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ADVANCES IN
Immunology

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The Clonotype Repertoire of B Cell Subpopulations

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I. Introduction

Hallmarks of the immune system include (1) the extraordinary diversity of clonally distributed recognition molecules, (2) the ability to discriminately recognize self versus nonself, and (3) cognate interactions that rely on subtle differences in the affinity of receptor–ligand interactions to delineate triggering versus nontriggering events and to provide powerful selective forces for the somatic evolution of responses. The keys to unraveling this intricate biosystem have been the elucidation of the clonal selection hypothesis (Burnet, 1959) and the consequent analysis of the immune system at the clonal and molecular levels. As of a decade ago, when the subject of the murine B cell clonotype repertoire was last reviewed in this series (Sigal and Klinman, 1978), the fundamental concept of antibody specificity arising from the interplay of highly selective stimulatory processes operating on an extremely diverse repertoire of clonally distributed specificities was already evident. However, at that time both clonal and molecular approaches were only beginning to impact on our understanding.

In the intervening years, remaining doubts concerning the monopotentality and integrity of clonal responses have dissipated. Most importantly, the analysis of monoclonal antibody-producing cell populations, particularly hybridomas, through the use of gene cloning technology has provided substantial gains in our understanding of (1) the molecular basis of the extraordinary diversity of the B cell repertoire, (2) the patterned developmental acquisition of the variable region repertoire, (3) the mechanism of isotype switching, (4) the molecular basis for polymorphic disparities in repertoire expression, and (5) the role of somatic mutation as the basis for the generation and selection of novel specificities subsequent to antigenic stimulation. At the same time, the availability of monoclonal antibodies against cell

surface markers, in conjunction with sophisticated cell separation technology, has provided a new awareness of the distribution of various phenotypic and functional subpopulations of B cells.

In spite of the remarkable gains in our understanding of B cell repertoire expression, many fundamental issues remain unresolved including the relative impact of evolutionarily predetermined versus random somatic events on primary B cell repertoire expression. Furthermore, it is not clear whether B cells develop as clones of identical sister cells or at what developmental stages clonal commitment is established. Additionally, although environmental determination of repertoire via such mechanisms as tolerance induction and antiidiotypic immunoregulation has been identified, the impact of these mechanisms in shaping the B cell repertoire has yet to be evaluated. Finally, and most important with respect to the topic of this review, we are only at the seminal stages of understanding the interrelationships of the various processes responsible for generating and shaping the expressed B cell repertoire and the differential utilization of these processes for repertoire establishment among various definable B cell subpopulations. Indeed, the major emphasis of this review will be an attempt to collate the information that is currently available and that will ultimately be required to provide an understanding of the evolutionary and functional significance of a B cell repertoire expressed disparately in functionally different B cell subpopulations. In order to permit a comprehensive analysis on the status of this issue, this review will be selective rather than detailed in its description of the molecular aspects of repertoire expression [which have been reviewed extensively elsewhere (Honjo, 1983; Perry, 1984; Max, 1984; Gearhart, 1982; Alt *et al.*, 1986; Storb *et al.*, 1986; Coleclough, 1983; Leder *et al.*, 1980; Tonegawa, 1981)] and will largely rely on the previous review of Sigal and Klinman (1978) for both historical perspective and the status of the methods used for approaching repertoire expression prior to the advent of hybridoma and gene cloning technology.

II. The Mechanics of Variable Region Gene Expression

Delineating the molecular mechanisms that are responsible for antibody diversity has been among the major accomplishments of molecular biology during the past decade. The intensive efforts of numerous laboratories have led to a comprehensive understanding of the genetic elements that contribute to antibody variable region expression, the molecular mechanisms responsible for the combinato-

rial association of these elements, and the processes by which these mechanisms contribute to diversification.

Immunoglobulin variable regions are generated as the combinatorial product of polypeptide chains encoded by five separate gene elements. The variable region of the heavy chain (V_H) is the product of three gene elements (V_h , D , and J_h) (Davis *et al.*, 1980; Maki *et al.*, 1980; Kataoka *et al.*, 1980; Schilling *et al.*, 1980; Early *et al.*, 1980; Bernard and Gough, 1980; Gough and Bernard, 1981; Sakano *et al.*, 1980, 1981; Kurosawa *et al.*, 1981; Kurosawa and Tonegawa, 1982; Kemp *et al.*, 1979) while immunoglobulin light chains represent the combinatorial product of V_L and J_L (Leder *et al.*, 1974; Seidman *et al.*, 1978a,b, 1979; Lenhard-Schuller *et al.*, 1978; Hamlyn *et al.*, 1978; Brack and Tonegawa, 1977; Hozumi *et al.*, 1981; Tonegawa *et al.*, 1978; Blomberg *et al.*, 1981; Miller *et al.*, 1981). In the mouse, all V_H regions are encoded by the same heavy chain variable region locus; however, two unlinked loci [κ (κ) and λ (λ)] contribute light chain variable regions. Repertoire diversity, per se, emanates from numerous levels within the process of V region gene generation. First, there is considerable diversity within certain gene elements themselves. In mice there are approximately 10^2 – 10^3 functional V_h gene segments (see below), any one of which can apparently combine with one gene segment of the various D and J_h gene elements to generate a functional V region gene (Davis *et al.*, 1980; Maki *et al.*, 1980; Kataoka *et al.*, 1980; Schilling *et al.*, 1980; Early *et al.*, 1980; Bernard and Gough, 1980; Gough and Bernard, 1981; Sakano *et al.*, 1980, 1981; Kurosawa *et al.*, 1981; Kurosawa and Tonegawa, 1982; Kemp *et al.*, 1979; Gearhart *et al.*, 1981). In general it appears that relatively homologous V_h gene segments (families) are clustered with respect to one another (Brodeur and Riblet, 1984; Brodeur *et al.*, 1984; Dildrop, 1984). The relative position of these gene families within the V region gene locus will be discussed below.

Numerous diversity (D) segments are also available for inclusion in the V region gene and, in addition, any given V region gene includes one of four available J_h segments (Davis *et al.*, 1980; Early *et al.*, 1980; Sakano *et al.*, 1981; Kurosawa *et al.*, 1981; Kurosawa and Tonegawa, 1982; Gearhart *et al.*, 1981). Thus, if all V_h , D , J_h gene combinations are possible, then solely by the combinatorial association among the numerous members of these gene elements, 10^4 – 10^5 V_H region genes could be generated.

Superimposed on this level of diversity is an additional level created by variation in the joining of the V_h – D gene segments and D – J_h gene segments. This junctional diversity emanates from recom-

ination occurring at varying nucleotide positions at the respective 5' and 3' termini, as well as nucleotide additions occurring between the combining gene segments (Alt and Baltimore, 1982; Tonegawa, 1983). Since the mechanisms responsible for this imprecision at the joining regions are still unknown, the exact contribution of junctional diversity to V_H diversity cannot yet be evaluated; however, junctional diversity could add several orders of magnitude to the already considerable degree of heavy chain variability created by the combinatorial association of the various V gene segments.

Kappa light chain variability also arises from the combinatorial association of four J region gene segments with an estimated 90–320 V region gene segments (Zeelon *et al.*, 1981; Cory *et al.*, 1981). Lambda light chain variability is more confined in that each of the two V region gene segments are associated with one or more J region segments (Tonegawa *et al.*, 1978; Blomberg, *et al.*, 1981; Miller *et al.*, 1981). For light chains, like heavy chains, variability is increased by junctional diversity (Tonegawa, 1983; Sakano *et al.*, 1979; Weigert *et al.*, 1980) and possibly nucleotide additions as well (Heller *et al.*, 1987).

Given all of these sources of diversity for V_H and V_L and the diversity generated by the combinatorial association of V_H with V_L at the polypeptide chain level, the potential for variable region diversity is considerably greater than the estimated 10^7 – 10^8 specificities that might comprise the primary B cell repertoire of any given murine strain (Sigal and Klinman, 1978; Kreth and Williamson, 1973; Klinman, 1972; Klinman and Press, 1975a; Klinman *et al.*, 1976a, 1977; Owen *et al.*, 1982; Cancro *et al.*, 1978). Regardless of whether junctional diversity is considered a somatic or a germline source of diversification (discussed in detail below), it is clear that the inherited potential for primary B cell repertoire expression is enormous even in the absence of additional diversity generated by somatic mutation, a mechanism which apparently diversifies the repertoire to an even greater extent subsequent to antigenic stimulation (see below).

In addition to the extensive molecular analyses that have established the multiplicity of gene elements that contribute to V regions and the rearrangements of these gene elements, a series of studies has been carried out using mainly transformed neonatal and adult bone marrow B cell progenitors to establish the sequence of events that leads to V region establishment (Alt *et al.*, 1981, 1984; Reth and Alt, 1984; Reth *et al.*, 1985). The earliest detectable event in pre-B cells appears to be a D – J_h joining. D – J_h joinings are followed by V_h – D joining and, if successful, this is followed by heavy chain expression. Preliminarily, it appears that once heavy chain synthesis is accom-

plished, further heavy chain rearrangements are prohibited, which may account, at least in part, for allelic exclusion (Alt *et al.*, 1986). Once the heavy chain is expressed, light chain rearrangement ensues which is rapidly followed by light chain synthesis and intact surface immunoglobulin molecule expression.

III. Unresolved Issues Concerning V Region Expression

Considering how little was known regarding the molecular basis of B cell repertoire expression a decade ago, the extensiveness of our current understanding of the molecular basis for variable region expression stands as a testament to the power of molecular biological approaches to fundamental biological issues and to the diligence of the researchers who applied these techniques to the understanding of antibody diversity. However, in spite of the impressive progress in this area during the past decade, substantive questions remain and the impact of these unresolved issues on our understanding of the mechanisms of B cell repertoire expression as it applies to various B cell subpopulations will be summarized in this section.

Although a considerable amount is now known concerning the structure and arrangement of V region gene segments within the genome, a complete understanding of repertoire expression will ultimately require a more comprehensive analysis of the total number of functional genes within each locus and the precise arrangement of these genes within the various loci. Although there is a fair amount of agreement that the number of V_{κ} gene segments is between 90 and 320 in the mouse (Zeelon *et al.*, 1981; Cory *et al.*, 1981), relatively little information is available as to the interrelationships between V_{κ} gene segments, the arrangement of these segments within the V_{κ} locus (Heinrich *et al.*, 1984), and whether they distribute, like V_{H} , in families of relatively homologous sequences.

At the current time, there is less agreement on the total number of functional V_{H} gene segments. Assessing the total number of V_{H} gene segments has been facilitated by the fact that these gene segments seem to be compartmentalized into families of closely related sequences (Brodeur and Riblet, 1984; Brodeur *et al.*, 1984; Dildrop, 1984). By using probes for each of these families at various stringency levels, it has been possible to estimate the number of V_{H} gene segments in each of the 10 families. In general, these estimates have indicated that there are approximately 100 V_{H} genes. This number has been corroborated by analyses of the proportionate representation of messenger RNA in the spleen of normal mice and in populations of

polyclonally stimulated cells (Dildrop *et al.*, 1985; Schulze and Kelsoe, 1987). Although there appears to be reasonable agreement on the number of V_h gene segments in most of the identified families, there is now considerable disagreement concerning the total number of V gene segments in the $V_h J558$ family. By banding patterns and proportionate representation, this family has been estimated to include between 60 and 100 gene segments (Brodeur and Riblet, 1980; Brodeur *et al.*, 1984; Dildrop, 1984). However, intensive investigation of one of the identified bands demonstrated that there may be multiple different V gene segments within given bands (Boersch-Supan *et al.*, 1985). A similar conclusion has been obtained using $C_{\delta}t$ analysis where the total number of V_h gene segments in the $V_h J558$ family was estimated to be at least 10^3 (Livant *et al.*, 1986). It remains uncertain how many of these genes may be functional. Nonetheless, these studies put considerable doubt on the approximation of 10^2 as the total number of V_h gene segments in the murine V_h gene locus. A difference of an order of magnitude in the number of functional V_h gene segments could greatly affect considerations of the generation of repertoire diversity.

A second aspect of V_h gene organization requiring resolution is the precise nature of the arrangement of V_h genes within families and the position of the V_h gene families with respect to one another. The grouping of V gene segments into families was accomplished by stringency analysis for DNA hybridization, and more precisely by sequence analysis and grouping of genes of similar sequence within the same family (Brodeur and Riblet, 1980; Brodeur *et al.*, 1984; Dildrop, 1984). The positioning of V_h gene segment families with respect to one another was initially accomplished by analyses of idiotype expression in inbred and recombinant mice and by the distribution within Igh recombinant strains of restriction enzyme fragment length polymorphisms (Brodeur and Riblet, 1980; Brodeur *et al.*, 1984). It should be noted that the formal positioning of V genes within families and between families will probably require precise positioning of V gene segments within genomic clones using phage vectors (Crews *et al.*, 1981; Bothwell *et al.*, 1981) and cosmid clones (Siu *et al.*, 1987; Cattaneo *et al.*, 1981) or by mapping using pulse field electrophoresis (Schwartz and Cantor, 1984), analyses which have not yet been accomplished. To date, little information is available utilizing DNA pieces that are large enough to include at least two V_h gene segments. In general this information corroborates the conclusion that similar V genes are proximal within V gene locus; however, some information indicates that this might not always be the case (Siu *et al.*, 1987).

Recently a second method of positioning V_h gene segment families has been utilized wherein cell lines expressing a single rearranged V_h gene are assessed for the presence or absence of putative upstream (present) or downstream (absent) V_h gene segment families (Rathbun *et al.*, 1987). The results from this analysis are in poor agreement with the aforementioned analyses using V_h recombinant strains. However, the cell line analysis agrees quite well with the sequential expression of V_h gene segments in fetal and neonatal mice (see below). Taken together these three approaches are in agreement that the V_h 7183 and V_h Q52 gene families are among the families most proximal to D . The cell line analysis places the V_h X24 family in the same complex and places the V_h 36-60 family as the next upstream family (Rathbun *et al.*, 1987). This positioning of the V_h 36-60 family is also strongly favored by the high level of its utilization in early neonates (Riley *et al.*, 1986; Wu and Paige, 1986). The cell line analysis also groups the S107, VGAM3-8, and J606 V_h gene segment families as being D proximal to the very large V_h J588 family and V_h 3609 family which appears most distal to D in some strains. Thus, the resulting V_h gene order and the estimated number of V_h gene segments in each family would be as depicted in Fig. 1. It should be stressed that this V_h gene segment arrangement is tentative and that specific positions may differ from strain to strain (Rathbun *et al.*, 1987). In addition, it will be difficult to draw conclusions from the positioning of V_h gene segment families as long as the extent of intermixing of V_h genes from different families remains unknown. Nonetheless, the positioning of the V_h gene segment families as depicted in Fig. 1 is consistent with the theory that fetal and neonatal V_h gene expression is nonrandom and favors the D most proximal genes (discussed in detail below). In addition, the positioning of the V_h gene families as depicted in Fig. 1 would be consistent with some findings concerning V_h gene expression in adults that indicate a continued propensity (though less dramatic than in neonates) for high expression of D proximal V_h genes and low expression of V_h genes distal to D (Rathbun *et al.*, 1987).

In addition to uncertainties concerning both the total number of V genes for V_k and V_h and the positioning of these genes within the chromosome, a considerable amount of uncertainty remains concern-

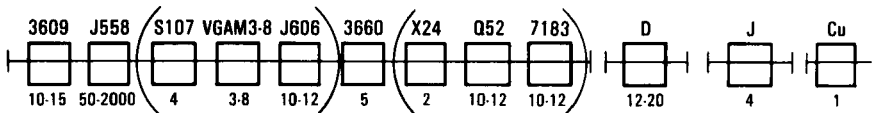


FIG. 1. Presumptive alignment and composition of mouse V_h gene segment families as per Rathbun *et al.* (1987).

ing the *D* region elements of the heavy chain locus. Current estimates indicate that there are between 12 to 20 *D* gene segments (Sakano *et al.*, 1981; Kurosawa *et al.*, 1981; Kurosawa and Tonegawa, 1982), however, the entire locus has not yet been examined. Furthermore, a considerable amount of sequence information is available from rearranged *V* genes that does easily fit with the known *D* region sequences. This indicates that either more *D* region sequences may be available than currently proposed, or that *D* region segments can be incorporated in tandem with unusual recombinational sites. Since the *D* region contributes substantially to the third hypervariable region of heavy chains and, thus, to antibody combining sites it is critical to our understanding of repertoire expression to completely understand the *D* region locus.

