human CML cells in contrast to the effects seen with MIP-1α (Cashman *et al.*, 1990; Holyoake *et al.*, 1993). Similarly, TNF-α and interferon are effective antitumor agents both *in vivo* and *in vitro*. Their mechanism of action, however, remains obscure but almost certainly extends beyond simple cell cycle inhibition.

C. Mechanisms of Tumor Resistance

The ability of tumor cells to escape the growth regulatory actions of feedback inhibitors represents a developmental strategy to maintain a growth advantage over normal tissues. The growth advantage achieved is a consequence of a number of factors and includes insensitivity to inhibitors and growth suppression of the normal counterpart. The growth suppression may result from tumor-derived or accessory cell-derived inhibitors or perhaps a combination of both sources. Progressive bone marrow failure frequently accompanies, for example, advancing CLL and may occur in advance of any physical "crowding out" effect by the increasing tumor burden. It appears that, in addition to the malignant expansion of monoclonal lymphocytes, patients with CLL also have a defective bone marrow microenvironment. Studies by Lagneaux *et al.* (1993) and Stryckmans *et al.* (1988) have revealed that stromal cells from patients with B-cell CLL have a deficient colony supportive activity with a decreased production of IL-6. TGF-β neutralizing antibody abrogated this suppression, probably because the malignant B-cells were inducing excess TGF-β production. IL-6 production

and the enhanced synthesis of TGF-β, with its secondary effects on normal hemopoiesis, may account for a number of features observed in CLL patients. Inhibition of normal hemopoietic progenitor cells by TGF-β probably contributes to the progressive bone marrow failure which is likely to be exacerbated by the deficiency in IL-6 production. IL-6 is also recognized to be an important signal for enhancing terminal differentiation of activated B-cells. Its shortfall may play a role in initiating and maintaining the differentiation block and the resultant hypogammaglobulinemia. Once again, the growth advantage of the B-cell clone may be augmented by a degree of TGF-β resistance (Israels *et al.*, 1990).

The mechanism(s) underlying the differential sensitivity between normal and tumor cells are far from clear and presumably exist at multiple levels. Alterations in the delicate balance between growth stimulatory and inhibitory regulatory signals may be involved. Thus, proto-oncogene overexpression may act to enhance the growth stimulus and override the feedback inhibitory circuit. Loss of tumor suppressor genes may result in a similar scenario. A major regulatory step governing the action of TGF-β is the activation of its latent, precursor molecule. Certain epithelial carcinoma cell lines have lost the ability to activate latent TGF-β despite the fact that they continue to secrete inactive TGF-β and bear receptors for the molecule (Keski-Oja *et al.*, 1987).

In addition to the variable effects of inhibitors on normal and transformed cells, neoplasms may also respond differentially to inhibitory molecules. CML is associated with a specific genetic rearrangement, BCR-ABL, that encodes a protein with a number of novel properties, including tyrosine kinase activity (De Klein *et al.*, 1982; Konopka *et al.*, 1984). This fusion gene is specific for CML and almost certainly plays a primary role in the pathogenesis of the disease and its hyperproliferative state. It is therefore interesting to speculate on the link between MIP-1α resistance and the fusion gene product. The continued response to TGF-β exhibited in the same leukemic cell suggests that the two inhibitors act through different signal transduction pathways. Alternatively, the BCR-ABL product may interfere with the action of MIP-1α at a site proximal to its convergence with the pathway that delivers the antiproliferative signal initiated by TGF-β.

VI. Clinical Perspectives

The enhanced production of inhibitory factors may account for a number of clinical observations that are relevant to both malignant and nonmalignant diseases. Under normal conditions, human bone marrow nucleated cells express low levels of MIP-1α mRNA (Maciejewski *et al.*, 1992). However,

there is a very significant increase in the level of MIP-1α transcripts in patients with aplastic anemia and myelodysplasia (Maciejewski *et al.*, 1992). It is perhaps possible, therefore, that an exaggerated production of MIP-1α plays a role in the underlying pathophysiology of bone marrow suppression. Similarly, it appears likely that certain inhibitors are responsible, at least in part, for malignancy-associated bone marrow failure, e.g., TGF-β in CLL (Lagneaux *et al.*, 1993; Stryckmans *et al.*, 1988). The overproduction of inhibitors observed in these pathologies suggests a potential role for neutralizing antibodies or antisense oligonucleotides against the inhibitory molecule. Conversely, malignant cells may maintain a growth advantage by developing inhibitor resistance. This scenario is best illustrated by the continued proliferation of CML progenitors in the presence of MIP-1α (Eaves *et al.*, 1993a,b; Holyoake *et al.*, 1993). In this instance, the addition of MIP-1α may provide controlled suppression of normal hemopoiesis and allow specific targeting of the malignant progenitor cell population by chemotherapy. This hypothesis is currently the subject of U.K. multicenter trial evaluating the MIP-1α analog BB10010 in patients with CML. It remains to be seen whether nonmalignant, hyperproliferative states such as psoriasis and the bone marrow myeloproliferative diseases may be similarly amenable to treatment with tissue-specific inhibitors.

A number of the hemopoietic proliferation inhibitors possess a wide spectrum of activity beyond cell cycle regulation. The polyfunctional nature of TGF-β, TNF-α, and the IFNs has resulted in these molecules being evaluated in such diverse conditions as wound healing (Sporn *et al.*, 1983), multiple sclerosis (Silberberg, 1994), and malignancy (Hieber and Heim, 1994; Quesada *et al.*, 1986a,b).

A. Bone Marrow Protection

Experimental studies and clinical trial data have shown that the tumor response to chemotherapy is directly proportional to the delivered dose. More precisely, the response is related to the intensity of delivery when expressed as per unit of time (dose/m^2/week). This concept of dose intensity is fundamental to treatment design and ultimately may represent the major variable in tumor response and survival. The major dose-limiting factor in cancer chemotherapy is bone marrow damage. Neutropenia and thrombocytopenia may result in significant patient morbidity and may prevent the delivery of curative chemotherapy regimens. Most of the clinically relevant chemotherapeutic agents produce reversible myelosuppression. This results from damage to the rapidly dividing progenitor cell compartment, which produces a delayed fall and recovery of the morphologically recognizable blood cells. The reduction in progenitor cell numbers is reflected in an

attenuated feedback inhibitory stimulus to the normally quiescent pluripotent stem cell pool. Recruitment of these latter cells into DNA synthesis replenishes the maturing populations with normalization of the blood picture. Unfortunately, the return to stem cell quiescence does not appear to mirror the recovery of mature cell numbers (Lord, 1988)—an index that historically has guided the decision for retreatment. At present, treatment programs are based on multicyclic schedules that almost certainly produce incremental damage to the more primitive self-renewing hemopoietic precursor cells. This is borne out by the clinical observations of delayed neutrophil recovery and increasing toxicity on megakarypoiesis. Ultimately this damage may produce a picture of delayed bone marrow failure, dysplasia, or secondary acute myloid leukemia (Testa *et al.*, 1990).

The introduction of hemopoietic growth factors has allowed a modest increase in dose intensity (typically less than twofold). The ability to accelerate chemotherapy delivery with G-CSF or GM-CSF has resulted in a greater response rate but it is unlikely, given the limited improvement in dose intensity, that this will be reflected in an improved survival or remission duration. Furthermore, this approach is hampered by increasing thrombocytopenia and additional dose-limiting toxicities, including mucositis. Experimental studies also suggest that the accelerated delivery of chemotherapy with growth factor support may increase stem cell damage, with the attendant risk of late complications (Hornung and Longo, 1992).

The functional assessment of proliferation inhibitors is based on their ability to reduce cell death from S-phase-specific agents such as tritiated thymidine (^{3}HTdR). This evaluation points to a potential role for stem cell inhibitors as chemoprotective agents. Murine models, utilizing MIP-1α in combination with hydroxyurea or cytosine arabinoside, have confirmed this protective effect (Lord *et al.*, 1992; Dunlop *et al.*, 1992). MIP-1α-treated mice showed a faster recovery of the CFU-S population and an earlier normalization of neutrophil numbers. This mode of therapy not only provides an alternative method for escalating dose intensity but also has the advantage of maintaining stem cell numbers and viability, which if confirmed with other cytotoxics, should result in less cumulative toxicity to megakaryopoiesis and possibly reduce the risks of long-term bone marrow damage.

Many of the clinically useful cytotoxic agents are not specific for cells in DNA synthesis, but target cells largely irrespective of their cycle status. It therefore remains to be seen whether inhibition of proliferation will have any useful therapeutic impact in this setting. Further experimental investigation is required but several observations suggest that there may be a therapeutic benefit for protection against all classes of cytotoxics. Non-S-phase-specific drugs such as doxorubicin and cyclophosphamide produce a greater cell kill when cells are synchronized in DNA synthesis (Kim and Kim, 1972; Dewys *et al.*, 1970). Also, the qualitative damage induced by alkylating

species is potentially reparable (Sancar and Sancar, 1988) if the cell has sufficient time to carry out gene housekeeping functions before it enters DNA synthesis. While rather esoteric, these observations suggest that cell cycle inhibition may at least attenuate the toxicity of noncycle-specific agents and possibly prevent the propagation of sublethal mutational damage.

At present there are only limited experimental data on the use of inhibitors with non-S-phase-specific drugs. The tetrapeptide AcSDKP has allowed a modest increase in survival in mice receiving lethal doses of doxorubicin (Ramirez *et al.*, 1994) or cyclophosphamide (Bogden *et al.*, 1991). The mechanism of this advantage has still to be elucidated, but our own observations show that MIP-1α-treated mice are subject to identical acute hemopoeitic damage following a range of chemotherapeutic insults, including cyclophosphamide, 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and busulfan (E. Marshall, unpublished observations). TNF-α has been shown to reduce the toxicity of 4-hydroxy-cyclophosphamide (4HC) on hemopoietic progenitor cells *in vitro* (Moreb *et al.*, 1990). The mechanism of this cytoprotection is unclear but it is unlikely to be a consequence of cell cycle arrest. Recently, however, TNF-α was reported to produce a three-fold increase in cytosolic aldehyde dehydrogenase, an enzyme that has been postulated to be responsible for 4HC resistance in hemopoietic stem cells (Dainiak *et al.*, 1994).