Although there are a substantial number of preliminary findings and some highly provocative theories already available, the mechanism by which *V* regions are selected for rearrangement remains an important uncertainty. Although it now appears the the utilization of *V* regions may correlate with the accessibility of given *V* regions to enzymes (Alt *et al.*, 1986; Blackwell and Alt, 1984; Blackwell *et al.*, 1984; Yancopoulos *et al.*, 1986), the mechanisms responsible for determining which *V* region segments should be available is not yet known. In addition, although a considerable amount of information is available on the rearrangement mechanisms per se, little is known concerning the operation of mechanisms responsible for junctional diversity (Desiderio *et al.*, 1984). Although some data indicate that once rearranged chains express their protein products further rearrangement and expression may be inhibited (Alt *et al.*, 1986; Yancopoulos and Alt, 1985), it remains uncertain whether this is the mechanism responsible for allelic exclusion (Coleclough *et al.*, 1981; Bernard *et al.*, 1981). To date, experiments using transgenic mice that carry rearranged V_H or V_L genes remain ambiguous on this issue (Grosschedl *et al.*, 1984; Ritchie *et al.*, 1984; Rusconi and Kohler, 1985). It is certain, however, that a comprehensive understanding of the mechanics of repertoire expression will ultimately require a thorough understanding of the aforementioned issues and it is to be anticipated that a considerable amount of information in this area will be forthcoming in the next few years.

IV. The Relative Role of Random Somatic Events versus Evolutionary Selection in *V* Region Expression

Since the acceptance of the proposition that the antibody specificity of a given B cell is determined solely by the genome of that B cell, the

relative contribution to repertoire expression of evolutionarily selective forces as opposed to random somatic events has remained unresolved. Given the well-known differences in mouse B cell repertoire expression associated with polymorphic differences in V_H (Brodeur and Riblet, 1984; Brodeur *et al.*, 1984; Lieberman *et al.*, 1974; Riblet *et al.*, 1975a; Eichmann and Berek, 1973; Brient and Nisonoff, 1970; Makela and Karjalainen, 1977; Riblet *et al.*, 1975b; Lieberman *et al.*, 1981; Trepicchio and Barret, 1985), V_L (Laskin *et al.*, 1977), and now D (Riley *et al.*, 1988) gene segments, there is, unquestionably, an evolutionarily determined influence on repertoire expression. However, since primary repertoire expression also appears to involve combinatorial events among multiple V region gene segments, multiple possibilities at the joining regions including nucleotide additions, and somatic mutations which accumulate in rearranged genes, there is obviously a contribution of somatic events within developing and stimulated B cells and at least some of these somatic events are likely to be random. Given the probability that both types of influences affect repertoire expression, it is critical to have an accurate evaluation of which aspects of repertoire expression are mainly contributed by one versus the other influence.

A. THE CONTRIBUTION OF SOMATIC MUTATIONS TO PRIMARY AND SECONDARY B CELL REPERTOIRE EXPRESSION

1. *The Contribution of Somatic Mutation to Secondary B Cell Repertoire Expression*

One aspect of repertoire expression that has a high probability of reflecting the consequence of the stochastic accumulation of somatic events is the role played by somatic mutation. Although a likely contribution of somatic mutations to repertoire expression was recognized over a decade ago, particularly in studies of variations in the sequence of the λ chain of antidextran antibodies (Weigert *et al.*, 1970; Tonegawa *et al.*, 1978), the enormous impact of the contribution of somatic mutations and the rapidity with which they accumulate in secondary B cells has been fully realized only in the past few years. The accumulation of somatic mutations in immunoglobulin variable regions during the course of an immune response has now been documented in a variety of antigen systems. The first evidence for this occurrence, in addition to the aforementioned studies with the λ chains of antidextran antibodies, involved analysis of hybridoma and myeloma antibodies of BALB/c mice specific for phosphorylcholine (PC) (Gearhart, 1982; Gearhart *et al.*, 1981; Malipiero *et al.*, 1987; Perlmutter *et al.*, 1984; Crews *et al.*, 1981). These findings docu-

mented a substantial number of somatic mutations in the rearranged heavy chain genes of these anti-PC antibodies and, at that time, a correlation was drawn between the accumulation of somatic mutations and the expression of isotypes other than IgM. Similar findings were obtained from an analysis of the light chain of anti-PC antibodies (Selsing and Storb, 1981). In both instances, the nucleic acid sequence of the putative ancestral germline gene had been established so that differences in nucleic acid sequence in the expressed immunoglobulins were clearly due to somatically accumulated mutational events.

The association of the rapid accumulation of somatic mutations during the course of the generation of secondary responses was first established by a series of elegant studies on the response of BALB/c mice to the hemagglutinin (HA) of the influenza virus PR8 (Clarke *et al.*, 1985; McKean *et al.*, 1984). Because of the availability of an extensive panel of viruses expressing variants of the HA molecule, it was possible to affix the specific recognition of given determinants and, thus, to analyze the antibodies of multiple hybridomas derived from the B cells of several individual mice specific for these determinants. These investigations used as the basis for clonal relationships not only the sequences of the rearranged V region gene but also the rearrangement patterns of nonexpressed V region genes. Some of the relevant conclusions from these studies can be summarized as follows: First, depending upon how the calculation is done, somatic mutations may be occurring as rapidly as 1×10^{-3} per base pair per generation. Second, somatic mutations can occur throughout the variable region and in both coding and noncoding regions and in any of the three nucleotides of any codon. However, mutations which cause amino acid replacements, particularly within hypervariable regions, appear favored, presumably by antigen selection. Third, the pattern of specific mutations within the antibodies of a single mouse at a given time suggests familial relationships among the antibodies generated, in that the relationships of antibodies to one another can be reasonably attributed within lineal trees. This suggests sequential and continued selection by antigens of preferred mutant sequences. Finally, the studies suggested that the particular germline used as the progenitor for the accumulated somatic mutants generally differs from mouse to mouse within a strain and individual mice might choose more than one progenitor to select and somatically mutate.

In general, studies analyzing the immune response of A/J mice to the arsonate (ARS) haptenic determinant (Manser *et al.*, 1984, 1985, 1987; Juszczak *et al.*, 1984; Manser and Geffer, 1986; Near *et al.*, 1985;

Wysocki *et al.*, 1987; Slaughter *et al.*, 1984; Capra *et al.*, 1982), the response to (3-hydroxy-4-nitrophenyl)acetyl (NP) (Bothwell *et al.*, 1982; Cumano and Rajewsky, 1985; Krawinkel *et al.*, 1983; Savkutzky *et al.*, 1985; Siekevitz *et al.*, 1987; Allen *et al.*, 1987; Berek and Milstein, 1987), and the response to 2-phenyl-5-oxazolone (phOX) (Berek *et al.*, 1985; Evan *et al.*, 1985; Griffiths *et al.*, 1984; Milstein *et al.*, 1986) are in agreement with most of the above findings. Each of these studies, however, has added information which has been important to our understanding of the process of somatic mutation, particularly as it pertains to the generation of secondary immune responses. As opposed to the aforementioned studies on PR8-HA, each of these latter studies was carried out in a system wherein a predominant cross-reactive idiotype (CRI) had been well established within the given response. Thus, the studies of the generation of somatic mutation were focused on the variable region gene composite responsible for the predominant set of specificities. In the ARS system, it was observed that the CRI was only one of many potentially ARS-responsive variable region gene sets in the primary response. Nevertheless, it was the idiotype which appeared most likely to accumulate somatic mutants which could increase affinity, thus favoring its preferential selection. In the response to NP of C57BL/6 mice and phOX in BALB/c mice, this did not appear to be the case in that the early predominant clonotype was not preferentially selected. In each of these cases the predominant clonotype represented only one specificity among many that could be somatically mutated and, therefore, was not generally responsible for the majority of the memory response. In all three of these systems, as well as the response to PC, it was noted that successful somatic mutants were generally associated with increased affinity and, thus, favorable antigen selection appeared to correlate with increased affinity of antigen binding.

Taken together, these and other studies concerning the accumulation of somatic mutations associated with immunization and the generation of secondary B cell responses have revealed the somatic mutation process as one which can provide an ongoing immune response with an almost limitless potential to generate new selectable specificities. Given the differences among the well-studied systems it appears that the immune system is relatively opportunistic in its selection of the mode by which its antibody products will be selected. Thus, in some instances no particular germline seems favored for this process and the selected product or products of individual mice vary enormously between individuals within a strain. In other, rarer

instances, the germline genes capable of giving rise to somatic mutations with increasing affinity for antigen appear to be relatively limited so that a predominant clonotype arises which bears close relationship from individual to individual. Alternatively, some clonotypes which predominate early in a response appear disfavored by the somatic mutation process and, thus, responses diverge. In all instances, however, some common threads exist. Somatic mutations appear to occur at a very rapid rate, particularly during the course of the generation of secondary B cells (Kim *et al.*, 1981; Wabl *et al.*, 1985). Somatic mutations are limited to rearranged variable region genes and to the flanking regions of these genes (Gearhart and Bogenhagen, 1983). Finally, somatic mutations appear to be possible in almost any nucleotide position; however, antigen selection appears to favor amino acid replacement mutations rather than silent mutations, particularly in hypervariable regions or antigen contact regions (Amit *et al.*, 1985).

2. *The Contribution of Somatic Mutation to Primary B Cell Repertoire Expression*

The somatic mutation process described above is an extraordinary biological mechanism, which may be relatively unique to the generation of memory B cells as, for example, it apparently does not apply to T cell receptors (Kronenberg *et al.*, 1986; Kabat *et al.*, 1987). This mechanism is largely responsible for enabling the rapid diversification of the repertoire of clonotypes specific for a given antigen within an individual mouse and among mice of a given murine strain. Although it is clear that this mechanism plays a highly important role in the generation and character of secondary responses, does it also obtain for the generation of the primary B cell repertoire? Because of the apparent stochastic nature of mutational events, the resolution of this question has fundamental implications to the understanding of the relative contribution of evolutionary versus somatic selective forces in the generation of the primary B cell repertoire. In addition, the resolution of this question has serious implications for an understanding of the total number of specificities that may be present in the primary B cell repertoire of individual mice and of a strain, and the potential role of tolerance induction or idiotypic recognition in shaping the primary B cell repertoire.

By and large, the information currently available on this issue would indicate that somatic mutation, as opposed to those changes in amino acid sequence that accompany variable region gene segment rearrangement, does not participate to any major extent in the genera-

tion of the primary B cell repertoire. Because this is a fundamental issue, we will review in some detail the evidence on this point as it now exists.

The first, and perhaps most important piece of evidence, comes from the analysis of nonrearranged variable region gene elements. Although a substantial number of germline variable region gene elements, both V_H and V_L , have been analyzed, no verified instance of a somatic mutation other than those attributable to potential strain variation has been identified (Kabat *et al.*, 1987; Bruggemann *et al.*, 1986; Sablitzky *et al.*, 1985; Rudkoff *et al.*, 1984). Thus, it would appear that of all the differentiative and divisional events leading to the generation of somatic cells, be they liver or lymphoid, none appears to be accompanied by an unusually high rate of the somatic mutation in V region gene elements.

Do somatic mutations occur after V gene rearrangement during the generation of the primary B cell repertoire? The clustering of somatic mutations within hypervariable regions clearly evidences the role of antigen selection in the generation of the secondary B cell repertoire (see above). Since antigen selection apparently plays little or no role in the generation of the primary B cell repertoire (Sigal and Klinman, 1978, see below), the relative absence of mutations in nonhypervariable regions of both primary and secondary antibodies would imply that somatic mutations contribute little to primary B cell diversification. More directly, numerous studies have attempted to assess the frequency of somatic mutations in antibodies generated as a consequence of either polyclonal stimulation of primary B cells or antibody generated during the early course of a primary response. In toto, these studies have revealed very few sequence changes potentially attributable to somatic mutations (Siekevitz *et al.*, 1987; Berek and Milstein, 1987; Manser *et al.*, 1987; Gearhart, 1987; Pech *et al.*, 1981; Bernard *et al.*, 1978).

Among the evidence that indicates somatic mutations contribute very little to the primary B cell repertoire, or even primary antibodies, is the finding that somatic mutations are only very rarely found in IgM antibodies (Gearhart *et al.*, 1981; Manser *et al.*, 1987; Manser, 1987). However, there have been exceptions (Riley *et al.*, 1986; Rudkoff *et al.*, 1984; Manser *et al.*, 1987). In instances where mutations in IgM have been found, they could in fact be attributed to any of several processes. First, there is a low rate of somatic mutation in genes in general and it is unlikely that rearranged immunoglobulin genes would be an exception to this. Second, in many instances the IgM antibodies in question were obtained after immunization or stimula-

tion and, thus, the somatic mutations could be the result of the standard process of somatic mutation occurring after immunization as opposed to preexisting in the primary B cell repertoire pool. Third, some of these mutations may be attributable to sequencing errors or to strain variations not previously identified. Finally, we have suggested (see Section VI) that primary B cell repertoire generation in neonates may be quite different from that which occurs in adults (Zharhary *et al.*, 1984). Thus, any finding of somatic mutations in the IgM antibodies of neonates may reflect some accumulation of somatic mutations that occurs in the generation of primary neonatal B cells. This potential exception is of great interest, but would not reflect the accumulation of somatic mutations in B cells as they generate in the bone marrow of adult mice and that are responsible for the primary B cell repertoire of adult mice.

In addition to the experimental findings listed above which strongly imply that somatic mutation in either unrearranged or rearranged variable region genes plays little if any role in primary B cell repertoire diversity, several biological aspects of the development of B cells in the adult bone marrow would lead to the same conclusion. As discussed above, somatic mutations are not generally associated with unrearranged V region gene elements. Studies from several laboratories have now indicated that cells of the B cell lineage developing in the adult murine bone marrow cease division at about the time of light chain rearrangement and, in fact, have little division potential subsequent to V_h rearrangement (Osmond, 1986; Osmond and Nossal, 1974; Landreth *et al.*, 1981). This being the case, there would be little opportunity for the accumulation of somatic mutations during the development of adult murine B cells. Less direct, but also consistent with the conclusion that somatic mutation plays little role in diversification of the primary B cell repertoire, are findings concerning tolerance susceptibility of newly developing B cells in the bone marrow of adult mice. These studies will be discussed in detail in Section VI, however, the conclusions of these studies are that the tolerance susceptible phase of developing bone marrow B cell precursor cells appears to coincide with the first expression of surface immunoglobulin (sIg), just after light chain rearrangement, and thus would coincide with the approximate time at which divisional expansion of B cell clones is ceasing. If B cells were to continue to divide subsequent to this developmental stage and if somatic mutations were to occur in conjunction with this division, new potentially antiself specificities would likely arise in cells destined to become primary B cells which would no longer be tolerance susceptible. Thus, the

correlation of the absence of somatic mutation in primary B cells, the fact that newly developing B cells do not divide after V_L rearrangement, and the timing of tolerance susceptibility are entirely consistent with the notion that tolerance functions to purge self-reactive specificities from the primary B cell repertoire.

B. JUNCTIONAL DIVERSITY AS A SOURCE FOR RANDOM SOMATIC DIVERSIFICATION

Shortly after the identification of the process of V gene rearrangement for the assembly of H and L chain variable regions, it became apparent that a considerable amount of diversity was generated at the joining regions between V_L and J_L , as well as V_h-D and $D-J_h$ (see Section II). Although a considerable number of available sequences of myeloma and hybridoma proteins represent the apparent combinatorial association with intact V gene segments in frame, a large percentage of sequences appears to include at least some element of junctional diversity. Given the variety of possible junctional variations, it would appear that combinatorial association of intact V gene segments in frame is far more likely to occur than any single variant junction. Nonetheless, diversity generated by these junctional processes is significant and, since it falls in the third hypervariable region of both the H and L chain, contributes considerably to the diversity of recognition phenotypes. At the present time, the exact enzymatic mechanisms responsible for V gene segment joining, and in particular N-nucleotide addition, are not known (Blackwell and Alt, 1984; Blackwell *et al.*, 1984; Yancopoulos *et al.*, 1986). However, since the junctional diversification process is one which occurs somatically, to the extent that this process may be random, it would add a random element to the total diversity of the primary B cell repertoire.

Several recent findings indicate that junctional diversification may be less random than previously thought. As discussed in Section II, given estimates for the numbers of V_h , D , J_h , V_k , and J_k gene segments, combinatorial association alone could account for a repertoire of 10^7-10^8 specificities and junctional diversity could vastly increase that estimate. However, since individual mice have approximately 2×10^8 B cells, and the best current estimates of repertoire size for any individual mouse are approximately 10^7 specificities (Sigal and Klinman, 1978; Klinman and Press, 1975a; Owen *et al.*, 1982; Cancro *et al.*, 1978), it is remarkable that even clonotypes represented by a low frequency of B cells in any individual mouse are often reproducibly expressed in all individuals of a murine strain

(Sigal and Klinman, 1978; Sigal, 1982; Sigal *et al.*, 1977; Accolla *et al.*, 1977; Gerhart *et al.*, 1977; Riley and Klinman, 1985; Stashenko and Klinman, 1980; Riley and Klinman, 1986; Nishikawa *et al.*, 1983). This is particularly surprising since certain specificities for which this is true include not only imprecise matching of junctional sequences but also N-nucleotide additions (Landolfi *et al.*, 1986; Slaughter *et al.*, 1984; Sigal, 1982; Riley and Klinman, 1986; Nishikawa *et al.*, 1983). That is, the antibodies obtained from different individual mice appear to have an identical V_H sequence and that sequence does not match the known germline sequences for V_h , D , or J_h . The best studied of these systems is the response of A strain mice to ARS. Expression of the CRI which predominates after immunization of mice of this strain is dependent on the expression of a single V_h , D , and J_h combination, as well as a germline V_κ - J_κ combination which has no junctional diversity (Wysocki *et al.*, 1987). The V_H region, however, has junctional diversity at both the V - D and D - J junctions and this diversity apparently requires a nucleotide addition (Manser *et al.*, 1985; Slaughter *et al.*, 1984; Landreth *et al.*, 1981). It has been shown that essentially every immunized A strain mouse expresses the CRI (Pawlak and Nisonoff, 1973; Tung and Nisonoff, 1975) and frequency analysis of clonal precursor cells for this idiotype indicates an average of approximately 1 in 10^7 B cells expressing this idiotype in mice of this strain (Sigal *et al.*, 1977). Similar findings exist for the response to NP in Igh^b mice. In this case, the response bears a λ light chain and the frequency of B cells that express cross-reactive idiotypic determinants as well as the private Ac146 idiotope is approximately 1 in 20,000-30,000 B cells (Riley and Klinman, 1986; Nishikawa *et al.*, 1983). Expression of the private idiotope requires not only the $\lambda 1$ light chain but also V_h 186.2, the Igh^b equivalent of the Igh^a DFL16.1 D region with a N-nucleotide addition at their junction (Cumano and Rajewsky, 1985; Boersch-Supan *et al.*, 1985). The reproducible occurrence of these, as well as other "major clonotypes" in all mice of a given strain, might imply that the process of junctional diversity and N-nucleotide addition is not a random one but one which may frequently accompany the combination of a given V_h and D , or D and J_h regions.

Further evidence that junctional diversity is not a totally random process comes from indications that it might be greatly influenced by a particular D region haplotype. This is implicit in the findings of Landolfi *et al.* (1986), wherein mice differing in their entire V_H haplotype cannot create the same junctional diversity as A strain mice. The effects of polymorphism in D has been shown directly using the

response of Igh^b mice to NP (Riley *et al.*, 1988). In these studies, C-B/R3 mice were analyzed at the clonal level for their responsiveness to NP-hemocyanin (Hy). These mice represent a recombinant between C.B20 (Igh^b mice) and BALB/c (Igh^a mice) and express the Igh^b V_h region and the Igh^a $D-J_h$ region. Igh^b mice have a high frequency of clonal precursor cells that give rise to anti-NP antibodies that bear the $\lambda 1$ light chain and are positive for the cross-reactive idiotype NP^b (Riley and Klinman, 1986; Nishikawa *et al.*, 1983; Klinman and Press, 1975a; Karjalainen *et al.*, 1980). Many of these antibodies are also positive for the private idiotype Ac146 which appears to require not only the appropriate V_H gene segment, 186.2, but also the appropriate D region segment, the Igh^b equivalent of DFL16.2 (Cumano and Rajewsky, 1985; Boersch-Supan *et al.*, 1985). BALB/c mice are negative for both the NP^b and Ac146 markers. In these studies, it was found that C-B/R3 mice have a relatively low frequency of λ -bearing B cells that can respond to the NP determinant. Thus, by merely switching the Igh^b $D-J_h$ region for the Igh^a counterpart, the overall frequency of this response was reduced. Most importantly, consistent with the V_h^b origin of the variable region segments, the λ -bearing NP-specific monoclonal responses of C-B/R3 mice were NP^b positive. However, none was Ac146 positive. These findings also held true for responses to (4-hydroxy-3,5-dinitrophenyl)acetyl (NPN) for which all tested strains have a much higher frequency of responsive λ -bearing B cells (Riley and Klinman, 1986). Thus, it appears that both the overall frequency of λ -bearing NP-responsive B cells and B cells expressing an idiotypic determinant dependent on an N-nucleotide addition can be affected by the D region haplotype. Since the V_h , λ , and J_h do not differ between C.B20 and C-B/R3 mice, only differences in their D regions would be likely to be responsible for the repertoire differences observed. The BALB/c counterpart for DFL16.1 appears to be extremely similar to the Igh^b DFL16.1 (Cumano and Rajewsky, 1985), but differences in DFL16.1, or other differences in the D region, apparently preclude the appropriate N-nucleotide addition to give rise to a high frequency of Ac146-positive anti-NP antibodies.

Although the aforementioned findings represent only a small proportion of all sequences found to vary by junctional diversity and N-nucleotide addition, they are provocative with respect to the control mechanisms responsible for junctional diversity. Nonetheless, far more research is necessary to determine the extent to which that junctional diversity may be a random versus an evolutionarily selected process. In addition, the above findings introduce the issue of

the role of haplotype differences in V region gene elements. Are the aforementioned polymorphic differences in V_h , D , and V_κ evolutionarily selected and, if so, is the selective process antigen dependent or might such selection be part of the process of internal recognition such as might be needed to create a functional idiotypic network?

C. RANDOM VERSUS NONRANDOM V REGION SEGMENT SELECTION

Estimates of potential repertoire derived from the combinatorial association of the various gene segments that give rise to the intact variable region generally assume that all functional V gene segments are equally available and that all combinations of V gene segments are equally likely to occur. Any limitation in these processes would not only favor the expression of certain V gene segment combinations and disfavor others, but, if certain combinations are not possible, the actual size of the repertoire could be greatly curtailed. The current information concerning this issue derived from a considerable amount of accumulated sequence information clearly shows that a given V_h gene segment can be expressed in combination with any of several different D and J_h gene segments and in different junctional constructs (see Section II). It is equally clear that various V_H combinations can be expressed with any of several κ chains, and, in certain instances, λ chains. These findings give the impression that combinations of various V gene segments are not totally limited and imply that all combinations are potentially equally possible. However, a considerable amount of information from a variety of experimental approaches is leading more and more to the conclusion that V gene segment combinations and selection are not merely a random assortment of all functional V gene elements.

With respect to the utilization of particular V_h germline genes or gene families and V_κ germline genes, at the present time insufficient information is available to make a final judgment as to the extent of equality or inequality that exists for expression of various gene segments. Recent studies now indicate that there is unequal utilization of J_κ gene segments in that $J_{\kappa 1}$ and $J_{\kappa 2}$ appear to be used more frequently than $J_{\kappa 3}$ and $J_{\kappa 4}$ (Wood and Coleclough, 1984). In addition, studies now indicate that at least for λ light chains certain functional V genes are normally expressed very rarely (Sanchez and Cazenave, 1987; Dildrop *et al.*, 1987).

In a similar vein insufficient information is currently available to determine whether certain V region gene combinations are more frequently expressed than others. One study has been conducted wherein the frequency of total B cells expressing V_{H1} (V_hT15) in

association with light chains of various light chain subgroups has been assessed (Bruggemann *et al.*, 1986). The results of these studies indicated that there is a preferential association of V_H with certain light chain subgroups.

1. *Assessing Antigen Responsive Cell Populations*

Before discussing randomness versus nonrandomness in the expression of antigen-responsive cell populations, it is necessary to review the two major approaches used to assess antigen-specific B cell populations: antigenic stimulation versus polyclonal mitogenic stimulation of B cells in limiting dilution. In both techniques, an attempt is made to have sufficiently few cells in each culture so that zero or one cell of the desired specificity or clonotype would be present. Generally, analyses for antibody production are carried out at the micro level unless hybridomas are generated following one of the two aforementioned stimulatory protocols. Polyclonal stimulation has the advantage of encompassing a very broad spectrum of the B cell population; however, since a sizable percentage of B cells responds, all discrimination must take place at the level of the assay used for evaluating antibody reactivity. Antigenic stimulation, on the other hand, can be far more discriminatory in the selection of participatory B cells and thus assays for antibody reactivity are generally necessary only for the screening of positive responses rather than for discriminating among responses. However, since both procedures are applied to the same B cell populations, it should be anticipated that, if applied properly, both procedures should yield essentially the same answers concerning repertoire expression. Indeed, this has been the case in the one situation in which equally discriminatory identification procedures were used to assess the clonotype of responding cells (see below). Unfortunately, in general, when polyclonal stimulation has been used for repertoire analysis, responding cells have been judged positive for antigen specificity solely on the basis that their antibody products bind to a given antigen using screening assays that detect low-affinity and high-affinity interactions equally well (Andersson *et al.*, 1977; Moller, 1976; Eichmann *et al.*, 1977; Moller *et al.*, 1976; Levy, 1984; Kelsoe *et al.*, 1980). This has generally led to the conclusion that the actual frequency or "potential repertoire" for reaction against given antigenic determinants is manyfold higher than can be revealed by antigenic stimulation. The conclusion that this may represent a "potential" rather than a utilized repertoire results from the finding that when assayed carefully, much of the response to mitogenic stimulation is comprised of antibodies that are not found in

serum responses, memory cells, or hybridomas generated by virtue of antigenic stimulation.

These differences in the assessment of precursor frequencies have led to confusion on a variety of issues in addition to the aforementioned disparities in precursor cell frequency. For example, since a given antigen would be anticipated to tolerize only B cells that could respond to immunization with that antigen, tolerance induction, even if highly effective, would not be expected to eliminate all cells whose antibody products could bind that antigen particularly with low affinity. Thus, the consequences of tolerance induction are much more dramatic when B cell populations are assessed by antigenic stimulation (see below) as opposed to mitogenic stimulation (Moller *et al.*, 1976).

In the one instance wherein the frequency of cells of a specific well-defined clonotype was assessed by both procedures, the frequency of cells of that clonotype responding to mitogenic stimulation was essentially the same as the frequency of those cells responding to T cell-dependent antigenic stimulation in fragment cultures. Nishikawa *et al.* (1983), using limiting dilution of LPS stimulated spleen cells, assayed for the frequency of precursor cells whose antibody product could bind the haptenic determinant NP. The findings were striking in several regards. One out of four λ -bearing B cells produced antibodies that bound NP. In spite of this exceedingly high frequency of antigen-binding cells, when the antibodies were assessed for their expression of idiotypic determinants characteristic of Igh^b NP responsive antibodies, the frequency was substantially lower than would have been anticipated from antigen responses *in vivo* or *in vitro*. Most striking was the small percentage of antibodies that bore the private Ac146 idiotypic determinant which is characteristic of a sizable minority of λ -bearing NP^b antibodies. Overall, these investigators estimated that approximately 1 in 25,000–100,000 B cells actually expresses λ -bearing antibodies that bind the NP haptenic determinant and express the Ac146 idiotypic determinant.

These experiments provide a basis for comparison of the frequency of B cells in the spleen of Igh^b mice that can respond to antigenic stimulation with NP in fragment cultures. One B cell in approximately $1 - 2 \times 10^5$ produced λ -bearing antibodies expressing the Ac146 idio type in response to stimulation of fragment cultures with NP conjugated to hemocyanin (Hy) (Riley and Klinman, 1986). However, a higher frequency of cells producing antibody bearing these characteristics responded when stimulated with NNP-Hy. NP^b antibodies in general have a higher affinity (heteroclitic) for the NP analog NNP

(Imanishi-Kari and Makela, 1973; Karjalainen *et al.*, 1980) so that it is not surprising that a higher frequency of B cells responded to NNP-Hy. Indeed, the response to NNP appears totally inclusive of the λ antibody response to NP and includes a large number of λ -bearing B cells in addition to those which can respond to NP. The frequency of splenic B cells of Igh^b mice that could respond to NNP and produce λ -bearing antibodies that bound NP and expressed the Ac146 idiotypic determinant was one in 20,000–30,000. This frequency compares very favorably to that obtained by Nishikawa *et al.* (1983), using limiting dilution LPS-stimulated cultures. Since a high-frequency of λ -bearing responses bound NP, it is unlikely that the latter studies missed very much in the way of NP-responsive B cells. Thus, these findings serve as strong evidence that when assays of sufficient discriminatory capacity and sensitivity are used, the population of cells assessed by antigenic stimulation and LPS stimulation is indeed quite similar and that T cell-dependent antigenic stimulation is no less efficient in stimulating cells of a given clonotype than is LPS stimulation. These findings also demonstrate the difficulty in determining the true antigen specificity of polyclonally stimulated cells. Thus, most of the B cells that had been judged NP specific were more likely NNP specific albeit their antibody products bound NP.

Both of the aforementioned approaches to repertoire expression utilize a similar strategy in that the diversity of repertoire expression is calculated using the proportion of responsive cells that is specific for a given antigenic determinant or that bears a given idiotypic determinant, normalized to the total population of B cells. Thus, B cells of that clonotype are judged to represent 1 in 10^7 of all B cells if, on the average, 1 in 10^7 B cells reveals characteristics of that clonotype. If many clonotypes reveal the same frequency, it may be assumed that the repertoire consists of at least 10^7 specificities. This is a lower limit estimate, since, in the absence of sequence information, it is possible using most assays to determine that the products of two B cells are different but not that they are identical. Conclusions reached by such calculations could be greatly affected if (1) only a small percentage of B cells was actually functional, or (2) if the majority of the repertoire were occupied by a relatively few “predominant” clonotypes, thus leaving only a small space within the B cell population to be occupied by the lower frequency clonotypes upon which repertoire estimates are based. Although neither of these caveats has been formally ruled out, the weight of evidence presently available disfavors both. Although responses of many murine strains have been found to include “predominant” specificities, at the clonal precursor

frequency level, the most frequent clonotype yet observed (T15) represents 1 in 50,000 BALB/c splenic B cells (Gearhart *et al.*, 1975a) and most predominant clonotypes are present in a much lower frequency than this (Sigal and Klinman, 1978). In addition, even families of clonotypes which can dominate responses do not appear to be present in frequencies greater than 1 in 20,000 B cells (Sigal, 1982; Riley and Klinman, 1986; Nishikawa *et al.*, 1983; Froscher and Klinman, 1985; Stohrer and Kearney, 1984). Thus, predominant clonotypes appear to occupy a very small proportion of the overall repertoire. Indeed, responses to most antigens, even those with a very high frequency, demonstrate an exceedingly high degree of diversity (Sigal and Klinman, 1978; Kreth and Williamson, 1973; Cancro *et al.*, 1978).