The protective effects of the MIP-1α analog BB10010 on hemopoiesis have been investigated using a murine model of repeated sublethal radiation (four cycles of 4.5 G γ-ray radiation every 2 weeks) (Lord *et al.*, 1995). BB10010 was administered before radiation, via an implantable osmotic pump that delivered a continuous infusion of the molecule over 7 days. Over the four treatment cycles, the results showed a significant cumulative enhancement of the CFU-S recovery in the BB10010-treated cohort despite an apparently small direct protective effect. Reduction in the duration of BB10010 exposure attenuated the response, suggesting that the major therapeutic benefit of MIP1-α in this setting probably arises from the stem cell self-renewing enhancement properties of the molecule (Lord, 1995; Verfaille *et al.*, 1994). Cell cycle inhibition played a secondary role in ameliorating some of the radiation-induced damage.

The increased use of intensified chemotherapy with hemopoeitic growth support has highlighted the problem of additional dose-limiting toxicities on other self-renewing tissues. Ulcerative stomatitis or mucositis is a common, painful condition that frequently accompanies chemotherapy. Increasingly, it has become a dose-limiting factor for a number of chemotherapy regimens. The etiology of mucositis is probably related to the cytotoxic effect of chemotherapy on the rapidly dividing cells of the basal oral epithelium. The development of an epithelial proliferation inhibitor might therefore afford protection to the oral and gut mucosa in a fashion similar to that

for the hemopoietic inhibitors. As a start, Sonis *et al.* (1994) found that the topical application of TGF-β3 reduced the fraction of oral epithelial cells undergoing DNA synthesis so that when applied prior to chemotherapy, the inhibitor resulted in a significant reduction in the incidence, severity, and duration of mucositis.

A limited number of observations suggest that MIP1-α may also possess growth-modulating properties on epithelial tissues (Graham and Pragnell, 1992; Lord *et al.*, 1993). In contrast to TGF-β, MIP1-α is extremely well tolerated, with a maximum tolerated dose not yet reached in clinical trials. This lack of toxicity offers a significant advantage in clinical protection studies. The recognition that MIP1-α may also influence DNA synthesis of spermatogonia (Hakovirta *et al.*, 1994) hints at an intriguing mode of protection for limiting gonadal damage during cancer chemotherapy.

B. Mobilization of Hemopoietic Progenitor Cells by MIP-1α

During the course of investigating the myeloprotective effects of the MIP-1α analog BB10010, Lord *et al.* (1995) observed an unexpected transient mobilization of leukocytes giving a four-fold increase over baseline cell numbers within 30 min. This was mirrored by progenitor cell release into the peripheral blood. A single subcutaneous administration of BB10010 doubled the circulating CFU-S(8), and CFU-S(12). When evaluated after 2 days of G-CSF priming, a single administration of BB10010 increased circulating CFU-S(8), CFU-S(12), and MRA to 38,-33, and -100-fold respectively. The mobilization of progenitor cells following chemotherapy also appears to be enhanced by BB10010 with or without G-CSF (E. Marshall, unpublished). These unexpected findings underline the difficulty or defining the specific physiological role of many of these molecules. However, the findings do have important clinical implications with the recognition of a novel agent that possesses both myeloprotective and mobilization potential. Current clinical practice necessitates approximately 5 days of G-CSF for optimal harvesting of sufficient hemopoietic progenitor cells for transplantation. Furthermore, the peak effect of this mobilization is also poorly defined, occurring between 24 and 30 hr postfinal G-CSF (Sato *et al.*, 1994). The report by Lord *et al.* suggests that the combined use of BB10010 and G-CSF may enhance progenitor cell numbers, improve the quality of the apheresis, and allow a more rapid and predictable time course for harvesting. These issues are the subject of several clinical trials now under way.

The mechanism of progenitor release following BB10010 is unclear but it appears to be the result of mobilization rather than sequestration. MIP-1α is known to have chemotactic properties on several mature leukocyte subsets and may act via adhesion factor expression. Interestingly, many

unrelated chemotactic factors also share this ability to produce an acute leukocytosis, possibly by producing morphological or cytoskeletal changes in the target cells or by modifying surface proteins involved in adhesion (Jagel and Hugli, 1992). Recently, a related chemokine, IL-8, has also been reported to produce acute mobilization of progenitor cells with remarkably similar kinetics (Laterveer *et al.*, 1995). At present there are no data showing a similar mobilization potential for other hemopoietic inhibitory molecules.

VII. Concluding Remarks

Despite initial scepticism, an increasing number of putative feedback inhibitors have now been described. As a consequence of improved biochemical separation techniques and advances in recombinant DNA technology, large quantities of pure factors are now available for evaluation. Extensive preclinical studies have produced a wealth of data and, as a result, several inhibitors are now in clinical trials with the therapeutic goal of alleviating chemotherapy-induced toxicity in oncology patients. However, the cellular response to inhibitory factors remains a remarkably difficult end point to assess. The response to the hemopoietic proliferation inhibitors, unlike the hemopoietic growth factors, may be subtle and only quantifiable following perturbation of the system. *In vitro* studies have highlighted the importance of the cell environment, with the results highly dependent on the combination of growth factors or the presence of accessory cells such as macrophages. Furthermore, evaluation is handicapped by the heterogeneity of bone marrow mononuclear cells even in apparently purified populations such as those enriched for $CD34^+$. The suicide technique using tritiated thymidine or S-phase-specific cytotoxic agents remains the major tool for assessing the proliferative index of cell populations. However, investigators must appreciate that the results require careful interpretation and that a sufficient number of experiments must be performed for adequate statistical analysis. In the future, increasingly sophisticated immunophenotyping may assist in defining and purifying the precise target cell population. Also, cell cycle analysis may be greatly enhanced by an improved knowledge of the signal transduction pathways and the effects on gene expression that follow inhibitor–receptor interactions.

Clinical evaluation of the inhibitors is likely to prove equally challenging. Conventional phase I studies are designed to define a dose that results in a required biological response or maximum tolerated dose. In steady-state conditions, the biological effect resulting from inhibitor treatment may not be apparent or may even be absent. The dose of inhibitor used in subsequent phase II trials may therefore be based solely on empirical pharmacodynamic

parameters. The recognition that inhibitor effects may follow a bell-shaped dose-response curve adds a further complication with the real possiblity that pharmacologically detectable doses may not reflect the optimal concentration required for adequate bone marrow protection.

The tetrapeptide AcSDKP was the first inhibitor to enter clinical trial (Carde *et al.*, 1992). A phase I/II study was undertaken in cancer patients undergoing consecutive cycles of monochemotherapy (Ara-c or Ifosfamide). The study was designed as a double-blind crossover protocol with one cycle "protected" by inhibitor and one cycle "protected" by placebo. The protective effect was assessed by comparing leukocyte recovery. The tetrapeptide had a modest dose-response effect, with the duration of neutropenia shortened from 7.1 to 5.7 days ($p < 0.04$). The study design had the advantage of avoiding interpatient variation—a significant problem when evaluating hemopoietic recovery. However, any clinically significant benefit may only be apparent following consistent protection over multiple cycles of chemotherapy. The alternative strategy of comparing inhibitor-treated patients with a placebo-controlled arm may represent a more attractive approach but will undoubtedly necessitate a large number of patients. The protective effect may not be easily defined using current cell cycle analytical techniques but instead may have to be assessed indirectly by quantifying the surviving progenitor cells and by comparing mature cell recovery kinetics.

The past 10 years have seen a marked improvement in the availability of pure inhibitory factors. The challenge for the future is to design appropriate experimental and clinical studies to help define more clearly their physiological role and any potentially useful clinical properties.

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The Incidence, Origin, and Etiology of Aneuploidy

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Aneuploidy, the presence of an extra or missing chromosome, is the most frequent cause of mental retardation and pregnancy loss in our species. Studies can be divided into those of incidence, origin, and etiology. Trisomy 21 is the most common aneuploidy among liveborns whereas monosomy X and trisomy 16 are the most frequent causes of pregnancy loss. Aneuploidy primarily arises by the process of nondisjunction in the first meiotic division of maternal meiosis; however, this varies among chromosomes in that some show a significant proportion of paternal and/or meiosis II errors. The most common etiological factor associated with aneuploidy is advancing maternal age and it is generally agreed that this is a result of the increasing likelihood of nondisjunction in the aging ovary. There has been intense debate as to the existence of of a paternal age effect and recent studies on human sperm suggest that there may be a small effect for the sex chromosomes. Furthermore, recent molecular studies on trisomic conceptuses have revealed a second etiological factor associated with nondisjunction, namely, reduced genetic recombination.

KEY WORDS: Aneuploidy, Trisomy, Monosomy, Nondisjunction, Age effect, FISH, Chromosome, Mosaicism

I. Introduction

Until recently, cytogenetic research was fundamentally a descriptive science. It was in the late 1950s that preparations of human chromosomes became good enough to reliably obtain karyotypes from a number of human

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tissues and this was largely thanks to the accidental discovery that hypotonic solutions spread the chromosomes adequately to allow analysis (Tjio and Levan, 1956; Ford and Hammerton, 1956). The normal human number of 46 chromosomes rapidly became apparent and deviations from this were soon associated with estabished clinical syndromes. For instance, trisomy 21 (three copies of chromosome 21) was associated with Down syndrome (Lejeune *et al.*, 1959), monosomy X (one copy of the X chromosome) with Turner syndrome (Ford *et al.*, 1959), and "XXY" sex chromosome trisomy with Klinefelter syndrome (Jacobs and Strong, 1959). In addition, other trisomic conditions [such as trisomy 18 with Edwards syndrome (Edwards *et al.*, 1960) and trisomy 13 with Patau syndrome (Patau *et al.*, 1960)] were described on the basis of chromosomal findings.

The ability to distinguish each chromosome became possible with the introduction of banding techniques; these revealed distinctive patterns for individual chromosomes and permitted every trisomy to be unequivocally determined. Furthermore, they allowed the detection of small structural rearrangements such as deletions, duplications, and translocations (Miller, 1974). Thus, cytogenetics rapidly became a clinical procedure, particularly for prenatal diagnosis, and research in this area involved a great deal of cataloging of interesting cases and calculating their incidence in the general population.

Lately things have changed: the union of molecular technology with classical cytogenetic approaches, principally the introduction of fluorescent *in situ* hybridization (FISH), has opened a new era in cytogenetic research. It is rapidly moving away from being principally a descriptive science and toward addressing fundamental questions about the origin, etiology, and mechanism of formation of chromosome abnormalities (Hassold and Schwartz, 1994).