The second issue, the percentage of total B cells that is functional is more difficult to assess. However, the majority of B cells can be shown to be responsive to a mixture of polyclonal activators, or stimulation with appropriately presented antiimmunoglobulin antibodies (Wetzel and Kettman, 1981; Andersson *et al.*, 1979; Parker *et al.*, 1979; Howard and Paul, 1983). In general, such stimulated B cells can be shown to proliferate and produce immunoglobulins and these immunoglobulins can be shown to bind one or several antigenic determinants. In addition, it appears that a very large proportion of B cells can bind, process, and present antigens for T cell activation (Kaikiuchi *et al.*, 1983; Lanzavecchia, 1985), thus indicating at least one type of functional relevance for most B cells.

2. *Predominant Clonotype Expression Is the Product of Nonrandom V Gene Segment Selection*

The first argument against a random or stochastic mechanism being responsible for repertoire expression is the existence of predominant clonotypes and predominant clonotype families. Whereas the vast majority of clonotypes are expressed at exceedingly low frequency within the repertoire (see above), some clonotypes are expressed at a high frequency and these disparities are reproducible from animal to animal within an inbred strain. It remains, however, a formal possibility that clonotypes which appear to be predominant are not expressed at a greater frequency than those which are rarer, but rather they could be either the product of developing B cell clones which are unusually large or the result of a postexpression environmental up-regulation. Several laboratories have now analyzed the frequency of precursors of "predominant specificities" in the newly developing bone marrow cell pool as compared to the splenic B cell pool (Riley and Klinman,

1986; Nishikawa *et al.*, 1983; Froscher and Klinman, 1985; Juy *et al.*, 1983; Klinman and Stone, 1983). It was reasoned that if predominant clonotype expression is the product of environmental up-regulation, then the frequency of representation of such clonotypes within the spleen should be significantly higher than their representation in the developing bone marrow pool where they exist prior to environmental influences. In all instances where this postulate has been tested, the frequency of B cells of predominant clonotypes was similar in the developing bone marrow cell pool as in the spleen. This finding is consistent whether assessed by polyclonal stimulation of limiting dilution B cell cultures (Nishikawa *et al.*, 1983; Juy *et al.*, 1983) or by antigenic stimulation in fragment culture (Riley and Klinman, 1986; Froscher and Klinman, 1985; Klinman and Stone, 1983). It should be noted that when mitogenic stimulation was used, bone marrow precursor cells were incubated *in vitro* for 5–7 days prior to their stimulation in order for them to become responsive to the stimulatory mitogen. In the fragment culture system, this does not seem to be necessary since B cell precursors isolated as sIg⁻ are transferred *in vivo* wherein they apparently express their sIg receptors (Neil and Klinman, 1987).

The possibility that predominant clonotypes might not be the product of a more frequent generation of a given specificity from within the repertoire, but rather could be the product of greater clonal division during B cell development, has been examined using the response to PC (Klinman and Stone, 1983) (see Table I). In these experiments, PC-responsive B cells of multiple individual leg bones of (CBA/N × BALB/c)_F₁ male mice were examined. The use of mice expressing the CBA/N immunologic defect made it possible to survey whole bone marrow populations, thus avoiding errors introduced by variability in the purification of sIg⁻ bone marrow cells. This is the case since one aspect of the CBA/N immunologic defect is the total absence of mature primary PC-responsive B cells in the bone marrow and spleen (Klinman and Stone, 1983; Quintans, 1977; Mond *et al.*, 1977). As will be discussed below, these mice have an intact population of PC-specific precursors in their sIg⁻ pool (Klinman and Stone, 1983), and approximately 40% of these PC-responsive sIg⁻ precursor cells bear the T15 idiotype. Extensive analyses of PC-specific antibodies that do not bear the T15 idiotype indicate that such antibodies represent a very heterogeneous (greater than 100) population of specificities (Owen *et al.*, 1982). By assessing the frequency and phenotype of PC-specific precursor cells in the bone marrow of individual leg bones (each of the two femurs and a combination of the

TABLE I
PC-SPECIFIC RESPONSES FROM INDIVIDUAL BONE MARROW ISOLATES OF (CBA/N × BALB/c)F₁ MALE MICE^a

Mouse number	Source ^b	Total responses	T15 ^{+c}	T15 ⁻	Mouse number	Source	Total responses	T15 ⁺	T15 ⁻
1	RF	0	— ^d	—	11	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	0	—	—		T	0	—	—
2	RF	0	—	—	12	RF	1	—	—
	LF	0	—	—		LF	3	—	—
	T	1	—	1		T	0	—	—
3	RF	0	—	—	13	RF	0	—	—
	LF	1	1	—		LF	0	—	—
	T	0	—	—		T	0	—	—
4	RF	2	2	—	14	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	3	—	3		T	0	—	—
5	RF	0	—	—	15	RF	0	—	—
	LF	2	—	1		LF	0	—	—
	T	0	—	—		T	0	—	—
6	RF	0	—	—	16	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	3	3	—		T	0	—	—

7	RF	0	—	—	17	RF	1	1	—
	LF	0	—	—		LF	0	—	—
	T	0	—	—		T	2	—	2
8	RF	0	—	—	18	RF	0	—	—
	LF	0	—	—		LF	0	—	—
	T	0	—	—		T	0	—	—
9	RF	2	—	2	19	RF	1	1	—
	LF	0	—	—		LF	0	—	—
	T	2	—	1		T	2	—	—
10	RF	0	—	—	20	RF	0	—	—
	LF	2	—	—		LF	0	—	—
	T	0	—	—		T	0	—	—

^a From Klinman and Stone (1983).

^b Twenty individual 2- to 3-month-old (CBA/N × BALB/c) F₁ male mice were sacrificed and cells were prepared individually from their femurs (F) and tibias (T). Cells from each femur (R, right; L, left) were injected into separate recipients and cells from both tibias were pooled and injected into a third recipient. Each of these three sources provided ~10⁷ cells.

^c Responses are reported as the total number of fragment cultures derived from each recipient spleen containing a clone producing >1 ng of anti-PC antibody per day over two collection periods.

^d In general, monoclonal antibodies that could not be designated as T15⁺ or T15⁻ were available in insufficient amounts for accurate quantitation of the T15 idiotype.

tibias) a distribution analysis of the frequency of occurrence of T15⁺ versus T15⁻ precursor cells was obtained. Although a minority of individual leg bones included any PC-specific precursor cells, a high proportion of those leg bones whose bone marrow populations yielded at least one response to PC in fact included more than one responsive cell. In addition, when the idiotype of monoclonal responses obtained from individual leg bones was analyzed, all responses of a given positive leg bone were either T15⁺ or, alternatively, T15⁻, i.e., mixed populations were not found in any individual leg bone. The jackpotting of specificities, either T15⁻ or T15⁺, in any given leg bone serves as evidence for clonal expansion during the B cell development (see below).

Another important finding was that the frequency of occurrence of leg bones with T15⁺ precursor cells versus leg bones with T15⁻ precursor cells was proportionate to the overall frequency of these specificities in the entire sIg⁻ population. In addition, the range of frequencies of precursor cells within any given T15⁺ or T15⁻ leg bone was approximately the same. Thus, it would appear that precursor cells giving rise to T15⁺ B cells (the predominant clonotype) do not expand to any greater extent than those giving rise to any of the multitude of T15⁻-responsive B cells. However, the frequency of occurrence of T15⁺ V gene segment rearrangements is substantially greater than the frequency of rearrangements of any single T15⁻ clonotype. If this finding holds true for other predominant clonotypes, then the only possible explanation for the unequal representation of clonotypes would be a disproportionate selection of variable region gene segments from within the V region pool which would be inconsistent with a random or stochastic process of V region selection.

3. The Acquisition of the Clonotype Repertoire during Fetal and Neonatal Development Is Nonrandom

The most clearly established situation in which the repertoire is expressed in a nonrandom fashion is repertoire expression during fetal and neonatal B cell development wherein repertoire expression is both highly restricted and reproducibly expressed among individuals of the same strain (Press and Klinman, 1974; Klinman and Press, 1975a; Sigal, 1977; Fernandez and Moller, 1978; Cancro *et al.*, 1979; Fung and Kohler, 1980; Cancro and Klinman, 1981; Denis and Klinman, 1983; Stohrer and Kearney, 1984; Teale and Kearney, 1986). The repertoire of fetal liver B cells and early neonatal splenic B cells has been analyzed with respect to responsiveness to several antigens both by fragment culture and hybridoma technology. These studies

have demonstrated that for certain antigens, such as DNP and TNP, the frequency of responsive B cells is similar to that of adult splenic B cells (Press and Klinman, 1974). However, the total number of responding clonotypes falls in the range of 3–10, whereas in adult mice several thousand clonotypes are available for responsiveness to these antigens (Klinman and Press, 1975b; Denis and Klinman, 1983). For some antigens, such as ARS, the frequency of neonatal responsive cells is somewhat higher than adults (Sigal, 1977), whereas for many antigens such as fluorescein (Press and Klinman, 1974), the frequency of responsive neonatal cells is much lower than would be anticipated from the frequency within the adult repertoire. Importantly, for some antigens responsiveness does not occur until relatively late in neonatal development (Sigal *et al.*, 1976; Stohrer and Kearney, 1984; Fung and Kohler, 1980; Fernandez and Moller, 1978). This latter finding is true for both responsiveness to PC and certain dextran-responsive cells, both of which are dominated in adult mice by recurrent predominant clonotypes (see Section II). Thus, in all of these systems, it appears that the neonatal clonotype repertoire is severely restricted, representing less than 1% of the total potential repertoire of adult individuals.

Importantly, when a population of neonates is examined, all neonates of the same strain and at the same age exhibit very similar repertoires. In an extensive series of studies in which the clonotype repertoire specific for the PR8–HA was analyzed in two murine strains (Cancro *et al.*, 1979; Cancro and Klinman, 1981; Thompson *et al.*, 1983) it was found that the neonatal repertoire was restricted both 1 and 2 weeks after birth, albeit the specificities present at 1 week of age were somewhat different than those present at 2 weeks. Additionally, it was found that F₁ heterozygous mice displayed a restricted repertoire that was not identical to either parent and included specificities not present in the repertoire of either parent (Cancro and Klinman, 1981). However, specificities of heterozygous mice that were typical of the reactivity pattern of one of the two parental strains were shown to bear the allotype of that parental strain, thus implying integrity of V_H gene repertoire control even in the heterozygous state.

Recent studies using hybridoma technology have confirmed the basic elements of the aforementioned findings. A series of hybridomas was constructed by the transfer fusion protocol using neonatal splenic B cells stimulated in irradiated carrier (Hy)-primed recipients with DNP–Hy (Denis and Klinman, 1983). Three of these hybridoma antibodies were studied extensively. Antiidiotypic antisera were raised against these antibodies in rabbits and, after appropriate

adsorption to render them highly specific, these sera were used to test a large number of monoclonal antibodies obtained from fragment cultures of neonatal spleen cells. A significant proportion of neonatal anti-DNP antibodies was found to be idiotypically indistinguishable from each of the three hybridoma antibodies. One of the antibodies, TF2-36 (μ, κ), was idiotypically indistinguishable from approximately 14% of monoclonal antibodies derived from spleen cells of 3-day-old BALB/c neonates. For a second antibody, TF5-139 (α, κ), the peak of responsiveness of B cells bearing this idiotype (10%) also occurred in spleens of 3-day-old neonates. The third antibody, TF2-76 (μ, κ), shared the idiotype with relatively few early neonatal B cell antibodies but its idiotype was shared by approximately 7% of BALB/c neonatal splenic B cells 1 week after birth. The sequence of occurrence of these clonotypes was verified by tolerance studies wherein tolerogen added to cultures of day 5 neonatal spleen cells eliminated the late arising and, thus, immature TF2-76 B cells but did not affect the more mature TF2-36 B cells. None of these three antiidiotypic sera recognized a significant proportion of monoclonal antibodies derived from adult BALB/c B cells or monoclonal antibodies from other strains. As will be discussed below, sequence analysis has been carried out on the rearranged heavy and light chain V regions of these hybridoma antibodies to affix the V region gene segment families that were used in these predominant neonatal specificities (Riley *et al.*, 1986). Other series of hybridoma antibodies have been raised by fusion of polyclonally stimulated neonatal spleen cells (Teale and Kearney, 1986). These hybridoma antibodies have been analyzed for the capacity to bind various antigenic determinants as well as having been subjected to antiidiotypic analysis (Kearney and Vakil, 1986). Again, the specificities of these antibodies are highly skewed from those which were obtained from adult splenic B cells by similar procedures.

In an effort to determine whether certain V_h region gene segments are utilized preferentially during fetal and neonatal development, two laboratories conducted extensive studies using AB-MuLV-transformed fetal liver pre-B cells and/or hybridomas derived from fetal or neonatal liver B cells (Perlmutter *et al.*, 1985; Yancopoulos and Alt, 1984). The findings indicated that the D most proximal gene family V_h 7I83 (see Fig. 1) rearranged with the highest frequency and, in particular, the V_h 8IX gene rearranged more frequently than any others in this family. The second most frequently rearranged V_h family was the V_h Q52 family which is the next most D proximal V_h gene segment family in the BALB/c mice and appears to be inter-

mixed with the 7183 V_h family in certain mouse strains. The extensive utilization in these hybridomas of the D most proximal V_h gene families led to the proposition that the skewing of the early neonatal repertoire was the result of a sequential utilization of V_h gene segments which followed from the D most proximal V_h gene segments upstream and ultimately all V_h region gene segment families. It should be noted that even within this overall skewing of V_h gene segment utilization there appears to be a preferential utilization of certain V_h region gene segments within the early rearranged V_h gene segment families. These findings were true not only for the AB-MuLV-transformed fetal liver pre-B cells but also for the hybridomas obtained from fetal liver and early neonatal spleens.

The analysis of the three aforementioned anti-DNP early neonatal hybridomas (Riley *et al.*, 1986) demonstrated that (1) the TF2-76 hybridoma, which represented clonotypes appearing relatively late in the neonatal spleen also utilized the 7183 V_h region gene segment family, and (2) the other two hybridomas, TF2-36 and TF5-139, which had been derived from different donor spleens, appeared to utilize the same V_h region gene segment, V_h 1210.7 a member of the 36-60 V_h family which had been thought to be relatively far upstream among the V_h region gene segment families. The original positioning of this family has been questioned by recent studies that have placed this V region family quite close to the 7183-Q52 V_h complex (see Fig. 1). If this is so, then all studies directed at analyzing V_h region gene segment utilization in neonates are consistent in that for AB-MuLV-transformed cells, polyclonally stimulated B cells and antigen-stimulated functional B cells, V_h region gene segment utilization during early development clusters toward the D proximal end of the V_h gene segment complex.

Since each of the three utilized V_h region gene segment families (7183, Q52, and 36-60) are substantially smaller than the V_h J558 family, which is utilized at only a very low frequency in neonatal B cells, it is clear that V_h utilization during fetal development and in early neonates is nonrandom. Although the V_h genes utilized by some of the early neonatal antibodies have been characterized (Riley *et al.*, 1986), insufficient data are available concerning the frequency of occurrence of these κ genes as well as the relative positioning of κ genes within the V_h locus to draw any similar conclusions.

These findings provide a molecular confirmation of the sequential expression of the B cell repertoire during neonatal development, as well as a strong indication that V_h gene segment utilization, at least during B cell ontogeny, is not a random process. While it could be

argued that this represents a special case, it certainly demonstrates the possibility that mechanisms exist which can favor the utilization of certain V region genes as opposed to others.