Chromosome abnormalities can be either structural (involving rearrangements of chromsomes) or numerical (involving whole chromosomes). Numerical abnormalities fall into two categories: those involving an extra or missing single chromosome (aneuploidy) and those involving an extra whole chromosome complement (polyploidy). This chapter reviews the past and present research on aneuploidy (monosomy and trisomy).

Aneuploidy largely arises as result of an error of chromosome segregation at cell division, namely, "nondisjunction." In this error, homologous chromosomes or sister chromatids, rather than segregating against one another in a balanced configuration, segregate in such a way as to produce unequal numbers of chromosomes in daughter cells. This can occur at meiosis I, meiosis II, or mitosis. However, as will be discussed in more detail later, it is principally an event associated with the first meiotic division of maternal meiosis. Less frequently, nullisomic gametes and hence monosomic offspring may arise through loss of a chromosome by "lagging" on the meta-

phase plate. Studies of aneuploidy in humans fall into three specific areas: incidence, origin, and etiology.

II. Incidence

A. Live Births

Liveborns are the most amenable population by which to asses the incidence of aneuploidy and they have hence been the most extensively studied. Down syndrome (trisomy 21) is among the most common of genetic diseases in humans and occurs in about 1 in 750 live births. Trisomies 18 and 13 (both of which are associated with severe clinical phenotypes) are found in 1 in 10,000 and 1 in 20,000 live births respectively. Hence trisomy (primarily of chromosome 21 but also to a lesser extent of chromosomes 18 and 13) is the most prevalent cause of mental retardation in our species (Hassold *et al.*, 1984). Each of the sex chromosome trisomies (XXX, XXY and XYY) confers less severe phenotypes and is also common, with an incidence of 1 in 650 live births, collectively, whereas the incidence of sex chromosome monosomy is 1 in 20,000 at birth. When mosaic trisomies (those with a mixed population of normal and abnormal cells) are taken into account, this means that about 1 in 300 of all babies are born with some form of aneuploidy. The precise figures for the incidence of each type of aneuploidy based on nearly 60,000 births is given in Table I.

B. Stillbirths

Comparatively little is known about the incidence of trisomy among stillbirths, i.e., fetal deaths occuring after 20 weeks postgestation, and studies have involved some 624 fetuses. It is estimated that trisomy occurs in 4% of all stillbirths (Hassold *et al.*, 1995). This is more than tenfold the incidence in liveborns. The trisomies seen in liveborns are also the most represented among stillborns; however, trisomies 9 and 22 have also been reported (Table I).

C. Spontaneous Abortions

Many studies have clearly correlated the incidence of spontaneous abortion (i.e. fetal death between 6 and 20 weeks' gestation) with numerical chromosome abnormalities—particularly aneuploidy. Two approaches have been

TABLE I

Incidence of Sex Chromosome Monosomy and Individual Trisomies in Different Populations of Clinically Recognizable Human Pregnancies, and Estimated Proportion Surviving to Term[a]

Chromosome constitution	Spontaneous abortions (n=4088) (%)	Stillbirths (n=624) (%)	Livebirths (n=56952) (%)	All clinically recognized pregnancies (%)	Liveborn (%)
Trisomy:					
1	—	—	—	—	—
2	1.1	—	—	0.16	0.0
3	0.3	—	—	0.04	0.0
4	0.8	—	—	0.12	0.0
5	0.1	—	—	0.02	0.0
6	0.3	—	—	0.04	0.0
7	0.9	—	—	0.14	0.0
8	0.8	—	—	0.12	0.0
9	0.7	0.2	—	0.10	0.0
10	0.5	—	—	0.07	0.0
11	0.1	—	—	0.07	0.0
12	0.2	—	—	0.02	0.0
13	1.1	0.3	0.005	0.18	2.8
14	1.0	—	—	0.14	0.0
15	1.7	—	—	0.26	0.0
16	7.5	—	—	1.13	0.0
17	0.1	—	—	0.02	0.0
18	1.1	1.1	0.01	0.18	5.4
19	very few	—	—	—	0.0
20	0.6	—	—	0.09	0.0
21	2.3	1.3	0.13	0.45	23.8
22	2.7	0.2	—	0.40	0.0
XXY	0.2	0.2	0.05	0.08	53.0
XXX	0.1	0.2	0.05	0.05	94.4
XYY	—	—	0.05	0.04	100.0
Mosaic trisomy	1.1	0.5	0.02	0.18	9.0
Double trisomy	0.8	—	—	0.12	0.0
Total trisomy	26.1	4.0	0.3	4.1	6.0
Sex chromosome monosomy (45, X)	8.6	0.3	0.005	1.3	0.3
Total aneuploidy	34.7	4.3	0.305	5.4	6.3

[a] Adapted from Hassold *et al.*, 1996.

used to analyze this material, namely, cultured preparations (Hassold *et al.*, 1980) and direct preparations (Eiben *et al.*, 1990). Both indicate that aneuploidy accounts for 35% of spontaneous abortions and hence is the leading cause of pregnancy loss in humans; unlike stillbirths and live births,

however, trisomies of all human chromosomes with the exception of chromosome 1 have been reported. Trisomy 16 is the most common trisomy, but the most common single aneuploidy is monosomy X. Despite being associated with a relatively mild clinical phenotype among liveborns, it is estimated that less than 0.3% of monosomy X conceptuses survive to term (Table I). Thus the incidence of aneuploidy seen in spontaneous abortions (35%) is nearly tenfold that seen in stillbirths (4%) and represents a 100-fold increase over that observed in liveborns (0.3%). The full details of the findings by Hassold and colleagues (the largest of these studies) on the incidence of individual aneuploidies in spontaneous abortions is given in Table I. It is therefore possible, given the incidences in stillbirths and liveborns, to estimate the probability that an aneuploid conceptus, once clinically recognized, will survive to term. These figures are also given in Table I.

D. Preimplantation Embryos

The advent of *in vitro* fertilization (IVF) techniques has made it possible to study human embryos at conception and assess aneuploidy rates at this developmental point. However, study of IVF embroys may or may not accurately represent the *in vivo* situation since they are generated from hyperstimualted ovaries and since the effect of *in vitro* culture on the incidence of aneuploidy is not known. Despite this, Jamieson *et al.* (1994) and Angell *et al.* (1986) adapted standard cytogenetic procedures to collectively study metaphases from more than 200 embryos and suggested that the incidence of aneuploidy in this material is about 20%. Furthermore, the trisomies identified were, as might be expected, primarily of chromosome 16 and the acrocentric chromosomes.

Other studies using FISH on human embryos (Griffin *et al.*, 1991, 1992; Munne *et al.*, 1994) have the advantage that most or all of the cells in the embryo may be assessed at interphase, not merely those that are capable of producing analyzable metaphases. The major disadvantage in using this approach is that only a few chromosomes can be examined because of the limited number of colored fluorochromes in the visible spectrum. Hence investigations have thus far been limited to chromosomes X, Y, 18, 13, and 21. Nevertheless, a number of interesting conclusions have emerged. First, autosomal monosomy appears to be as frequent as trisomy at this stage. This finding is not entirely suprising since monosomies and trisomies are thought to be the results of reciprocal events at meiosis and thus supports the idea that autosomal monosomies all abort prior to being clinically recognized (Munne *et al.*, 1995). Second, the incidence of aneuploidy appears to increase with increasing maternal age, which is consistent with

most of the theories regarding the effect of maternal age on trisomy (see subsequent sections). Finally, aneuploidy increases with decreasing embryo quality and thus poorer quality embryos tend to be more chromosomally abnormal (Munne *et al.*, 1995). Therefore another reason to view data with caution when assessing the absolute incidence of aneuploidy is that these embryos are surplus to requirements from IVF clinics; they are of suboptimal quality (the better quality embroys are transferred to the uterus) and therefore, by implication, perhaps more likely to carry chromosome abnormalities.

E. Oocytes

IVF techniques have also allowed cytogenetic analysis of human oocytes and provided a means to look at chromosome abnormalities in the female gamete. These oocytes are recovered following therapeutic superovulation and are arrested in meiosis II; thus only errors that have occurred during the first meiotic division can be assessed. Taking all the major studies into account (Jacobs, 1992), rates of aneuploidy (calculated as twice the disomy rate since nullisomic preparations may reflect artifactual loss) in the range of 13.2% (Table II) have been reported; however this would presumably have been higher if MII errors could have been detected. As with studies of human embryos, these figures need to be viewed with caution because (1) the oocytes are generated from hyperstimulated ovaries, (2) they are largely those remaining unfertilized after exposure to sperm, and (3) they are recovered from women of greater than average reproductive age. For these reasons, it has been suggested that they are unlikely to accurately represent *in vivo* conditions (Jacobs, 1992).

F. Sperm

The majority of studies on the incidence of aneuploidy in the male gamete have used the human-hamster or "humster" fusion technique to prepare

TABLE II
Summary of Chromosome Studies of Human Oocytes and Sperm[a]

Cell type	Total no. studied	No. hypohaploid (%)	No. hyperhaploid (%)	2 X hyperhaploid (%)
Oocytes	1024	189 (17.8)	70 (6.6)	140 (13.2)
Sperm	20895	448 (2.1)	146 (0.7)	292 (1.4)

[a] Adapted from Jacobs (1992).

metaphases. This technique was introduced in the late 1970s (Rudak *et al.,* 1978) and more than 20,000 metaphases have been analyzed since then. The overall aneuploidy rate (calculated as twice the disomy rate since nullisomic preparations may reflect artifactual loss) is cited as 1–2%, about one tenth of the rate in oocytes; furthermore it appears that disomies of autosomes 1, 9, 16, 21, and the gonosomes are overrepresented (Holmes and Martin, 1993). This may be artifactual since these chromosomes are among those most readily identifiable in suboptimal preparations (Jacobs, 1992) but also may indicate that there are differences among chromosomes in rates of nondisjunction. It is unlikely, however, that the humster technique, due to its highly technical nature, will ever produce sufficient aneuploid metaphases to allow accurate estimates of chromosome-specific rates of nondisjunction in sperm. This is borne out by the fact that fewer than 150 disomic sperm have been identified since the approach was introduced in 1978! A much more robust way to address this question involves the use of fluorescent *in situ* hybridization. Using a FISH-sperm assay to estimate the proportion of disomic cells, a large number of meiotic products can be examined in a short time; further, the limitation of only being able to visualize two or three chromosomes in each sperm head does not present a problem since there is a virtually unlimited supply of cells and multiple aliquots can be used. Provided multicolor FISH is employed, disomic sperm can be distinguished from diploid sperm and it is possible, for the sex chromosomes, to distinguish meiosis I from meiosis II errors (Williams *et al.,* 1993; Griffin *et al.,* 1995). The results of the major multicolor FISH studies in this area are summarized in Table III. There is some variation among authors in calculated rates of disomy and this presumably reflects relative stringencies with regard to scoring criteria. Nevertheless, it seems clear that the sex chromosomes are particularly prone to nondisjunction, as suggested by the humster studies. Furthermore, our own studies, like those of the humster studies, suggest that chromosome 21 also has a high rate of nondisjunction. Both FISH and humster studies in human sperm make it clear that the incidence of aneuploidy in male gametes is about 2%.

G. Incidence of Aneuploidy at Conception

Extrapolation from data on all clinically recognized pregnancies indicates that at least 5% of all human conceptions are aneuploid. This is, however, almost certainly an underestimate since it is likely that many aneuploid conceptuses, such as all the autosomal monosomies, arrest before they are clinically recognized. For the reasons mentioned, studies on oocytes and embryos may not provide an accurate estimate of the *in vivo* situation.

TABLE III

Summary of Chromosome-Specific Rates of Disomy (%) from Two- and Multicolor FISH Sperm Studies[a]

	Chromosome disomy														
Study (No. of donors)	1	3	4	6	7	8	10	12	15	16	17	18	XY	XX	YY
Han *et al.* (1993)													0.28	0.21	0.21
Wyrobeck *et al.* (1993)						0.07							0.04	0.04	0.09
Williams *et al.* (1993) (9)										0.13		0.08	0.04	0.06	0.09
Bischoff *et al.* (1994) (2)		0.38	0.28	0.11	0.06	0.09	0.22	0.30	0.20	0.39	0.13	0.25	0.38	0.08	0.13
Spriggs *et al.* (1995) (5)	0.10							0.16	0.11			0.11	0.07	0.21	0.15
Griffin *et al.* (1995) (24)												0.04	0.13	0.02	0.03

[a] Adapted from Spriggs *et al.* (1995).

However, it is not unreasonable to hypothesize that the load of trisomy is somewhere in the region of a fifth to a quarter of embryos at conception. Figure 1 gives, estimates of the incidence of aneuploidy at various developmental stages on the basis of available data. Recently, Wolstenholme (1995) has provided an audit for the incidence of trisomy 16 (the most common human trisomy) at the various stages of development, including gametogenesis, conception and beyond; these values are also included in Fig. 1.

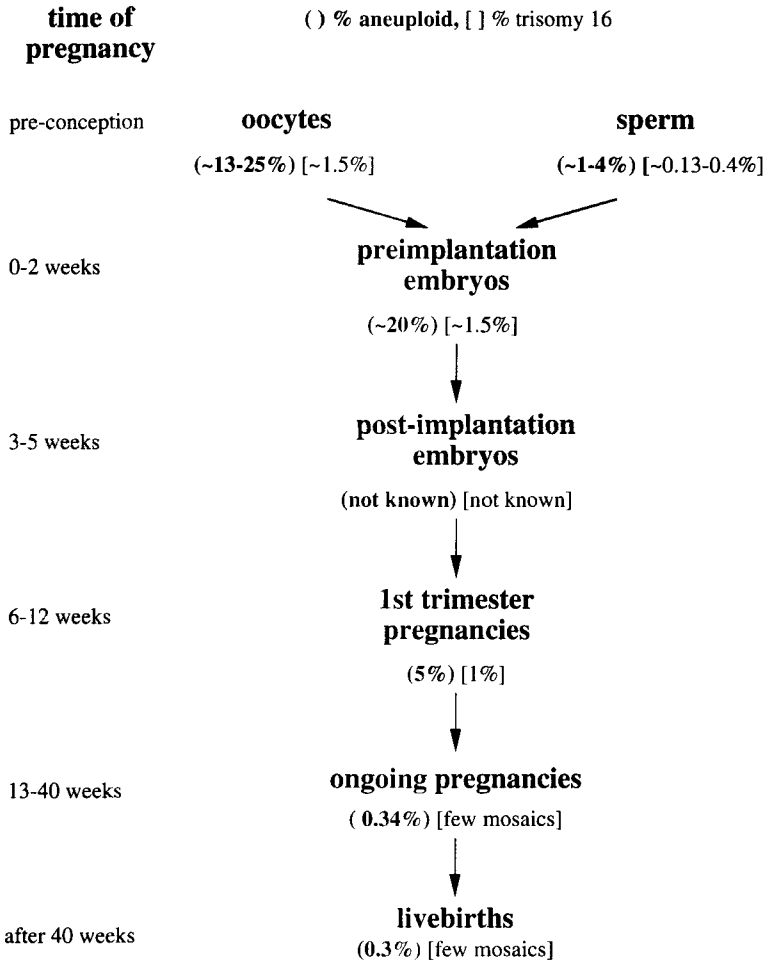

FIG. 1 Incidence of aneuploidy at various developmental stages.

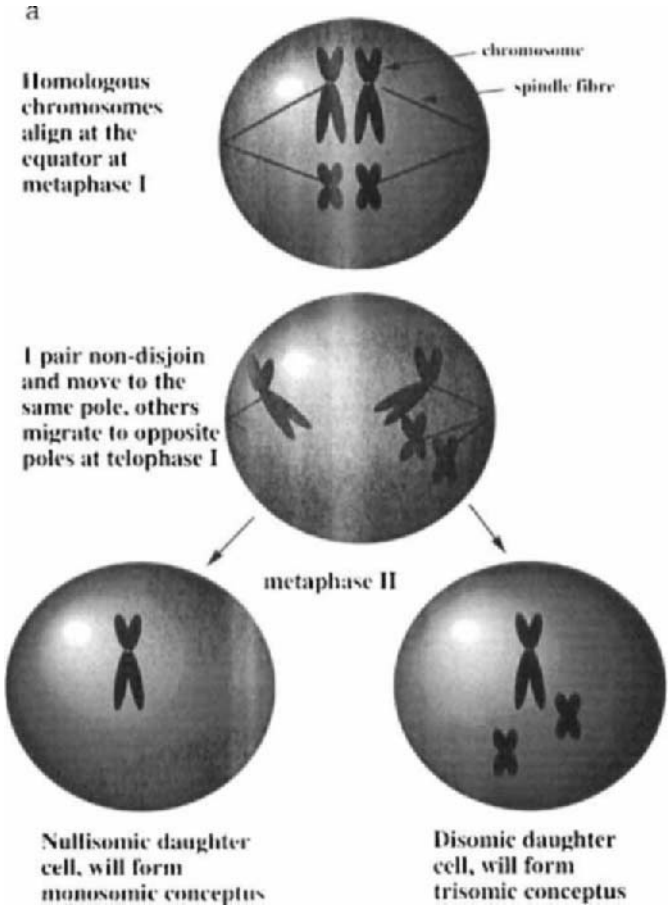

FIG. 2 Models of nondisjunction. (a) Classical model. (b, c) Angell model.

III. Origin

Initial attempts to distinguish maternal from paternally derived trisomies (and hence detemine the parent of origin) using cytogenetic polymorphisms, such as satellites on the acrocentric chromosomes, were superseded by less subjective and more accurate molecular approaches (Warren *et al.*, 1987). In early work, this involved restriction fragment length polymorphism studies, but highly polymorphic microsatellite markers have proven to be more informative. Work largely by Hassold and colleagues demonstrated that autosomal trisomy arises much more frequently in the female gamete (Hassold and Sherman, 1993). Nevertheless, paternally derived trisomies are

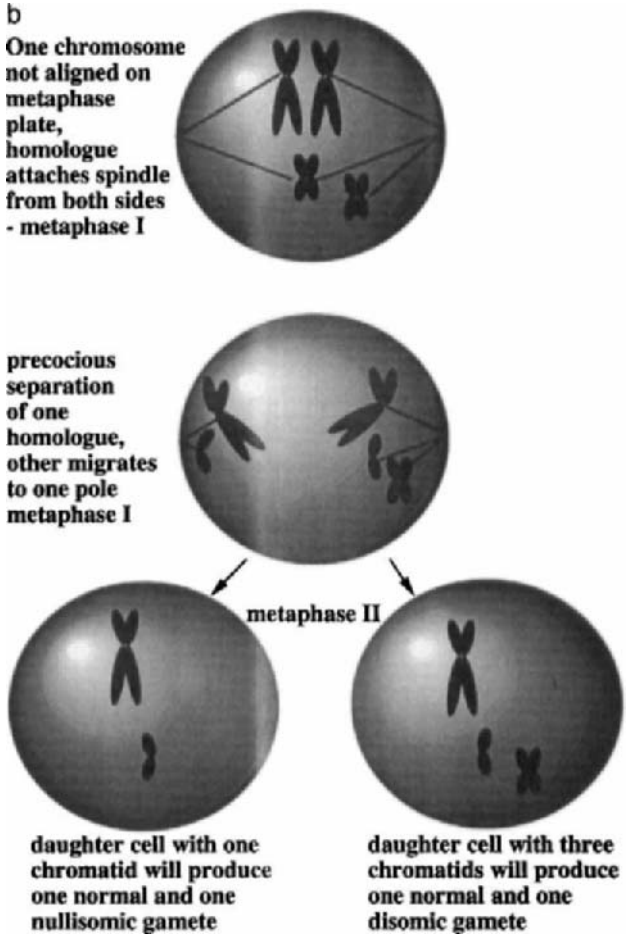

FIG. 2 (*continued*)

significant, contributing 7% of trisomy 21 cases and more for trisomies of thelarge chromosomes. Indeed, there is much variation among chromosomes in the parental origin of aneuploidy. For instance, 50% of XYY trisomies arise from nondisjunction in the sperm; paradoxically, paternally derived trisomy 16s are hardly ever seen. Furthermore, there is considerable variation in the stage of meiosis at which aneuploidy arises, namely, virtually all trisomy 16s arise as a result of an error in meiosis I, whereas one-third of trisomy 21 cases and the majority of trisomy 18 cases arise as a result of an error in the second meiotic division. The details for parental origin of all trisomies are given in Table IV.