4. V Gene Segment Utilization in Aged Mice Differs from That of Young Adult Mice

Recently, a second, equally dramatic, demonstration of nonrandomness in V_H region gene segment utilization has been identified in studies of B cell repertoire expression in aged BALB/c mice. Aged mice display a general reduction in immunologic reactivity (Price and Makinodan, 1972). Although the major deficits in these mice appear to be related to decreased T cell function (Krosgrud and Perkins, 1977; Segre and Segre, 1976; Thoman and Weigle, 1987), there is a concomitant decrease in B cell function (Doria *et al.*, 1978; Zharhary and Klinman, 1986a; Callard *et al.*, 1977; Goidl *et al.*, 1976; Kishimoto and Yamamura, 1971; Kishimoto *et al.*, 1976; Klinman, 1981a; Zharhary and Klinman, 1983, 1987). An analysis of the clonotype repertoire of aged mice has demonstrated that the frequency of responsive B cells to many antigenic determinants is decreased within the splenic sIg⁺ cell population (Zharhary and Klinman, 1983, 1986a). Since the frequency of newly generated B cells in the bone marrow of these same specificities is normal in aged individuals, it would appear that B cells of aged mice have a relatively shortened functional half-life (Zharhary and Klinman, 1987). Therefore, the representation of functional B cells is diminished in mature B cell populations. It should be noted, however, that the functional cells which remain appear to be normal in almost every respect (Zharhary and Klinman, 1983).

Although the frequency of responsive B cells in aged BALB/c mice specific for several haptenic determinants is reduced, the frequency of splenic B cells responsive to influenza virus determinants appears to be normal (Zharhary and Klinman, 1984). In addition, the frequency of cells responsive to PC appears to be elevated (Zharhary and Klinman, 1986b). The age-associated increased frequency in PC-responsive splenic B cells was found to be the result of a markedly increased frequency in the generation of PC-specific B cells within the sIg⁻ pool of bone marrow cells from aged BALB/c mice. Thus, when sIg⁻ bone marrow populations (see below) of BALB/c mice were compared, the overall frequency of newly generating PC-specific B cells was severalfold higher in aged as compared to young mice. This increased responsiveness included an increase in frequency of the normally predominant T15 specificity. However, a greater increase in frequency of newly generated cells was noted

among the PC-specific precursors that did not express the T15 idiotype.

Normally, PC-responsive B cells in BALB/c mice fall into two categories. The majority (Type I) of responses are truly PC specific in that they bind PC more avidly than the linker *p*-diazonium phenylphosphorylcholine (DPPC), whereas Type II PC-specific B cells bind PC with a relatively low affinity (Perlmutter *et al.*, 1984). Of the Type I antibodies, there are two subsets: (1) those that are identical to the T15 myeloma protein (60–80%), and (2) those that differ from the T15 myeloma protein and, in general, are not recognized by anti-T15 antiidiotypic antibodies (T15⁻) (Owen *et al.*, 1982). In an analysis of dozens of PC-specific myeloma and hybridoma antibodies of these specificities, in several murine strains, the heavy chains of all Type I anti-PC antibodies both T15⁺ and T15⁻ were found to be either *V_h S107* or, rarely, another member of the *V_h S107* gene segment family (Perlmutter *et al.*, 1984). To determine whether the T15⁻ Type I antibodies generated by bone marrow cells of aged BALB/c mice were also of the *V_h S107* gene segment family, we generated a series of hybridomas using the transfer fusion protocol with bone marrow cells of aged BALB/c mice (Riley *et al.*, 1988). Antiidiotypic assays for T15 as well as cDNA probes for the *V_h S107* and various other *V_h* gene families were used to assess *V_h* gene segment utilization in these hybridomas. It was found that all tested PC-specific hybridoma antibodies obtained from bone marrow cells of young mice utilized the *S107 V_h* gene segment. Although both T15⁺ and some T15⁻ antibodies obtained from fusions of bone marrow cells of aged mice also used *S107 V_h* gene segments, the majority of PC-specific hybridoma antibodies derived from bone marrow cells of aged mice were T15⁻ and utilized *V_h* gene segments of families other than *S107*. Several expressed *V_h* gene segments of the *V_h 7183* family, and others used *J558* and *X24 V_h* gene segments. In addition, preliminary studies of the light chains utilized by these hybridomas identified light chains that had not heretofore been observed among anti-PC antibodies (S. C. Riley, B. G. Froscher, K. Marcu, and N. R. Klinman, unpublished observations). Although these studies are preliminary and must be extended to other murine strains, it appears that *V_h* genes other than the *V_h S107* can indeed give rise to bona fide Type I PC-specific antibodies. However, in young mice of all tested strains, these *V* region combinations are not expressed within the normal B cell repertoire. The reasons for the late expression of anti-PC antibodies utilizing non-*S107 V_h* gene segments is currently the subject of intensive investigation. Nonetheless, these findings serve as strong

evidence for the nonrandom utilization of V_h genes in certain combinations within the normal B cell repertoire of young mice.

D. THE INTERRELATIONSHIP OF CLONAL EXPANSION AND V GENE EXPRESSION

Although we have gained a considerable amount of new information concerning the cellular and molecular aspects of B cell repertoire expression, two very important aspects of this process remain relatively unexplored. The first of these issues concerns whether B cells are expressed as clonal populations of identical sister cells, or whether they are expressed as individual B cells. Given the fact that a fixed number of B cells emanate daily from the bone marrow (Osmond, 1986), the number of new specificities entering the repertoire will be inversely proportionate to the clone size of newly generated cells. This issue has been addressed in the experiments described above, wherein individual leg bones of (CBA/N \times BALB/c) F_1 mice were examined for the expression of T15⁺ versus T15⁻ clonal precursor cells (see Table I). The findings demonstrated that there was jackpotting of the two specificity categories in any given leg bone that yielded at least one fragment responsive to PC (Klinman and Stone, 1983). Since the frequency of precursors of each of these bone marrow populations was approximately the same whether they are T15⁺ or T15⁻, the conclusion drawn above from these experiments was that T15⁺ precursors occurred at a much higher frequency than any given T15⁻ specificity, but that the clone size of these responses was approximately the same. Another aspect of the finding of jackpotting of B cells of one type within a given responsive leg bone is the fact that there was a strong tendency for leg bones that contained any responsive B cells to contain several. This implies that clones are expanded at the level of developing sIg⁻ precursor cells. This is particularly so in the experiments mentioned since mice bearing the CBA/N immunologic defect have no mature primary precursor cells specific for PC (Klinman and Stone, 1983; Quintans, 1977; Mond *et al.*, 1977). This demonstration of expanded clones at the immature sIg⁻ cell stage is the only published finding which directly addresses the substantive issue of whether B cells are expressed in the bone marrow as clones of sister cells.

The importance of the finding of identical V gene commitment among sister cells of a clone is manifold. First, the demonstration that B cells are expressed as clones implies that the repertoire of any given mouse would be substantially smaller than the total number of B cells (approximately 2×10^8) present in the reticuloendothelial system of

that individual. For example, if the average clone size is 10^2 , then any mouse would have 2×10^6 different clonotypes within its mature B cell pool. Furthermore, since the estimated number of newly generated B cells per day is approximately 5×10^7 (Osmond, 1986; Osmond and Nossal, 1974) and if B cells are expressed as clones, then it may be anticipated that fewer than 10^6 different clonotypes could be expressed per day in a mouse. Thus, 2-month-old mice would have expressed fewer than 10^8 total specificities. The experimental data above are far from being sufficiently precise to allow estimates of clonal size within individual leg bones. If one assumes homing and stimulation efficiencies of approximately 2–4% for these cells, the results would be consistent with a clone size of anywhere from 20 to 400 cells.

The second important aspect of the conclusion that clones are expressed as multiple identical sister cells comes from the finding that B cells have limited division potential after light chain rearrangement and sIg expression (Osmond, 1986; Landreth *et al.*, 1981). Thus, if multiple B cells from the same clone express exactly the same specificity, then, perforce, they must express the same light chain. Since cells divide little if at all after light chain rearrangement, then multiple daughter cells of the same clone must have rearranged the same light chain. If this is the case, then the finding of multiple copies of the same specificity could be due either to a precommitment to a specific light chain rearrangement or a limited set of rearrangements, or, alternatively, the existence of enormous clones expressing the same rearranged V_H with a relatively random rearrangement of light chains such that each potential light chain rearrangement would be expressed in many cells. Given the time course of B cell development (Osmond, 1986) the latter explanation seems unlikely.

During the course of their studies of bone marrow B cell development *in vitro*, Witte and Whitlock identified the fact that certain B cells could develop to the point of heavy chain rearrangement *in vitro* but could not progress beyond that point (Whitlock *et al.*, 1983; Ziegler *et al.*, 1984). When clones of such cells were subjected to AB-MuLV virus transformation, some of these cells went on to express light chains and ultimately an immunoglobulin product. An analysis of the light chains rearranged by clones of B cells demonstrated that while different light chains were utilized, the choice of light chains for any given clone of heavy chain rearranged cells was somewhat restricted.

The second issue of great importance to our understanding of B cell repertoire development and expression that remains relatively unex-

plored concerns the relationship of particular stages of V region gene segment rearrangement with clonal development and expansion. Although at the present time the sequence of molecular events (D to J_h , V_h to D_h , V_L to J_L) appears to be emerging (see Section II), the correlation between each of these events and B cell development has not yet been carefully explored. In particular, it is not yet possible to know the extent of division potential of cells that have undergone each of these molecular events. The importance of this issue is reflected in questions such as to what extent do developing B cells of various specificities in a given bone marrow population bear the same D - J joining and/or the same V_h - D joining and, as mentioned above, to what extent do they share light chains? Furthermore, it is not yet clear whether, once a given D - J_h pair has been selected, its opportunities for various V_h chain rearrangements is unlimited and whether all such pairings have the same ultimate division potential. Until the relationships of such events are fully revealed, our understanding of the mechanism of B cell repertoire expression will remain limited.

V. The Impact of Environment on Primary B Cell Repertoire Expression

The expressed B cell repertoire is the product of the molecular events responsible for generating the clonally distributed population of B cells as well as effects of environmental influences in up- or down-regulating various preexisting specificities and participating in the generation and selection of new specificities. During the past decade our progress in understanding the contribution of environmental influences on repertoire expression and in understanding the mechanisms which underlie these contributions has been steady but far less dramatic than the progress in understanding the molecular basis for repertoire expression. It is now clear that antigenic stimulation, particularly T cell-dependent antigenic stimulation, of B cells can be highly instrumental in initiating and furthering the somatic mutation process. In addition, antigen appears crucial for the selection of the most appropriate somatic mutants (see Section III). However, these events appear to be mainly involved in the generation and selection of the population of secondary B cells and will therefore be discussed more fully in Section VI in the discussion of B cell subpopulations.

No role for antigenic stimulation in the generation of the primary B cell repertoire has yet been identified. Indeed, the major environ-

mental influences which have been identified that affect primary B cell repertoire expression remain, as they were 10 years ago, tolerance induction and antiidiotypic regulation (Sigal and Klinman, 1978). Although it is likely that both of these mechanisms do indeed play a role in shaping the expressed primary B cell repertoire, formal definitive proof for any contribution of these mechanisms to primary B cell repertoire expression remains elusive. Nonetheless, as will be detailed below, a considerable amount of evidence for an important role for tolerance induction in shaping the primary B cell repertoire does exist and evidence for some participation of idiotypic regulation in shaping the primary B cell repertoire is becoming available.

In order to assess the affects of environmental regulation on primary B cell repertoire expression, this laboratory and others have chosen to compare the repertoire as expressed in the sIg⁻ population of bone marrow cells with the repertoire expressed in the mature B cell populations. It was reasoned that developing B cells, prior to their expression of surface immunoglobulin, could not have interfaced with sIg receptor-specific mechanisms such as antigenic or antiidiotypic triggering events. Since the fragment culture protocol permits transferred sIg⁻ B cells to develop their sIg receptors subsequent to cell transfer (Neil and Klinman, 1987) and other protocols permit the development of sIg⁻ bone marrow cells *in vitro* in the absence of most self environmental antigens (Nishikawa *et al.*, 1983; Juy *et al.*, 1983), experimental procedures are available to permit this comparative analysis.

A. ASSESSING REPERTOIRE EXPRESSION IN sIg⁻ BONE MARROW PRECURSOR CELLS

Because of the importance of these issues, this laboratory has carried out an extensive comparison of the repertoire as expressed in sIg⁻ murine bone marrow cells in comparison to that expressed in mature sIg⁺ bone marrow cells as well as splenic B cells (Riley and Klinman, 1985, 1986; Froscher and Klinman, 1985; Klinman and Stone, 1983; Riley *et al.*, 1983; Klinman *et al.*, 1980, 1981, 1983; Morrow *et al.*, 1987; Wylie and Klinman, 1981a). These studies have covered a variety of antigenic determinants and have been carried out in several murine strains. As mentioned above sIg⁻ cells isolated from the bone marrow by rosetting away sIg⁺ cells using anti-Ig-coated erythrocytes (Walker *et al.*, 1979), upon transfer to irradiated carrier-primed recipients will acquire their sIg receptors and be responsive to antigenic stimulation in fragment cultures (Neil and Klinman, 1987). Overall, the proportion of B cell precursors that are responsive in fragment culture appears to be approximately 5% of total bone

marrow cells or 6% of sIg⁻ bone marrow cells. For comparisons to splenic B cells, wherein responsive sIg⁺ cells represent approximately 40% of the total population, a normalization factor of seven has generally been used for comparison of the two cell populations. It should be noted that the isolation of sIg⁻ cells that are also positive for the B cell marker B220, recognized by the 14.8 monoclonal antibody (Coffman and Weissman, 1981; Kincade *et al.*, 1981), yields a population that is at least 80% responsive in the fragment culture system (Neil and Klinman, 1987). This has made it possible to verify frequencies obtained using the total sIg⁻ population in terms of the frequency of precursor cells responsive to various antigens.

Responsive sIg⁻ cells generally yield clones producing less antibody than mature B cells and the antibodies tend to be devoid of IgG isotypes which are characteristics of immature B cell populations (Klinman *et al.*, 1974, 1980; Teale *et al.*, 1981). In addition, in several systems it has been shown that the sIg⁻ B cells, like immature neonatal and fetal B cells, are susceptible to tolerance induction by treatment of fragment cultures for 24 hours with a hapten presented on a noncognate carrier (a carrier for which the recipients have not been primed) whereas mature B cell populations are not inactivated by this treatment (see below). By all of these criteria, the cells isolated as sIg⁻ cells appear to be the immature precursors to sIg⁺ B cells and, as such, presumably display a representation of the repertoire prior to the interfacing of developing B cells with the environment through their sIg receptors.

B. ENVIRONMENTAL INFLUENCES DO NOT AFFECT PRIMARY B CELL REPERTOIRE DIVERSIFICATION

Using the aforementioned normalization for the frequencies of sIg⁻ versus splenic precursor cells, the findings for several antigen responses reveal that the frequency of responsive cells in the sIg⁻ bone marrow pool is essentially the same as the frequency of cells responsive to those antigens in the sIg⁺ bone marrow or splenic B cell pool. The antigens for which this is true are summarized in Table IIA. It can be seen that for antigens such as DNP, PR-8, Dextran, PC, and NNP, the frequency of responsive sIg⁻ precursor cells is approximately 5- to 10-fold lower than the frequency of splenic precursor cells and, thus, fit well within the normal range used to obtain the normalization factor of seven for comparisons of these two populations (Klinman *et al.*, 1981, 1983; Zharhary *et al.*, 1983; Riley *et al.*, 1983; Riley and Klinman, 1985; Klinman and Stone, 1983; Froscher and Klinman, 1985). From these observations it would appear that the frequency of

TABLE II
 FREQUENCY OF SPLENIC VERSUS sIg⁻ BONE MARROW PRECURSOR CELLS SPECIFIC FOR
 VARIOUS ANTIGENIC DETERMINANTS

	Clonal responses per 10 ⁷ injected sIg ⁻ bone marrow cells	Clonal responses per 10 ⁷ injected spleen cells	Ratio sIg ⁻ /spleen
A. Normal range expression of mature B cells			
DNP (BALB/c) ^a	5.7	23	0.24
NP (BALB/c) ^b	0.8	6	0.13
NNP (C.B20) ^c	4.6	28.4	0.16
NIP (C.B20) ^c	1.8	10.4	0.17
PC (BALB/c) ^d	0.53	5.7	0.09
PC (CBA/N × BALB/c F ₁ female) ^d	0.44	4.6	0.09
α1-3-Dextran (BALB/c) ^e	0.17	2.1	0.08
PR8 influenza virion (BALB/c) ^f	1.7	7	0.24
PR8-hemagglutinin (BALB/c) ^f	0.4	1.9	0.21
SV40-B6 (C57BL/6) ^g	0.71	2.76	0.25
SV40-B6 (BALB/c) ^g	0.3	2.12	0.14
B. Impaired expression of mature B cells			
Gly-Gln-Ala-Ala-Gly-Phe-Ser-Tyr- Thr (murine cyt c 41-49) (BALB/c) ^h	0.3	0.5	0.6
NP (homoclitic κ bearing responses) (C.B20) ^b	0.3	0.01	30
PC (CBA/N × BALB/c F ₁ male) ^d	0.4	0.1	4

^a Zharhary and Klinman (1983).