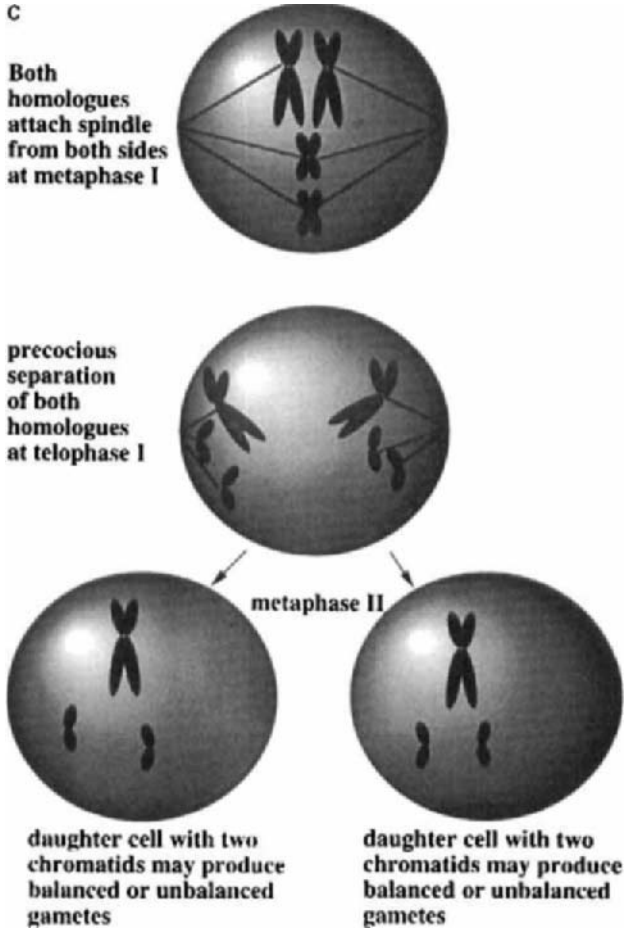

FIG. 2 (*continued*)

IV. Etiology

Despite nearly 40 years of cytogenetic research, relatively little is known about the underlying causes of nondisjuntion—so little in fact that it is almost embarrasing! Infrequently, aneuploidy arises genetically as a result of an unbalanced chromosomal translocation inherited from one parent. However, these conceptuses are rare compared with those arising via nondisjunction. Nondisjunction is primarily a *de novo* event and two major correlates have emerged as being associated with its incidence; these are advancing parental age and aberrant genetic recombination. Furthermore,

TABLE IV

Molecular Studies of Parental and Meiotic Stage of Origin in Autosomal and Sex Chromosome Trisomies[a]

Trisomy	No. of informative cases	Paternal			Maternal			Maternal (%)
		I	II	I or II	I	II	I or II	
2-12	16			3			13	81
13-15	54	1	4	2	12	8	27	87
16	62				51	1	10	100
18	73			3	16	35	19	96
21[b]	436	5	24		306	101		93
22	11		2		6	11		89
XXY	133	58			40	13	22	56
XXX	47		2		24	10	10	94

[a] From Hassold and Sherman (1993).

[b] For trisomy 21, we have presented only those cases having information on both parents and meiotic stage of origin of trisomy.

the classical theory with regard to the mechanism of nondisjunction in maternal meiosis I has been recently challenged by Angell and co-workers, based on their observations in human oocytes.

A. Mechanism of Nondisjunction

1. Classical Model

It has been long believed that nondisjunction in the first meiotic division occurs when homologous chromosomes, rather than segregating to opposite poles, segregate to the same pole, producing both disomic and nullisomic daughter cells. This is illustrated in Fig. 2a.

2. Angell Theory of Precocious Separation

In analyses of 179 meiosis II oocytes, Angell and co-workers (Angell, 1991; Angell *et al.*, 1994) found 64 that were chromosomally abnormal. However, rather than observing preparations with an extra or a missing chromosome (as might be expected by the classical model of nondisjunction), they observed either cells with 22 or 23 chromosomes and an extra chromatid, or those with 22 chromosomes and two extra chromatids. This led the authors to suggest that the predominant nondisjunction mechanism involves premature division of the centromere rather than migration of a whole chromosome to the wrong pole. This is illustrated in Figs. 2b and 2c. Opponents of this model, however, suggest that centromere separation as observed by

Angell and colleagues arises as a result of prolonged time in culture conditions since these oocytes often remain in culture for 3 days or more.

B. Effect of Maternal Age

The relationship between advancing maternal age and increasing incidence of Down syndrome was discovered in the 1930s (Penrose, 1933), long before its chromosomal basis was known. Indeed, the incidence of vitually all trisomy conditions has since been found to be associated with maternal age and it has been argued that maternal age is possibly the most important etiological factor in any human genetic disease. Among women aged 20–25 years, approximately 2–3% of all clinically recognized pregnancies are trisomic, but for women in their forties, this value exceeds 30% (Hassold and Chiu, 1985). For trisomy 21, the age-related increase is exponential and this seems to be the case for most of the other trisomies. For trisomy 16, however, it seems that this increase is a linear one, thus suggesting that trisomy 16 may be entirely dependent on maternal age whereas in other trisomies additional factors may be involved (Hassold *et al.*, 1995). Graphs for the incidence of trisomies 16 and 21 in association with maternal age are given in Fig. 3. There is less certainty about the relative importance of

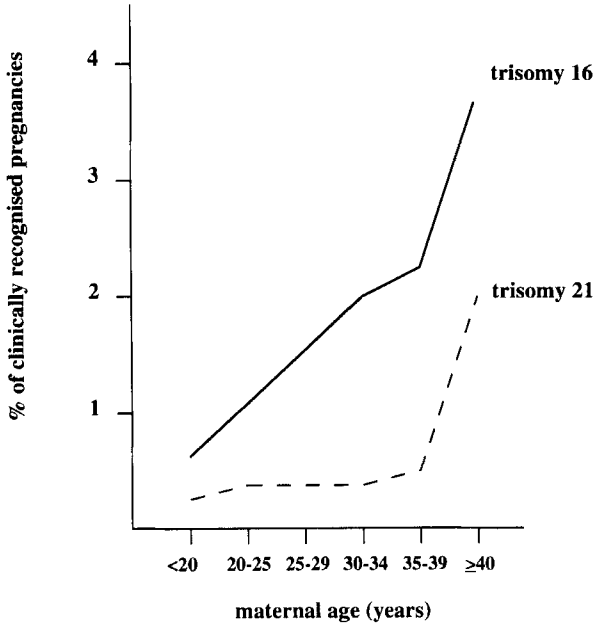

FIG. 3 Maternal age effect for trisomies 16 and 21.

the effects of maternal age on errors at meiosis I and II, respectively; that is, in maternally derived sex chromosome trisomy, the maternal age effect appears to be limited to the first meiotic divsion (MacDonald *et al.,* 1994). However, age effects are implicated in studies of trisomies 21 and 18 for both meiosis I and II (Fisher *et al.,* 1995; Sherman *et al.,* 1994). It may be the case therefore that there are chromosome-specific differences with respect to the effect of maternal age. There have been a number of models to explain the effect of maternal age and these are discussed in the following paragraphs.

1. Production-Line Hypothesis

This was proposed by Henderson and Edwards in 1968 and to date has been the most popular hypothesis to explain the maternal age effect. It is based on observations of declining frequencies of chiasmata in older mouse oocytes and suggests that subsequent increased univalent formation in older individuals leads to increased nondisjunction and thus explains age-dependent trisomy. It is known, however, that chiasma formation occurs prenatally in the female and thus the authors were forced to postulate the existence of a gradient in the fetal ovary such that the oocytes entering meiosis first are ovulated first several years later. Those entering meiosis last (those reportedly with fewer chiasmata) are ovulated last and it is those that are more susceptible to nondisjunction. Subsequent similar studies have confirmed these observations (Polani and Jagiello, 1976; Sugarawa and Mikamo, 1983; Luthardt, 1977) and there is some evidence to suggest that those oocytes entering meiosis first are those that are ovulated first (Polani and Crolla, 1991). However, despite several attempts, other studies have not been able to demonstrate a relationship between chiasma frequency, univalent formation, and aneuploidy (Speed and Chandley, 1983).

2. Limited Pool Hypothesis

This model suggests that since older women have a decreasing number of antral stage follices per cycle (Peters and McNatty, 1980), they ovulate oocytes that are "overripe" and thus more prone to nondisjunction (Warburton, 1989). Therefore, the suggestion is that it is the depletion of oocytes, not the chronological age of the mother, that accounts for the maternal age effect. Indirect evidence for this hypothesis is provided by the fact that unilaterally ovarectomized mice and Turner syndrome mosaics show increased levels of aneuploidy (Brook *et al.,* 1984; King *et al.,* 1978).

3. Local Factors Hypotheses

There are several models that propose that hormonal changes in the local ovarian environment as the woman ages accounts for an increase in nondisjunction rates. Crowley *et al.,* (1979) suggest that, with age, meiosis slows down in response to hormonal changes, thereby impairing bivalent separation. Sugawara and Mikamo (1983) suggest that there is an age-related decrease in spindle-forming ability while Eichenlaub-Ritter *et al.,* (1988) suggest an age-related increase in meiotic cell cycle length. Recently, Van Blerkom (1995) provided direct evidence of a relationship between low intracellular pH, spindle-forming ability, and aneuploidy in aging oocytes, thus providing direct evidence for a local factors model. This is consistent with a hypothesis put forward by Gaulden (1992), who proposed that the maternal age effect resulted from compromised microcirculation around growing follicles, thus subjecting the oocytes to hypoxic (low oxygen, high carbon dioxide and therefore lower pH) conditions.

4. Relaxed Selection Hypothesis

In 1982, Ayme and Lipman-Hand conceived a model that had little to do with oocytes. They claimed that the maternal age effect arises as the aging uterus becomes increasingly unlikely to abort trisomic conceptions. Recently however, this model has been put to rest because (1) paternally derived trisomies and those of mitotic origin do not show a maternal age effect, and (2) studies of oocytes and preimplantation embryos have recently demonstrated an increase in aneuploidy rate associated with maternal age. Thus it is almost certain that the relaxed selection hypothesis is incorrect.

5. Hawley's First Hypothesis

Recently, Hawley *et al.,* (1994) produced two models to directly explain the age-related mechanism of nondisjunction. They are based on studies of recombination and trisomy in humans (reviewed here) and comparisons with nondisjunction in *Drosophila.* They are unique in that they are the first to produce mechanistically distinct models for different chromosomes. The first model is based on the work of Sherman *et al.* (1994) on chromosome 21. Hawley and colleagues point out similarities between maternal meiosis I nondisjunction of human chromosome 21 and the nod^{DTW} mutation in Drosophila. The nod^{DTW} mutation impairs segregation of both chiasmate and achiasmate bivalents in that it is defective in maintaining the ability of chromosomes to sustain contact with themselves and with the meiotic spindle. Therefore Hawley and colleagues hypothesize that, as females age, their capacity to form a spindle diminishes (perhaps via an

analog of the NOD kinesin protein). Therefore achiasmate bivalents or those with distal chiasmata are more susceptible to nondisjunction in the presence of a suboptimally functioning spindle whereas bivalents with two crossovers or those with proximal chiasmata are more likely to undergo normal segregation.

6. Hawley's Second Hypothesis

In the same paper, Hawley *et al.* (1994) produced a very different model to explain the age-related increase of X chromosome nondisjunction. This is based on the work on meiosis I nondisjunction of the human X chromosome (MacDonald *et al.*, 1994) and its association with recombination. In this case similarities are drawn with spontaneous nondisjunction of the X chromosome in *Drosophila* females (Merriam and Frost, 1964). In both humans and flies when normal bivalents are compared with nondisjoining ones, (1) there is an increase in achiasmate and bichiasmate bivalents but a decrease in monochiasmate ones, and (2) there is a suprising increase in exchanges in pericentromeric regions. This led the authors to conclude that some of the meiotic exchanges seen in fly X chromosomes (and hence, by implication, in humans) are not the products of chiasmata but are the result of transposon-induced breaks. The rationale for proposing this is because (1) by definition these exchanges do not ensure segregation as do chiasmata, (2) they do not appear to be sensitive to the normal regional controls at the exchange level, (3) they do not display interference as do chiasmata, and (4) they are associated with the appearance of X-linked mutations (as in the case with some other transposons). The proposed model therefore is as follows: Some classes of transposons move at a high frequency during meiotic prophase. The excision and integration events generate a high frequency of chromosome breaks, and some of these are repaired as exchange events that are not mature chiasmata. Thus some meiotic exchange events are chiasmata and some are not; since a normal function of chiasmata is to ensure segregation, those that are not serve to interlock the chromosomes and *impair* segregation. It is further proposed that these transposon-mediated events increase in likelihood and frequency with the length of meiotic prophase. Since human eggs are arrested in meiotic prophase from the time they enter meiosis prenatally until ovulation, those ovulated later have been in prophase longer and are thus more likely to undergo nondisjunction.

C. Paternal Age Effect

Given that there is undoubtedly a maternal age effect on trisomy, the obvious question arises of whether there is also an effect of increasing

paternal age. Both epidemiological and molecular approaches have been used to address this question; however, neither has satisfactorily resolved it.

1. Epidemiological Studies

Before the chromosomal basis for Down syndrome was known, initial studies (Penrose, 1993) suggested that there was no paternal age effect for this condition, and this view has been supported by most subsequent epidemiological studies of trisomy 21 (Hook and Cross, 1982; Cross and Hook, 1987; Hook, 1987b; Erickson, 1978). Other groups, however (Sandler, 1981; Stene *et al.*, 1977; J. Stene and Stene, 1977, 1978, 1981), have consistently reported the existence of a parernal age effect, and this difference of opinion has led to a considerable amount of controversy in the literature (E. Stene and Stene, ab, 1989; Hook, 1987a, Carothers, 1988; Hook *et al.*, 1990). With regard to sex chromosome trisomies, similar epidemiological studies have provided little evidence for a paternal age effect on the incidence of these conditions. Indeed, a small inverse parental age effect has been reported for "XYY" trisomy (Carothers *et al.*, 1978). Therefore, generally speaking, epidemiological studies have provided little support for a paternal age-related increase in the rate of trisomy. Clearly, however, since only a small number of trisomies arise via nondisjunction in the sperm, subtle age effects might go undetected unless only those cases that are paternally derived are separated out and considered.

2. Molecular Studies

As mentioned, the subjective approaches of looking at chromosome heteromorphisms to determine the parental origin of trisomy were superseded by the use of DNA polymorphisms (Warren *et al.*, 1987; Sherman *et al.*, 1991) and thus it became possible to ask directly whether paternal age is elevated in trisomies of paternal origin. Several groups have reported on this question, with conflicting results. For example, Petersen *et al.* (1993) recently analyzed the parental ages of paternally derived cases of trisomy 21 and noted a small increased in paternal age in cases of meiosis I origin. Similarly, Schintzel and colleagues (Lorda-Sanchez *et al.*, 1992) reported a significant increase in paternal age in paternally derived XXY trisomies and, in other studies, they observed increases in paternal age in paternal uniparental disomy 15 (Robinson *et al.*, 1993b) and in paternal trisomy 18 (Ya-gang *et al.*, 1993). In contrast, MacDonald *et al.* (1944) observed no effect of increasing paternal age in their analyses of paternally derived 47, XXYs and 47, XXXs, nor did Zaragoza *et al.* (1994) in a small series of paternally derived acrocentric trisomies. Molecular studies have the inher-

ent limitation that the effort required to ascertain even a few paternal trisomies is considerable—fewer than 150 paternally derived trisomies have thus far been identified. Again, therefore, subtle effects of age might go undetected since these individuals were collected from different populations with different mean paternal ages.

3. Sperm Studies

Clearly, an alternative approach to address this question would be to ask whether rates of nondisjuction are higher in the sperm of older men than in their younger counterparts. As mentioned earlier, the classical approach to obtain cytogenetic information from sperm was to used the "humster" assay, but because few aneuploid metaphases can be recovered, the statistical power of this approach is limited (Martin and Rademaker, 1987). It has been superseded by the use of the FISH-sperm assay (Williams *et al.*, 1993; Bischoff *et al.*, 1994; Griffin *et al.*, 1995). Preliminary studies in this laboratory involving 400,000 sperm obtained from 24 men aged 18–60 years have revealed that there is a significant effect of age on the incidence of disomy for the sex chromosomes but not for chromosome 18 (Griffin *et al.*, 1995) (Fig. 4). Rough extrapolations from our data suggest that men

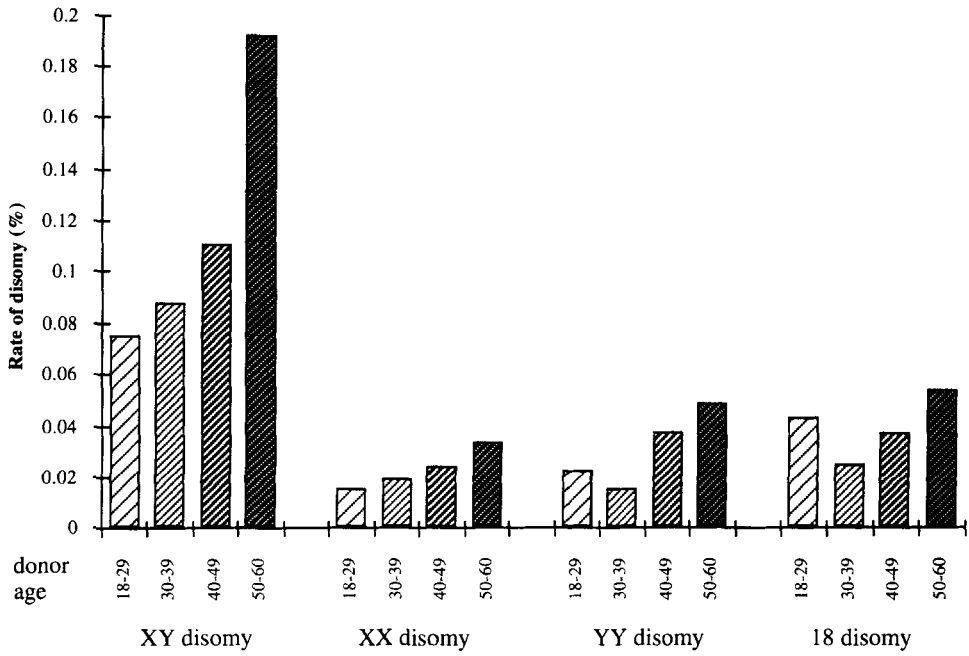

FIG. 4 Age-related disomy levels in human sperm.

in their 50s may be twice as likely to have sex chromosome trisomic offspring than men in their twenties. We found no such effect, however, for disomy 18 sperm and hence suggest that trisomy 18 is unlikely to be subject to a paternal age effect, particularly since molecular studies reveal that all paternal trisomy 18 conceptuses thus far discovered are consistent with a postzygotic mitotic nondisjunction error (Fisher *et al.,* 1995).

D. Aberrant Genetic Recombination

In yeast and female *Drosophila* there is a well-established relationship between errors in meiotic recombination and nondisjunction. Indeed, mutants that have reduced recombination invariably have increased frequencies of nondisjunction and chiasmata (at least proximal chiasmata) are usually thought to ensure proper segregation (Hawley and Theurkauf, 1993; Rockmill and Roeder, 1994). The availability of DNA polymorphisms has made it possible to study the relationship between recombination and trisomy since both the chromosomes that have recombined in the parental gamete are present in the trisomic offspring. Accordingly, it may be that homologous chromosomes that undergo meiosis I nondisjunction do not possess chiasmata. This certainly was the implication in the initial studies—namely those by Hassold *et al.,* (1991), who showed that in male X-Y nondisjunction the sex chromosome bivalent failed to recombine in the pseudoautosomal region in 33 out of 39 cases of paternally derived XXY trisomies; and those of Warren *et al.* (1987), who showed a general reduction in recombination in trisomy 21 individuals. As is often the case, however, things were not quite that simple; more detailed subsequent studies showed that most nondisjunction arose from chiasmate bivalents. Nevertheless, in each of the chromosomes studied. chromosomes 21, 16, and X have shown reduced and aberrant levels of recombination associated with nondisjunction in trisomic individuals.