^b Riley and Klinman (1985).

^c Riley and Klinman (1986).

^d Klinman and Stone (1983).

^e Froscher and Klinman (1985).

^f Riley *et al.* (1983).

^g Froscher and Klinman (1986).

^h Morrow *et al.* (1987).

B cells responsive to most antigenic determinants is determined by the frequency with which B cells of these specificities have emerged from the developing bone marrow progenitor pool and appears to be influenced little, if at all, by the environmental milieu of the developing B cells.

This conclusion is convincingly reinforced by a more detailed

analysis of the clonotypic composition of the responding precursor cell populations. For example, in the response to PR8-HA, it is possible to delineate the diversity of the specificity repertoire of responding precursor cells by using a large panel of cross-reacting hemagglutinin H1 variants (Riley *et al.*, 1983; Cancro *et al.*, 1979; Gerhard *et al.*, 1975; Braciale *et al.*, 1976). Table III displays the reactivity patterns available among a surveyed population of sIg⁻ bone marrow B cells obtained from BALB/c mice in comparison with a similar survey carried out using mature splenic B cells of BALB/c mice. It can be seen that the distribution and diversity of specificities are quite similar between the two populations. Indeed, calculations of diversity determined by this type of analysis indicate that the reper-

TABLE III
RELATIVE FREQUENCIES OF ANTI-PR8-HA RPs OBTAINED FROM ADULT sIg⁻ BONE MARROW VERSUS SPLENIC B CELL RESPONSES^a

Heterologous virus										
BH			+	+	+	-	+	-	-	-
WSE			+	+	-	+	-	+	-	-
MEL			+	-	+	+	-	-	+	-
WEISS	CAM	BEL								
+	+	+	<u>10.0</u>	<u>3.3</u>	x	x	x	<u>1.7</u>	x	x
			7.0	1.6	x	1.6	x	x	x	x
-	+	+	x	<u>5.0</u>	x	x	x	x	x	x
			3.1	x	x	0.8	x	0.8	x	2.3
+	-	+	<u>1.7</u>	<u>3.3</u>	<u>1.7</u>	x	x	x	x	x
			1.6	x	0.8	x	0.8	x	x	1.6
+	+	-	<u>1.7</u>	<u>1.7</u>	x	<u>1.7</u>	<u>3.3</u>	x	x	x
			2.3	x	2.3	x	x	x	x	x
-	-	+	x	<u>3.3</u>	x	x	<u>5.0</u>	x	x	<u>1.7</u>
			x	x	0.8	x	x	2.3	0.8	2.3
-	+	-	x	<u>3.3</u>	<u>1.7</u>	x	x	x	x	<u>1.7</u>
			0.8	0.8	0.8	x	x	2.3	4.7	1.6
+	-	-	<u>1.7</u>	<u>1.7</u>	<u>3.3</u>	x	x	x	<u>1.7</u>	<u>3.3</u>
			1.6	x	x	x	0.8	5.5	1.6	1.6
-	-	-	x	<u>6.7</u>	x	x	x	<u>3.3</u>	<u>3.3</u>	<u>21.7</u>
			5.5	2.3	1.6	7.8	3.1	6.2	5.5	14.0

^a Relative frequencies are given as the percentage of the total anti-HA response and are based on 60 adult sIg⁻ bone marrow and 129 primary splenic anti-HA monoclonal antibodies. Numbers above the line refer to sIg⁻ bone marrow responses; numbers below the line refer to primary splenic responses. x, RP not observed. From Riley *et al.* (1983).

toire of sIg⁻ cells is not less diverse than that of splenic B cells (Fig. 2). This finding, if typical of the repertoire in general, would indicate that environmental influences have little impact on the frequency of B cells responsive to given antigens, and play little role in increasing the diversity of the primary B cell repertoire. This conclusion is consistent with findings in the response to the NP haptenic determinant (Riley and Klinman, 1985, 1986), as will be discussed in more detail below.

C. ENVIRONMENTAL INFLUENCES DO NOT CONTRIBUTE TO PREDOMINANT CLONOTYPE EXPRESSION

A second aspect of B cell repertoire expression wherein environmental influences were thought to play a potentially crucial role was in the establishment and expression of "predominant clonotypes."

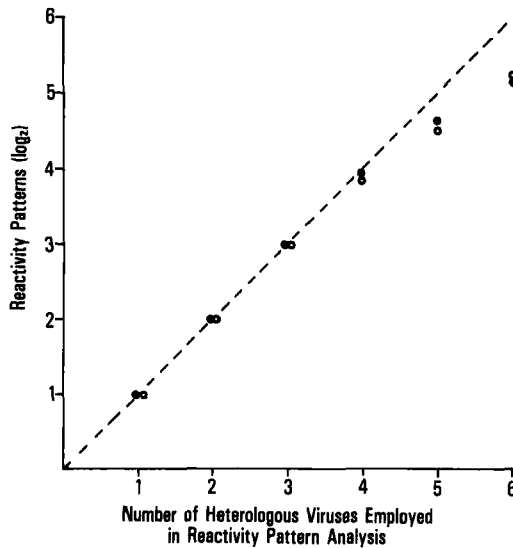


FIG. 2. Estimation of the number of HA-reactive clonotypes for adult primary spleen and sIg⁻ bone marrow cells. The number of anti-HA clonotypes (RP) expressed by BALB/c B cell populations is plotted against the number of heterologous H1N1 viruses used in the reactivity pattern analysis. When five or more viruses were used in the panel, the number of RP was estimated by the method of Briles and Carroll (1981). Closed circles represent the number of anti-HA RP for primary adult spleen; open circles are values for sIg⁻ bone marrow cells. The broken line indicates the maximum number of RP definable with a given number of heterologous viruses. The order in which the heterologous viruses were compared had little effect upon the calculated values (Riley *et al.*, 1983).

Studies to date using the fragment culture system as well as other systems (see Section III) have indicated that, consistent with the lack of environmental influence on the generation of primary B cell diversity, environmental influences play little or no role in the establishment of predominant clonotypes (Riley and Klinman, 1986; Nishikawa *et al.*, 1983; Froscher and Klinman, 1985; Juy *et al.*, 1983; Klinman and Stone, 1983). However, the term "predominant clonotype" applies to a variety of circumstances wherein immunized animals of a given murine strain ultimately produce an antibody population that contains a high representation of a single clonotype or clonotype family. In certain instances, such as B cells of A strain mice bearing the major CRI in response to the ARS haptenic determinant, the representation of B cells of the predominant clonotype in the primary B cell repertoire is quite low ($1-3/10^7$ B cells) (Sigal, 1982). In this situation, the cross-reactive idiotype is found to dominate only after extensive antigenic stimulation (Manser *et al.*, 1987; Near *et al.*, 1985) and clearly requires environmental selection for its expression at high levels. Such environmental influences, however, must be considered an aspect of the generation of secondary B cell responses and, in that regard, would not obtain to the establishment of the primary B cell repertoire.

In most instances, the predominance of a given clonotype or clonotype family can be observed within primary antibody responses and it can be demonstrated that the frequency of B cells in the spleen of unimmunized animals contains an exceptionally high percentage of B cells of these clonotypes or clonotype families. Several instances have now been studied in an effort to determine whether this high frequency of representation in the splenic B cell population is reflective of a high frequency with which such B cells are generated from within the bone marrow population. Concerning the issue of predominant clonotype expression, three antigen systems have been examined with respect to the relative expression in sIg⁻ bone marrow versus splenic precursor cells. In all three systems the frequency with which the predominant clonotype is expressed in the spleen is entirely reflective of the frequency with which the clonotype emanates from the generative cell pool.

The response to PC in BALB/c mice is dominated by B cells expressing an antibody whose V region sequence is identical to the T15 myeloma protein (see above). The frequency of T15⁺ B cells in BALB/c spleens ranges from approximately 1 in 20,000 to 1 in 100,000 and averages 1 in 50,000 (Gearhart *et al.*, 1977; Klinman and Stone, 1983; Sigal *et al.*, 1975). This is manifold higher than the representa-

tion of most clonotypes including those which comprise the highly diverse T15⁻ population of PC-responsive B cells in BALB/c spleens (Owen *et al.*, 1982). An analysis of the sIg⁻ bone marrow population of BALB/c mice revealed a distribution of T15⁺ and T15⁻ PC-responsive precursor cells similar to that observed in the splenic B cell populations of the BALB/c mouse (Klinman and Stone, 1983). In addition, the overall frequency of T15 bearing PC-responsive precursor cells was only slightly lower than that found in the spleen after normalization (see Table IIA). As discussed in greater detail in Section III (Table I), other experiments demonstrated that the high frequency of sIg⁻ T15⁺ B cells is apparently the result of a high frequency of recurrence of clones of this clonotype from within the generative cell pool as opposed to the generation of relatively large clones of cells bearing this specificity. This being the case, it would seem that the expression of predominant clonotypes is not only a reflection of B cell generative processes as opposed to environmental up-regulation, but the expression of predominant clonotypes is also the result of the frequent recurrence of certain V region segment combinations from within the generative cell pool.

A second predominant specificity that has yielded similar conclusions is that represented by the family of clonotypes characterized by BALB/c B cells responsive to the α ,1-3 dextran determinant that bear λ 1 light chains (λ -DEX) (Blomberg *et al.*, 1972). λ -DEX-responsive B cells are present as approximately 1/10⁵ B cells within the spleen (Froscher and Klinman, 1985; Stohrer and Kearney, 1984). As can be seen in Table II, the frequency with which these cells occur in the sIg⁻ bone marrow pool of BALB/c mice falls within the normalization range, and, thus, like B cells responsive to PC, the high frequency of λ -DEX B cells in the splenic B cell population of BALB/c mice appears to be the result of a high frequency of their recurrence within the sIg⁻ precursor population.

Finally, the response to the NP hapten in Igh^b mice is dominated by a clonotype family which characteristically bears the λ light chain and expresses idiotypic cross-reactivity with a prototype hybridoma antibody B1-8 (NP^b) (Imanishi-Kari and Makela, 1973; Boersch-Supan *et al.*, 1985). In the fragment culture system, responses with these characteristics are maximized by stimulation with protein conjugates of analogs of NP such as NNP and (4-hydroxy-5-iodo-3-nitrophenyl) acetyl (NIP) (Stashenko and Klinman, 1980; Riley and Klinman, 1986). It can be seen in Table IIA that as with responses of BALB/c mice to DEX and PC, the predominant λ NP^b-positive clonotype family, characteristic of responses to NNP and NIP in Igh^b mice, is present at

the same frequency (after normalization) in the sIg⁻ pool of the bone marrow and the mature B cell pool of the spleen. Thus, for all predominant clonotype sets analyzed, it would appear from studies using the fragment culture technique that predominant clonotype expression within the primary B cell pool reflects a high frequency of the generation of given specificities from within the generative pool of the bone marrow as opposed to environmental up-regulation as cells pass from this newly generated pool to the mature splenic B cell pool.

In terms of B cells whose antibody product binds to the NP determinant and its analogs, the findings for responses in the fragment culture system are consistent with findings obtained using limiting dilution B cell suspension cultures stimulated with LPS (Nishikawa *et al.*, 1983). These studies indicated that the frequency of precursor cells whose progeny produced λ -bearing antibodies that bound NP and expressed the NP^b idiotypic determinant was the same in splenic B cell populations as it was in bone marrow cell populations obtained by cultivating sIg⁻ bone marrow cells for several days prior to LPS stimulation. As stated above, the conclusions from these studies were that antiidiotypic up-regulation was not responsible for the high frequency of occurrence of λ NP-specific B cells. Similar conclusions were drawn from studies of responses of precursor cells whose clonal progeny produced antibodies that bound DNP and bore idiotypic determinants cross-reactive with those which are recognized by antiidiotypic antibodies specific for the MOPC 460 myeloma protein (Juy *et al.*, 1983). Thus, for all systems studied, where predominant clonotypes could be assessed at both the sIg⁻ precursor cell level and the mature splenic B cell level, the expression of predominant clonotypes appears to be independent of environmental up-regulatory mechanisms.

D. ENVIRONMENTAL DOWN-REGULATION IN CLONOTYPE REPERTOIRE EXPRESSION

In contrast to the lack of evidence for environmental participation in either repertoire diversification or the up-regulation of the frequency of B cells of predominant clonotypes, there are now several examples wherein the frequency of mature splenic B cells of a given specificity or clonotype subset is significantly lower than anticipated from the normalized frequency of cells of the same specificity or clonotype subset present in the sIg⁻ bone marrow precursor cell pool. Several examples of this are presented in Table IIB. Given the set of arguments which served as the *raison d'être* for the comparative study

of sIg⁻ versus splenic precursor B cell populations, the finding of disparities such as those shown in Table IIB between the mature splenic B cell population and the sIg⁻ population should be attributable to environmental down-regulation. However, it is much less straightforward to attempt to affix a specific mechanism such as tolerance or antiidiotypic regulation, as the basis for a diminution in the frequency of given specificities within the spleen versus sIg⁻ pools. For example, in order to formally demonstrate that the absence of specificities in the splenic B cell population that are present in the sIg⁻ pool is actually the consequence of tolerance to self-antigens, it would be necessary to demonstrate definitively which self-antigenic determinant was responsible and that, in the absence of that particular self-antigenic determinant, the disparity no longer exists. Thus, in the situation where the presumptive tolerogen was not present, the frequency of those antigen-specific cells from the mature splenic B cell pool would be the same (normalized appropriately) as the frequency in the newly generated sIg⁻ precursor cell pool from the bone marrow. Unfortunately, this set of circumstances does not yet exist for any of the antigenic responses for which disparities have already been demonstrated, and, thus, none of the examples in Table IIB can be considered as formal proof of the existence of an effective self-tolerance mechanism.

Nonetheless, several of the presented examples are highly indicative that a tolerance mechanism does exist since either a presumptive self-antigen can be identified or, in any case, there is no reason to believe that any other mechanism would be as likely to be responsible for the observed disparities in the two repertoires. Indeed, only in response to DEX has any evidence accumulated that would indicate that disparities in the two precursor cell pools may, in some instances, be due to an alternative mechanism such as antiidiotypic recognition. This set of circumstances and the presumptive role of antiidiotypic recognition in regulating the frequency of B cells responsive to DEX will be discussed below.