Sherman *et al.* (1994) in a study of trisomy 21 liveborns demonstrated that there is an overall reduction in recombination in nondisjoined bivalents; however, there is a highly significant increase in recombination in the terminal portion of the chromosome (Fig. 5a). This was the inspiration for Hawley's first hypothesis, which suggested that achiasmate bivalents and those with distal chiasmata were more likely to undergo nondisjunction (particularly in older women) than bichiasmate bivalents or those with proximal chiasmata. In similar experiments on the X chromosome, MacDonald *et al.* (1994) examined 172 maternally derived XXY and XXX trisomic individuals. Again, they reported a general reduction in recombination; however, there was a significant increase around the centromere. This finding is surprising since recombination is though to be absent at the

centromere and it prompted Hawley and colleagues to propose their second hypothesis, which suggested that these recombination events were not the products of mature chiamata. Recently Hassold *et al.,* (1995) have performed similar experiments on trisomy 16 spontaneous abortions. In this case again there was an overall reduction in recombination in nondisjoined bivalents, but this time it could be almost entirely accounted for by a massive reduction around the pericentromeric region (Fig. 5b). It would be interesting to see how Hawley and colleagues interpret this. Levels of recombination are best expressed as map lengths in centimorgans and Figs. 5 a and b show the differences between map lengths in normal chromosomes and those that undergo a nondisjunction event for trisomies 16 (Fig. 5a and 21 (Fig. 5b).

E. Environmental Factors

A multitude of environmental factors have been specifically implicated in the genesis of aneuploidy. These include irradiation, oral contraceptives, spermicides, fertility drugs, smoking, and alcohol abuse. Despite more than a decade of study, however, none have been unequivocally linked with the incidence of trisomic offspring. This is largely due to the limitations in the design of epidemiological studies and the inability to characterize the nondisjunctional process in any with any statistical reliability. Recently, however, Wyrobek and colleagues have begun using the FISH-sperm assay to investigate the levels of disomy in men exposed to various mutagens. This method has the advantage of being able to detect a large number of meiotic products and hence nondisjunctional events. In preliminary studies, they have reported increases in the rates of nondisjunction associated with men who have recently undergone chemotherapy and those who are heavy smokers (Wyrobek *et al.,* 1995).

F. Genetic Factors

In addition to the extrinsic factors thought to be associated with the genesis of aneuploidy, certain intrinsic factors have also been implicated. These include rare α-1-antitrypsin haplotypes, consanguinous matings, thyroid antibodies, and chromosome polymorphisms. As with environmental factors, however, none of these has been conclusively linked to trisomy for similar reasons. The FISH-sperm assay, in addition to asking questions about chromosome-specific nondisjunction, paternal age effect, and effect of environmental agents, is also a useful tool for investigating the effect of genetic factors on nondisjunction. The ability to examine several thousand

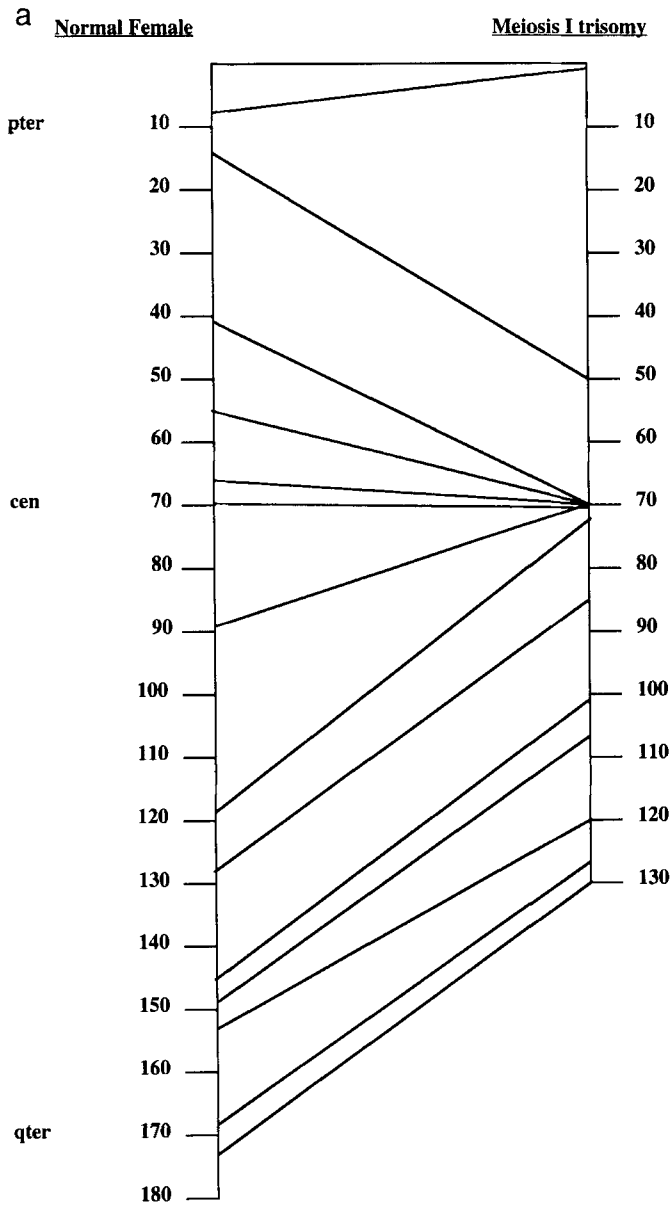

FIG. 5 Normal and trisomic (maternal MI) map compared. (a) Chromosome 16. (b) chromosome 21.

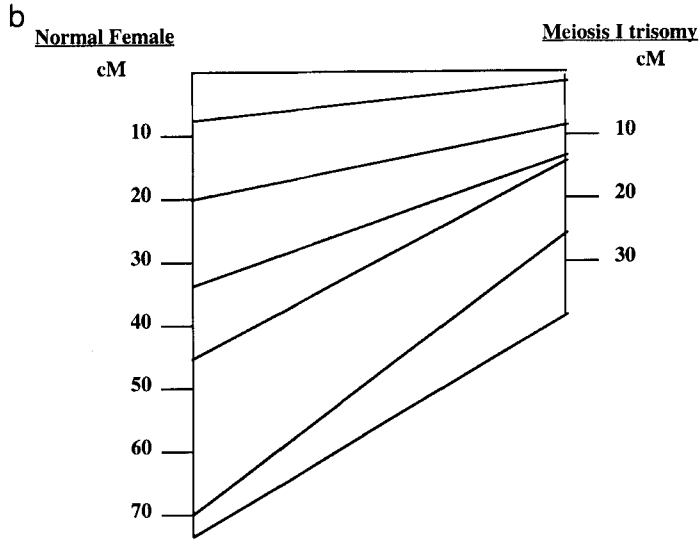

FIG. 5 (*continued*)

products of meiosis from any one individual gives this approach considerable statistical power over other approaches.

1. Association with Centromere Size

One of the major goals in our laboratory is to use the FISH-sperm assay to investigate the effect of variation in chromosomal structures on the rate of nondisjunction of certain chromosomes. In a preliminary study, we investigated the relationship between the size of the α-satellite array and the rate of nondisjunction of the Y chromosome (Abruzzo *et al.*, 1996). Since the centromere is an obvious determinant of chromosome segregation and since the α satellite has been shown to be a functional part of the centromere, we hypothesized that smaller centromeres may be less efficient at segregating a chromosome. We chose the Y because (1) it has the smallest α-satellite array, (2) arrays fall into two distinct size groups in different men, (3) these two size groups are also thought to represent sequence-specific haplotypes,and (4) the FISH assay will distinguish meiosis I from meiosis II errors involving this chromosome. In the event, we found no such effect in a population of 14 males; however, when we eliminated males over 40 years from our calculations (since our paternal age studies showed that they had significantly elevated rates of disomy), we observed a small increase in nondisjunction for males with larger centromeres. Clearly, this

is the opposite of our hypothesis and suggests that there is perhaps some effect of centromere haplotype rather than size per se on the rate of nondisjunction. Using similar approaches, we intend to investigate chromosomal variation and its effects on the rate of nondisjunction and thereby search for genetic components associated with this phenomenon.

2. Subfertile Males

FISH-sperm assay studies on males with the subfertility syndrome oligoasthenoteratozoospermia have revealed that these individuals are specifically prone to nondisjunction. That is, the rates of disomy in all these individuals are some tenfold higher than in normal individuals (e.g., Pang *et al.,* 1995). Clearly, study of these men and the mechanism by which nondisjunction occurs more frequently in them may provide valuable insight in studies of aneuploidy.

G. Sex Ratios in Trisomy

The ratio for the proportion of males to females at birth is 1.06 : 1; however, trisomic conditions show marked deviations from it. That is, it has been suggested that trisomy 21 probands are more likely to be male (median sex ratio 1.2 : 1.; Huether, 1990) whereas trisomy 18 liveborns are more likely to be female (Baty *et al.,* 1994). One developmental timepoint at which this could be determined is at the gametic stage. Indeed, Petersen *et al.* (1993) suggested that the excess of males seen in Down syndrome was likely to be due to a meiotic event in the testis since there is a large excess of males (3.5 : 1) in trisomy 21 cases of paternal origin. In order to test this hypothesis directly, we examined a population of disomy 21 sperm and asked how many were X-bearing and how many Y-bearing. We found that there was a significant excess of Y-bearing sperm (69 Y-bearing, 44-bearing) thus providing direct evidence for the existence of such a meiotic event. In similar experiments on disomy 18 sperm, we found no sex ratio difference with 55 Y-bearing and 53 X-bearing. Hence the large excess of females seen in Edwards syndrome cases is likely to be explained by differential selection against male conceptuses, particularly since all paternally derived trisomy 18 cases described to date are consistent with a postmeiotic error.

V. The Role of Chromosomal Mosaicism in Humans

Thus far, we have dealt with aneuploid conceptuses that have a uniform aberrant karyotype in all fetal cell lineages. A proportion, however, are

"mosaics," i.e., they have a mixed population of both normal and abnormal cells. Individuals with mosaicism typically display milder phenotypes than their counterparts with full-blown trisomy and often a have longer life expectancy. However, initial assumptions that any mosaicism in fetal tissues would usually be reflected in the extraembryonic cell lineages such as the cytotrophoblast and extraembryonic mesoderm (EEM) of the placenta, surprisingly, proved to be inaccurate. That is, it was found that abnormal cells are more likely to be *unevenly* distributed among the various embryonic and extraembryonic cell lineages (see Wolstenholme, 1995).

A. Incidence and Origin

1. Liveborns and Ongoing Pregnancies

In addition to being used for prenatal diagnosis, chorionic villus sampling (CVS) provides a means of assessing mosaicism by determining the karyotypic status of cytotrophoblast, extraembryonic mesoderm (EEM) and fetal cell lineages. A dichotomy in the chromosomal findings between one or more of these cell layers occurs in about about 2% of all clinically recognized conceptions. In the great majority of continuing pregnancies, the abnormality is confined to the placenta—so-called confined placental mosaicism or "CPM." Early experience suggested that most CPM pregnancies proceed uneventfully to term; however, a small subset of cases was found to be associated with a greatly enhanced risk of fetal loss, intrauterine death (IUD), intrauterine growth retardatiion (IUGR), early delivery, and/or possibly excessively high birthweights. These are usually characterized by very high levels of abnormal cells, often in both the cytotrophoblast and EEM.

Additionally, certain trisomies show chromosome-specific differences in terms of compartmentalization and likely origin. For instance, individual cases of trisomy 7 and 9 CPM can be equally restricted to either the cytotrophoblast or EEM, whereas trisomy 3 cells are almost always restricted to the cytotrophoblast, and trisomy 2 or 8 cells are found predominantly in the EEM. Futhermore, CPM trisomies involving chromosomes 16 and 22 are primarily meiotic in origin whereas CPM for trisomies 2, 3, 7, 8, and 9 largely arises as postzygotic mitotic errors.

2. Studies of Spontaneous Abortions

In comparison with studies of viable pregnancies, the examination of noncontinuing pregnancies (which constitute the majority of abnormal conceptions) is, suprisingly, a lightly researched area. Furthermore, analyses

have usually been limited to cells in either the EEM (Hassold *et al.,* 1980) or the cytotrophoblast (e.g., Eiben *et al.,* 1990). As mentioned earlier, aneuploid conceptuses account for 35% of fetal losses and about 5% of these have been reported as mosaic, but since both lineages were not assessed, this may not reveal the full extent of mosaicism. For instance, the incidence of nonmosaic trisomies 3 and 22 is significantly greater in the cytotrophoblast series than in the EEM series; conversely, trisomy 2 is more prevalent in EEM-derived material. This would indicate a considerable degree of confined mosaicism, which is not detectable by analysis of a single cell lineage. Few studies have provided details of the karyotypes of both cytotrophoblast and EEM cells from the same sample and they are too small for drawing any major conclusions. Nevertheless, examples of confined mosaicism, including trisomy 3 in cytotrophoblast cells and trisomy 2 in the EEM, are apparent. These studies report the incidence of confined mosaicism in all spontaneous abortion material as 4.6, 10, and 13% (Griffin *et al.,* 1996b; Kalousek *et al.,* 1992; Lombardi and Dev, 1994, respectively) and suggest that, unlike viable pregnancies, the incidence of normal cytotrophoblast and abnormal EEM is much more likely in this material.

3. Likely Incidence of Mosaicism

It seems clear therefore that traditional views on the incidence of trisomy may warrant some revision. The combined levels of confined mosaicism in continuing pregnancies and in spontaneous abortion material suggest that as much as 20–30% of the total load of trisomy relates, not to uniform trisomy, but to various combinations of aneuploid and euploid cells, i.e., mosaicism. In addition, for trisomies 2, 3, 8, and possibly 22, there is strong evidence for a consistent nonrandom distribution of aneuploid cells.

B. Mechanism of Formation

In order to affect significant numbers of cells in one or more cell lineage, confined mosaicism must have been in place from a very early stage postconception, i.e., at the blastocyst stage or earlier. Patterns of compartmentalization presumably arise during the early delineation of trophoblast and inner cell mass (ICM) lineages or as a result of separation of ICM-derived cells into the remaining extraembryonic cell lineages (including the EEM) and the fetus proper. There are two possible mechanisms by which confined mosaicism could apper in the blastocyst: (1) The abnormal cell line could be formed by a mitotic nondisjunction event in an initially normal conception. (2) The conception could be trisomic as a result of a parental meiotic

error and subsequently produce a normal cell line by a correcting event—so-called trisomy rescue.

Where studied, the majority of IUD and severe IUGR pregnancy outcomes have been associated with the second of these mechanisms where the euploid fetus possesses both copies of one chromosome pair derived from one parent—so-called uniparental disomy or "UPD." Two clinically distinct syndromes have been associated with UPD of chromosome 15 namely, Prader-Willi syndrome (where there are two copies of the maternal chromosome) and the less frequent Angelman syndrome (two copies of the paternal chromosome). Indeed, Prader-Willi and Angelman syndromes can often be characterized by trisomy 15 cells in the placenta (Cassidy *et al.,* 1992). In addition, UPD for other chromosomes such as 7, 11, and 16 has been associated with clinical syndromes (Kaluosek *et al.,* 1992). Less dramatic adverse outcomes probably relate to placental insufficiency due to the huge load of abnormal cells, and are likely to be a mixture of cases of corrected meiotic errors without UPD and those of mitotic origin. Conversely, it has been hypothesized that normal cells in the placenta may facilitate survival of an aneuploid fetus; for instance, many trisomy 13 and 18 term deliveries have been found to contain high proportions of karyotypically normal cells in their placentae.

VI. Concluding Remarks

Nondisjunction manifested as aneuploidy and uniparental disomy is the leading cause of mental retardation and pregnancy loss in humans. It is associated with several clinical phenotypes and has been implicated in intrauterine growth retardation, intrauterine death, stillbirth, early delivery, and high birth weight. It has frequently been described as the most problematic genetic hazard facing mankind and it is essential to fully understand the mechanistic basis of this phenomenon. The incidence of aneuploidy reflects the balance between the rate which the chromosome nondisjoins and the rate at which the abnormality is selected out during fetal development. Studies of conceptuses with confined mosaicism will elucidate which trisomies are compatible with survival in which fetal cell lineages. The first and most important etiological factor is maternal age and this was appreciated for Down syndrome in 1933. Identification of the second factor, namely, aberrant genetic recombination, took another 60 years. It is to be hoped that it will not take another 60 years before others are identified!

Interestingly, two of the newer hypotheses, namely, the Angell theory of precocious separation and Hawley's first hypothesis for the maternal age effect, seem to be compatible with one another. As Fig. 6 shows,

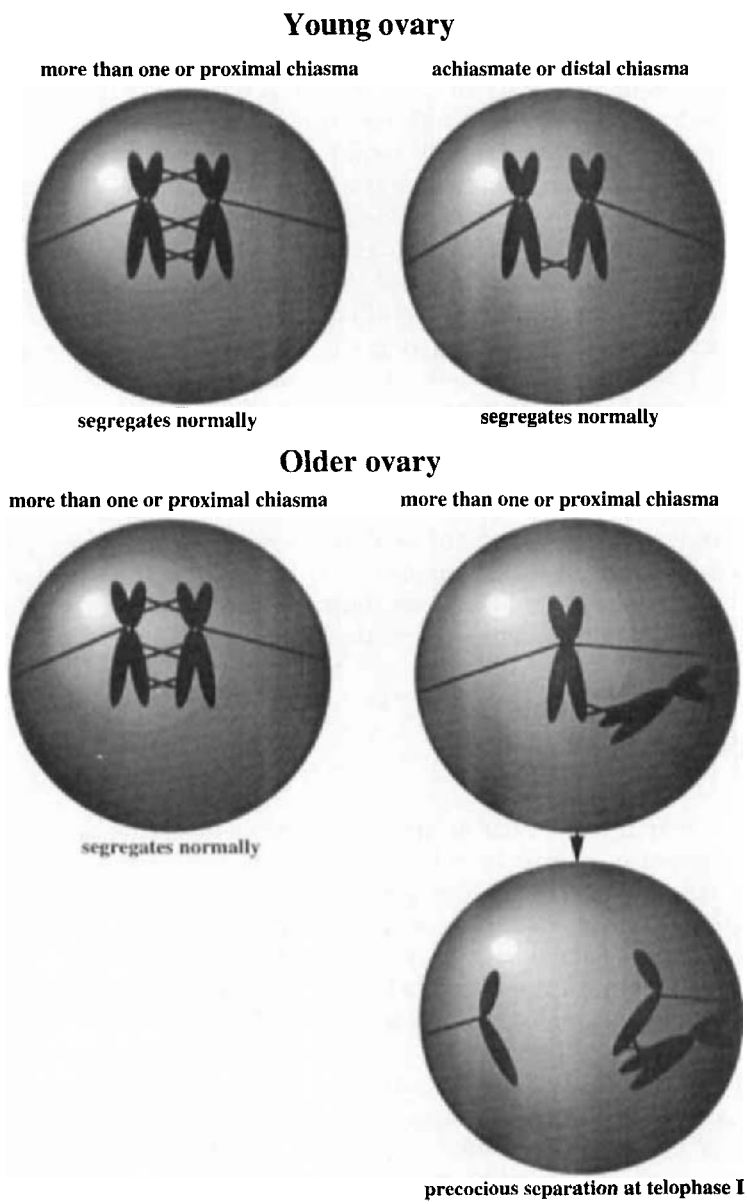

FIG. 6 Proposed model linking Angell model and Hawley's first hypothesis.

bivalents with distal chiasmata might be more prone to precocious separation in older ovaries which, as postulated by Hawley and colleagues, might have compromised spindle assemblies; bichiasmate bivalents or those with a proximal chiasma, on the other hand, might not. This also seems to agree with the recent work by Van Blerkom (1995), who correlated maternal age, spindle formation, aneuploidy, and intracellular pH. This will certainly not be the last word on the maternal age effect but it does provide a reasonable alternative to biologically implausible explanations such as the production line hypothesis.

Another interesting finding from recent work is that chromosomes seem to behave very differently from one another. They clearly have different rates of nondisjunction; some are more prone to errors at MI, others at MII; some may be more likely to nondisjoin by the classical mechanism, others by Angell's model. Indeed, Hawley and colleagues suggested that the age-related mechanisms of nondisjuntion may vary widely between chromosomes 21 and X. Furthermore, it is apparent that there are distinct compartmentalization patterns of certain trisomies in certain fetal lineages. The extent to which this reflect patterns laid down in the early embryo or the survival rates of trisomies in certain lineages warrants much investigation.

A combination of standard cytogenetic, molecular, and cytological techniques will continue to elucidate the secrets of meiosis and nondisjunction. For instance, newly discovered proteins associated with cell division, such as the centromere-binding proteins, promise to be invaluable. Model systems such as mouse, *Drosophila,* and yeast might provide insights when human material cannot be used for ethical reasons. We have suggested that a study of male meiosis in humans, i.e., sperm, might provide a model system for asking certain questions of female meiosis when sufficient meiotic products cannot be obtained from ovaries. Clearly, there is much work to be done.

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