1. The Effects of Tolerance to "Self-Antigens" on the Expression of the Primary B Cell Repertoire

Perhaps the system wherein a role for a self-antigen in shaping the B cell repertoire is most convincing is in the response of mouse B cells to murine cytochrome *c* (cyt *c*) (Morrow *et al.*, 1987; Jemmerson *et al.*, 1982; Jemmerson and Margoliash, 1979; Cooper *et al.*, 1988). Mice immunized with murine cyt *c* in various forms can mount an immune response to this antigen, although in general, the serum antibodies are

found at much lower levels than would be found with immunization with horse cyt *c*. In addition the anti-self cyt *c* antibodies, in general, appear to bind cyt *c* with a relatively low affinity (Cooper *et al.*, 1988). Indeed, it has been argued that the antibodies that one raises against murine cyt *c* in mice recognize only denatured determinants (Jemmerson, 1987a). Since it is difficult to prime T cells sufficiently to provide adequate carrier-primed T cell help to permit responses in the fragment culture system, in order to study the response to this antigen, the strategy used was to synthesize peptides inclusive of amino acids 41–49 of the murine cyt *c* sequence (Morrow *et al.*, 1987; Jemmerson *et al.*, 1982). This is a β bend region of cyt *c* that tends to be highly immunogenic among mammalian cyts *c* in general (Jemmerson and Margoliash, 1979). By coupling this peptide to carrier proteins, either bovine γ -globulin (BGG) or Hy, it was possible to obtain responses both *in vivo* and in fragment cultures to the peptide. Indeed, it was possible to demonstrate that this peptide could be recognized by antibodies generated against cyt *c* per se and that antibodies against this peptide had the capacity to recognize intact cyt *c*. These studies demonstrated that the frequency of mouse splenic B cells that could respond to this peptide was extremely low (Table IIB). This was particularly true for B cells whose antibody product discriminated murine cyt *c* from horse cyt *c* which differ in this region by two amino acid residues. Such B cells represented only approximately 1 in 10^6 splenic B cells. However, sIg⁻ precursor cells in the bone marrow of the same mice had a normalized frequency of precursor cells with these specificity characteristics at least 20-fold higher (Table IIB). Thus, a substantial diminution in the frequency of B cells recognizing a given antigenic determinant as cells matured from the pre-B cell pool to the mature splenic B cell pool was clearly demonstrated. In addition, the determinant used to assess this process was a derivative of a self-antigenic constituent. However, since these responses were generated against a peptide determinant, it remains a possibility that such antibodies see only denatured native protein structures (Jemmerson, 1987a; Hiragama *et al.*, 1985). Indeed, to date there remains no evidence that precursor cells within the sIg⁻ bone marrow pool can recognize this region when it is presented as a determinant within native intact murine cyt *c* (Morrow *et al.*, 1987). Thus, formal evidence that intact self-cyt *c* is responsible for elimination of B cells recognizing the β bend region of murine cyt *c* remains elusive. Nonetheless, the likelihood that this phenomenon represents evidence of self-tolerance remains high.

A second instance in which a marked disparity exists between the

frequencies of responsive cells in the sIg⁻ precursor cell pool and the mature B cell pool, which may be attributable to an environmental antigen, is found in responses to PC in cells from mice bearing the CBA/N immunologic defect. Among the immunologic deficiencies noted in CBA/N mice, or (CBA/N × BALB/c)_F₁ male mice, is a lack of B cell responses to a variety of antigens, particularly those that are likely to be abundant in the environment (Quintans, 1977; Mond *et al.*, 1977). Among these antigens is PC, and, to date, extensive studies in numerous laboratories have led to the conclusion that mice with the CBA/N defect not only respond poorly to immunization with PC, but also have few, if any, mature splenic B cells that recognize PC (Klinman and Stone, 1983). It should be noted that these mice can respond to PC protein conjugates by making Type II (see above) anti-PC antibodies (Kenny *et al.*, 1983), but only under special circumstances do these mice respond to immunization with PC by the production of truly PC-specific Type I antibodies (Clough *et al.*, 1981). In an extensive analysis of PC-specific precursor cells in (CBA/N × BALB/c)_F₁ male mice (Klinman and Stone, 1983), it was found that splenic B cells from these mice have an exceedingly low frequency of cells responsive to PC when conjugated to Hy and, as yet, none of these responses has been Type I anti-PC antibodies. On the other hand, as shown in Table IIB, when the sIg⁻ bone marrow cells of these mice were analyzed, there was an abundant population of PC-specific precursor cells. Indeed, Type I PC-responsive precursor cells, including those of the T15 idiotype, were present in a frequency comparable to that of their normal female littermates. Since B cells of mice expressing the CBA/N immunologic defect remain tolerance susceptible even as splenic B cells (McKearn and Quintans, 1980; Metcalf *et al.*, 1980) and since PC is an abundant environmental antigen, it is possible that the elimination of this specificity as B cells emerge from the immature pool in mice with the CBA/N defect is due to interaction with environmental antigen. If this is the case, then the absence of mature PC-specific B cells in CBA/N mice would represent an additional example of a role for tolerance in shaping the primary B cell repertoire. However, this also cannot be considered formal proof of a role for tolerance in shaping the B cell repertoire since the CBA/N defect is not yet fully understood. It is possible, for example, that the sIg⁻ B cells of these mice represent a separate B cell subpopulation which cannot mature in CBA/N mice. If so, the absence of mature PC-responsive B cells would represent a maturational deficit rather than evidence of tolerance to a self-antigen.

There are currently several other examples of disparate representa-

tion of B cells in the splenic B cell versus sIg⁻ precursor cell pools. Although these responses do not fulfill the criteria used to attribute clonotype diminution to antiidiotypic regulation (see below), it is difficult to attribute these disparities to tolerance since there is no obvious responsible environmental antigen. An example of this phenotype is seen in the response of Igh^b precursor cells expressing κ -bearing sIg receptors specific for the NP haptenic determinant (see Table IIB). As detailed above, the response of Igh^b mice to the haptenic determinant NP is characterized by serum antibodies predominated by B cells bearing the λ light chain and which have a lower affinity for NP than for many analogs of NP. In an analysis of sIg⁻ bone marrow precursor cells specific for NP obtained from Igh^b mice, it was found that the frequency of precursor cells whose progeny produced κ -bearing antibodies was considerably higher than would have been anticipated from the frequency of such cells obtained from the splenic B cell pool (Riley and Klinman, 1985).

As shown in Fig. 3, the differences in the two responses were more dramatic when high-affinity precursor cells were analyzed. In this case, all of the splenic B cells that expressed κ -bearing anti-NP antibodies produced antibodies with relatively low affinity, whereas, in the sIg⁻ pool, a substantial number of precursor cells yielded κ -bearing antibodies of high affinity. Again, particularly because of the affinity dependence of the disparity between sIg⁻ bone marrow precursor cells and mature splenic B cells, it is tempting to speculate that the elimination of high-affinity κ -bearing NP-specific B cells during B cell maturation in Igh^b mice is due to tolerance induction. Nonetheless, since the self-antigen that would be so closely related to NP has not been identified, this also does not constitute formal evidence of a tolerance mechanism shaping the B cell repertoire. It is interesting to note, however, that this finding does, at least in part, explain the unusual phenotype of responsiveness of Igh^b mice to the NP haptenic determinant (Imanishi-Kari and Makela, 1973; Karjalainen *et al.*, 1980). It would seem that the unusual dominance of λ heteroclitic antibodies specific for NP is, in part, the result of the elimination of high-affinity homoclitic κ -bearing B cells specific for NP that seems characteristic of antibodies obtained from mice of other heavy chain haplotypes. The distribution of λ - and κ -bearing antibodies in the pre-B cell pool of Igh^a mice, for example, is fairly similar to that of Igh^b mice. However, since κ -bearing Igh^a antibodies are also present as receptors on mature splenic B cells, responses of Igh^a mice to the NP haptenic determinant include κ homoclitic antibodies which are of higher affinity than the λ antibodies and rapidly do-

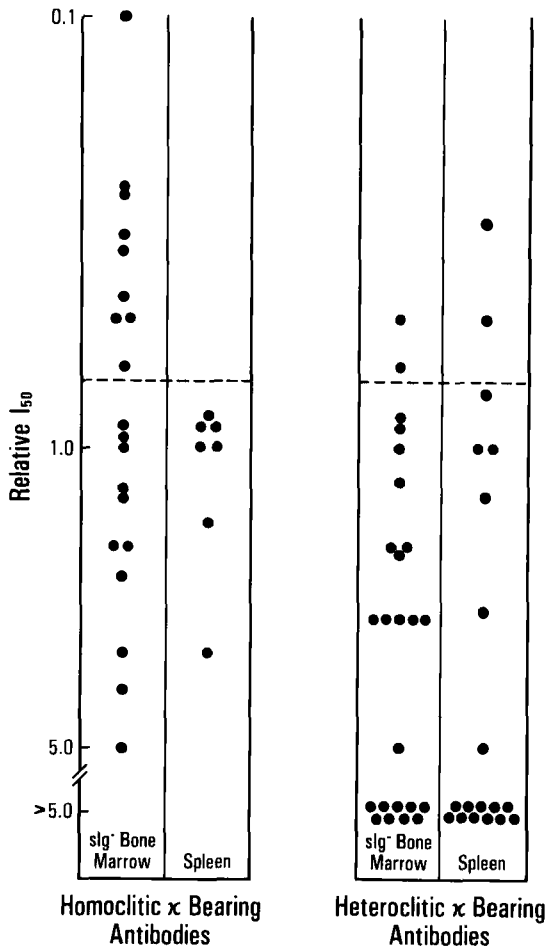


FIG. 3. The affinities of anti-NP κ bearing antibodies derived in splenic fragment cultures were assessed by determining the concentration of NP aminocaproic acid (NPCapOH) required to inhibit the binding of antibody to an NP-coated polyvinyl plate by 50% (I_{50}). Relative I_{50} values were calculated after normalization of the NPCapOH concentration resulting in 50% inhibition (I_{50}) of binding for the antibody tested to the I_{50} concentration of hapten determined for the polyspecific BALB/c anti-NP antiserum employed as a standard in the assays.

$$\text{Relative } I_{50} = \frac{I_{50} \text{ test antibody}}{I_{50} \text{ standard antiserum}}$$

Antibodies with relative I_{50} values <0.7 (dotted line) were considered to be high-affinity antibodies (Riley and Klinman, 1985).

minate the response (Imanishi-Kari and Makela, 1973; Stashenko and Klinman, 1980).

Taken together, the above findings provide convincing, if not compelling evidence, that tolerance to self or environmentally abundant antigens plays a significant role in shaping the expressed mature primary B cell repertoire. That tolerance would play a role in shaping the B cell repertoire is certainly a probability since the mechanism exists for B cell inactivation by antigen particularly when B cells are passing through an early developmental stage (Riley and Klinman, 1986; Klinman *et al.*, 1980, 1985; Nossal and Pike, 1975; Metcalf and Klinman, 1976, 1977; Stocker, 1977; Cambier *et al.*, 1976; Teale *et al.*, 1978; Metcalf *et al.*, 1977, 1979; Etlinger and Chiller, 1979). The developmental stage dependence of tolerance induction was deduced by Lederberg three decades ago (Lederberg, 1959) and provides an adequate explanation for tolerance to a broad range of self-antigens that presumably constitute a tolerance gauntlet through which B cells must pass during their maturation. The advantage of this theory is that once B cells are mature, they are no longer subject to tolerance induction and no special antigenic attributes are required to discriminate tolerogenic versus immunogenic signals. It is clear from data already available that the tolerance trigger is an active process requiring energy metabolism and macromolecular biosynthesis by the subject B cell (Teale and Klinman, 1980, 1984). In addition, it appears there is a requirement for surface receptor interlinkage (multivalent antigens or multivalent antigen presentation) as a requisite for tolerance induction which may obviate tolerance induction even by some environmentally abundant self-antigens (Teale and Klinman, 1980; Metcalf and Klinman, 1976).

Recent evidence also indicates that there is an affinity threshold for receptor-ligand interaction that must be exceeded in order for the tolerance process to proceed (Riley and Klinman, 1986). Indeed, it now appears likely that the affinity required for receptor-antigen interaction to tolerize (or stimulate) immature bone marrow precursor cells is higher than the affinity required to stimulate the same cells once they have fully matured. This disparity would dictate that B cells could escape the tolerance gauntlet because their affinity for a self-antigen was too low to enable tolerance induction, yet the affinity of the sIg receptors once the cell is mature could be sufficient to permit responses to the same self-antigen. The process by which affinity thresholds for successful triggering change during B cell maturation could represent an important biological mechanism for preserving repertoire diversity. However, the price paid for this

diversity would be the persistence of anti-self B cells in the mature B cell pool, albeit B cells with relatively low affinity for self-determinants. If this is the case, then the phenomenon of increased permissiveness of stimulation as B cells mature could account not only for the persistence of B cells reactive to self-antigens (Harris *et al.*, 1982; Jemmerson *et al.*, 1985), but also for the fact that when animals are experimentally tolerized, they retain some low-affinity antigen-responsive B cells (Pickard and Havas, 1972; Siskind and Benaceraff, 1969). Finally, this phenomenon could, in part, be responsible for some instances of autoimmunity.

The experimental evidence that led to the conclusion that the affinity requisite for tolerizing or stimulating immature B cells is higher than the affinity required to stimulate B cells once matured is presented in Table IV (Riley and Klinman, 1986). The frequency of mature splenic λ -bearing B cells of Igh^b mice responsive to NP was found to be 2- to 3-fold higher than would have been anticipated from the frequency of sIg^- precursor cells of this phenotype (Riley and Klinman, 1985). Although it was initially thought that this represented a unique example of a specific up-regulation of the frequency of a given clonotype family during B cell maturation, this is not the case. Instead, it appears that the appropriate precursors are present in the sIg^- pool at a frequency equivalent to that in the sIg^+ mature B cell pool, but that these precursors cannot be stimulated by NP as

TABLE IV
STIMULATION AND TOLERANCE INDUCTION OF λ -BEARING C.B20 PRECURSOR CELLS
SPECIFIC FOR NP OR NIP

Cell Population	Antigen	Tolerogen ^a	λ -Antibody secreting clones/ 10^7 injected cells
Spleen	NP-Hy	—	3.2
	NIP-Hy	—	3.1
	NP-Hy + NIP-Hy	—	2.6
sIg^- bone marrow	NP-Hy	—	0.2
	NIP-Hy	—	0.7
	NIP-Hy	NP-BSA	0.6
	NIP-Hy	NIP-BSA	0.2

^aTolerogens were added at the initiation of culture at 1×10^{-6} M hapten and remained in culture for 24 hours. After removal of tolerogen, fragments were cultured in the presence of NIP-Hy (1×10^{-6} M for 2-4 days). From Riley and Klinman (1986).

immature B cells. It should be remembered (see Section III) that the λ -bearing precursor cells that are responsive to the NP haptenic determinant in Igh^b mice have a relatively low affinity for NP and a much higher affinity (heteroclitic) for analogs of NP. It was reasoned, therefore, that λ -bearing B cells that respond to NP should also respond to carrier conjugates of analogs of NP including NIP.

In experiments carried out to assess this point it was found that, indeed, NIP stimulated the same frequency of λ -bearing splenic B cells as did NP and, in fact, the proportion of these B cells that bore NP^b idiotypic determinants as well as the Acl46 private antigenic determinant was identical to that found when stimulation was carried out with NP. Additionally, the fine specificity as assessed by reactivity of the antibody to hapten analogs was the same in the two populations. The final evidence that NP and NIP stimulated essentially the same population of λ -bearing B cells was obtained by studies in which the frequency of responsive cells was assessed using either NP-Hy or NIP-Hy alone, or the two antigens in concert. In this situation, as seen in Table IV, the frequency of B cells stimulated by both antigens together was the same as the frequency stimulated by either antigen alone. Since for other antigens tested in a similar fashion, such as DNP and TNP (Klinman *et al.*, 1973), and even for responses of κ -bearing B cells to NP and NIP (Riley and Klinman, 1986) the addition of two antigens yields additivity of responses, the complete overlap of the λ -responsive B cells to NP and NIP in Igh^b mice is a strong indication that these two haptenic determinants stimulate the same population of B cells. It should be noted that the λ -bearing B cells that are responsive to both of these antigens have a substantially higher affinity for NIP than they do for NP.

When sIg^- precursors were assessed for responsiveness to NIP carrier conjugates, it was found that the frequency of these precursor cells responsive to NIP was 4-fold higher than the frequency responsive to NP (see Table IV). Thus, the very same precursors which could be stimulated as mature B cells by both NIP and NP could be stimulated as immature precursor cells only with the higher affinity hapten NIP. An analysis of reactivity patterns and relative capacity for inhibition indicated that the populations of cells which did respond to NP among the sIg^- cells were those that had the highest relative affinity for NP. Thus, the majority of NIP-responsive sIg^- B cells had too low an affinity for NP to respond whereas these very same cells could have responded once they had matured to sIg^+ splenic B cells. This finding constitutes the first indication that the affinity threshold for precursor cell stimulation differs when the cells are present as

immature precursor cells in the bone marrow as compared to their triggering thresholds when they are mature splenic B cells.

Importantly, as is also shown in Table IV, when NP was used as a tolerogen for immature sIg⁻ bone marrow B cells, it was a much less efficient tolerogen than NIP in its capacity to inactivate NIP-responsive precursor cells. Thus, the capacity to tolerize B cells is comparable to the capacity to stimulate those cells and is equally affinity dependent. These findings constitute evidence that the affinity required to tolerize B cells as they mature through the tolerance gauntlet of self-antigen is a higher affinity than that ultimately required to trigger these cells once they mature. This extrapolates to the conclusion that the mature splenic B cell population could include a substantial number of cells that recognize self-antigenic determinants but have too low an affinity for these antigens to have been tolerized as immature B cells.

In toto, the above findings indicate that if tolerance induction plays a significant role in shaping the primary B cell repertoire, the effects of this induction would be highly clonotype dependent. Thus, it would be anticipated that B cells would persist in the mature B cell repertoire that could recognize (1) self-antigens that did not access the developmental milieu within the bone marrow of maturing tolerance susceptible B cells, (2) antigens that did access this milieu but at insufficient concentration to interact sufficiently with the developing B cell population, (3) antigens that are present in sufficient concentration but could not be presented in the multivalent fashion, and (4) antigens recognized by immature precursor cells whose receptor affinity is too low to enable tolerance induction. Because of all these constraints on the tolerance mechanism, it should be anticipated that mature splenic B cells persist that could react to self-antigens. Thus, it is likely to be extremely difficult to prove formally that tolerance induction has a significant impact on shaping the B cell repertoire. It should also prove difficult to detail precisely the specificity of the mechanisms that govern tolerance induction to self-antigens.

2. The Role of Antiidiotypic Recognition in the Expression of the Primary B Cell Repertoire

As in the case with tolerance induction, experimentally induced antiidiotypic suppression can markedly affect the expression of the primary B cell repertoire, particularly with respect to B cells whose sIg receptors express the idiotypic determinants in question (Kearney and Vakil, 1986; Kelsøe *et al.*, 1980; Klinman and Stone, 1983; Jerne, 1971; Rodkey, 1974; Kohler, 1975; Hart *et al.*, 1972; Eichmann, 1978;

Trenkner and Riblet, 1975; Bluestone *et al.*, 1982; Accolla *et al.*, 1977; Kohler *et al.*, 1979; Hiernaux *et al.*, 1981; Rubenstein *et al.*, 1982). Thus, assuming that a naturally occurring antiidiotypic mechanism does exist, to the extent that such a mechanism resembles experimentally induced antiidiotypic recognition, the parameters of this regulatory phenomenon may have already been defined. By and large, the experience has been that the most effective way of purging the repertoire of B cells expressing a given idiotype is to present the antiidiotypic during neonatal development. As with tolerance induction, it is far more difficult to eliminate responsive B cells from the fully mature B cell repertoire. On the other hand, it has been possible to increase the representation of B cells of a given idiotype by treatment of adult individuals with antiidiotypic antibodies (Kelsoe *et al.*, 1980; Kearney and Vakil, 1986; Trenkner and Riblet, 1975; Bluestone *et al.*, 1982). In this case, one might anticipate that the resultant B cells would have many of the characteristics of memory B cells, although this issue has not yet been addressed.

Treatment of neonates with antiidiotypic antibodies can lead to the elimination of a given clonotype from the mature B cell repertoire for several months thereafter, presumably long after the injected anti-idiotype has been eliminated from the system (Kelsoe *et al.*, 1980; Kohler, 1975; Kearney *et al.*, 1981; Kohler *et al.*, 1979; Cerny *et al.*, 1983; Accolla *et al.*, 1977). Given the fact that clonotypes continuously emanate from within the B cell pool of adult animals (see Section III) and that sIg⁻ T15⁺ precursors are present in normal frequency in the bone marrow of the adult mice suppressed as neonates with anti-T15 (Klinman and Stone, 1983), the mechanism for such long-lived suppressive effects remains undefined. This is particularly true since long-lived suppression appears to be a characteristic even of idiotype-suppressed nude mice (Etlinger, 1981). Although it is possible that T cells participate in maintaining the continued suppression of B cells of given idiotypes, it is likely that the presence of functional T cells is not required of this process.

Figure 4 demonstrates the results obtained by antiidiotypic suppression of neonatal BALB/c mice with antibodies specific for the T15 idiotype. It can be seen that while the non-T15 portion of PC-responsive cells is not affected by this treatment the population of mature splenic B cells responsive to PC that bear the T15 idiotype used for suppression is markedly diminished for several months after neonatal treatment (Accolla *et al.*, 1977). Findings similar to these have been obtained by several laboratories using the same or other antiidiotypes specific for T15, as well as antiidiotypes against other dominant

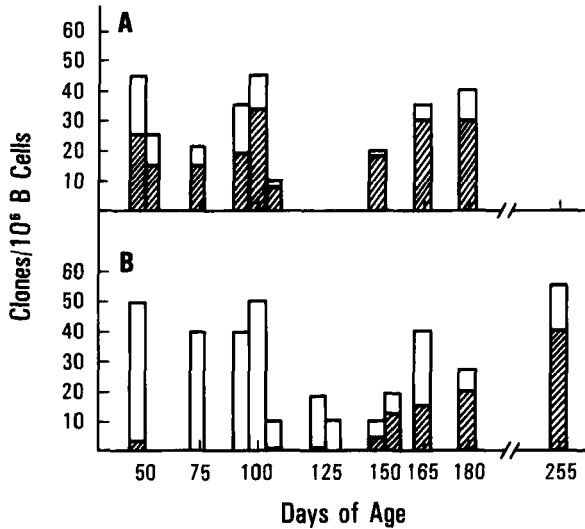


FIG. 4. Age-dependent expression of T15⁺ clones in anti-PC response of normal (A) and suppressed mice (B). Columns represent the total response in terms of PC-specific clones/10⁶ B cells for individual donor mice. Shaded areas represent the portion of clones expressing the T15 idiotype. Suppressed mice were injected with undiluted anti-T15 serum between 1 and 6 days of age (Accolla *et al.*, 1977).

clonotypes in a variety of antigen-responsive systems (Kelsoe *et al.*, 1980; Kearney *et al.*, 1981; Cerny *et al.*, 1983). One critical element of the set of experiments depicted in Fig. 4 is that the timing of the injection of antiidiotype antibodies was extremely important. As mentioned above, PC-responsive B cells, including those which bear the T15 idiotype, first appear in BALB/c neonates toward the end of the first week of postnatal life (Sigal, 1982). It is at this period in particular that PC-responsive cells are most susceptible to tolerance induction (Metcalf *et al.*, 1977). In addition, it appears that at the time that PC-responsive cells are first appearing in their spleen (days 5–8), neonatal mice are most susceptible to anti-T15 suppression. Thus, if antiidiotypic antibodies are given later than this time, the suppression is only partial and not long-lasting (Accolla *et al.*, 1977). In addition, if antiidiotype antibodies are given prior to this time, their effectiveness can be ablated by injecting large quantities of the idiotype prior to but not after days 5–7. Thus, in order to be effective, antiidiotype antibodies have to be present during a crucial period of time in the development of idiotype expressing cell clones.

The second aspect of antiidiotypic suppression is that experimen-

tally induced antiidiotypic suppression is relatively specific. As seen in the experiments presented in Fig. 4, while the T15⁺ response is markedly affected by treatment of neonates with anti-T15 antibodies the T15⁻ populations is not affected. Indeed, data have been presented that even clonotypes closely related to T15, including those that use the same heavy chain variable region gene elements, are not affected by this treatment (Accolla *et al.*, 1977). This conclusion is consistent with the findings from several other antigen systems including the treatment of Igh^b mice with anti-NP^b idiotype (Kelsoe *et al.*, 1980), and treatment of A strain mice with the antibodies to the CRI of anti-ARS antibodies (Manser and Gefer, 1986). Indeed, in the latter instance, even memory responses utilizing the variable region gene combinations necessary to constitute the CRI are eliminated by this treatment. This finding has been used as evidence that precursors to memory B cells do not themselves include somatic mutations which would eliminate recognition by anti-CRI antibodies. It is possible that the relatively long-lasting effects of antiidiotypic suppression, as well as reports of suppression of B cells that do not bear the idiotype in question but which are specific for the same antigenic determinant, are both reflections of an independent consequence of antiidiotypic suppression. It is possible that by the elimination of clonotypes that normally dominate a response, experimentally induced antiidiotype suppression creates an imbalance in the relative concentrations of antigen and idiotype normally present in the system. In addition, it has been proposed that treatment of neonates with antiidiotypic antibodies, or with antibodies bearing certain idiotypes, sets up a chain of events that eventually creates a network of reactivities that impacts not only on the neonatal repertoire but also the repertoire well into adulthood (Kearney and Vakil, 1986).

It is obvious from the above discussion that the potential role that network regulation may play in repertoire expression is, at the present time, highly hypothetical. Indeed, there are very few examples thus far identified wherein the level of clonotype expression could be accounted for by a naturally occurring idiotype-specific regulatory phenomenon. If, as demonstrated for experimentally induced antiidiotypic suppression, naturally occurring antiidiotypic suppression were highly specific, then, since murine strains expressing different V_H haplotypes express significantly different repertoires, it might be anticipated that mice could suppress their own idiotypes but not the idiotypes of Igh allogeneic mice. This expectation was born out in fragment culture experiments wherein BALB/c recipient mice were primed with both Hy and DNP-Hy and compared to mice primed

only with Hy in terms of their capacity to support B cell responses of syngeneic versus Igh allogeneic donor B cells (Pierce and Klinman, 1977). It was reasoned that if antigenic stimulation generated not only antibodies against the immunogen but also antiidiotypic antibodies specific for the idiotypes of the antigen-responsive B cells, then the presence of this antiidiotypic recognition could suppress B cells subsequently transferred to the antigen-primed recipients. If antiidiotypic suppression were Igh haplotype specific, then the suppression would be found for Igh syngeneic donor B cells but not Igh allogeneic donor B cells.

As can be seen in Fig. 5 these predictions turned out to be correct. Although Hy-primed BALB/c (Igh^a) irradiated recipients supported responses of BALB/c (Igh^a) and C.B20 (Igh^b)-primary DNP-specific donor spleen cells, BALB/c recipients primed with DNP-Hy as well as Hy supported only the response of the C.B20 donor B cells. As many as 75% of BALB/c donor spleen cells were unable to respond in the presence of this presumptive antiidiotypic network generated in recipients primed with DNP. The fundamental conclusion from these experiments, that antigenic stimulation induces not only antibody formation but an antibody-specific immunoregulatory mechanism, has been confirmed by several experiments wherein similar questions were asked (Thompson *et al.*, 1983; Herzenberg *et al.*, 1983). Among the important conclusions to be deduced from the above experiments are that antiidiotypic suppression induced by antigenic stimulation has specificity characteristics similar to that induced experimentally by the injection of antiidiotypic antibodies, and that this antiidiotypic suppression is long lived.

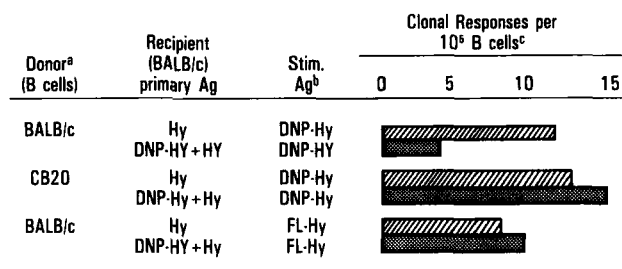


FIG. 5. Antibody specific immunoregulation. ^a Primary B cells were transferred into carrier-primed versus hapten plus carrier primed recipients. ^b Recipient mice of the BALB/c strain received 4×10^6 donor spleen cells. ^c All cultures were stimulated *in vitro* with hapten Hy conjugates at 10^{-6} M hapten. Positive foci were detected by assaying culture fluid for antibody specific for the *in vitro* stimulating hapten. From Klinman (1981b).

However, several other important conclusions can be drawn from the studies presented in Fig. 5. When similar experiments were carried out using syngeneic secondary donor B cells, antiidiotypic suppression was not observed in antigen-primed recipients. Thus, either the antiidiotypic specificities present in the antigen-primed recipients did not recognize the idiotypes of the secondary B cells, or secondary B cells are much less subject to antiidiotypic suppression than are primary B cells. This latter conclusion is consistent with findings that secondary B cells are more responsive than primary B cells in the presence of idiotypic-specific suppressor T cells (Owen and Nisonoff, 1978). Such resistance to antiidiotypic suppression of secondary B cells might be highly advantageous to immunized individuals, since immunized individuals would be able to respond secondarily to a new contact with the immunizing antigen, albeit, their primary B cells might be suppressed by the antiidiotypic regulation induced by the primary response.

In addition to these conclusions, a more subtle but equally important conclusion can be drawn from the data presented in Fig. 5. It can be seen that the frequency of both syngeneic, BALB/c, and allogeneic, C.B20 primary B cells was the same in BALB/c recipients that had been primed with Hy alone. This particular finding has been extended to other antigen systems and strain combinations (Riley and Klinman, 1985; Froscher and Klinman, 1985), and in these instances as well, the frequency of Igh allogeneic and Igh syngeneic precursor cells appears to be unaffected by the Igh haplotype of the carrier-primed recipient. If an antiidiotypic network preexisted within the recipient animals, then assuming the same specificity characteristics of any naturally occurring network regulation, one might have anticipated that syngeneic primary B cells might be suppressed in a syngeneic recipient as compared to an Igh allogeneic recipient. The absence of such down-regulation of syngeneic primary B cells in recipients that were not overtly antigen primed constitutes strong evidence against the existence of a naturally occurring idiotypic network capable of suppressing the responses of primary B cells. This would also make it less likely that naturally occurring networks play a role in the expression of the primary B cell repertoire. Alternatively, as proposed above, if such regulatory mechanisms do exist, they might exist in balance such that the stimulation of most primary B cells would not be affected.

Two examples of the presence of naturally occurring antiidiotypic regulatory mechanisms that do not require any overt experimental manipulation for their establishment now exist. The first of these was

identified in an analysis of immune responsiveness of aged BALB/c mice. As discussed above, aging is accompanied by a multifactorial decline in immune responsiveness. Among the immunologic functions that are diminished in aged individuals are helper T cell function and the frequency of B cells responsive to certain antigens. In experiments intended to determine whether aged mice also display antiidiotypic down-regulation, splenic B cells were obtained from normal young BALB/c donors and transferred to irradiated Hy-primed aged (2 years) versus young (3–6 months) BALB/c recipients (Klinman, 1981a). The frequency of responsive BALB/c splenic B cells was substantially lower in the aged recipients than young recipients. To rule out a trivial explanation for this finding, splenic B cells from normal young B10.D2 (Igh^b) and DBA/2 (Igh^d) mice were also transferred to aged versus young BALB/c recipients. In this case, the frequency of responsive cells was not significantly different in aged versus young recipients. Additionally, it was found that irradiated carrier-primed young mice repopulated with high numbers of spleen cells from aged mice also suppressed syngeneic but not allogeneic primary B cell responses.

The Igh haplotype specificity of this suppression parallels that observed in the aforementioned antibody-specific immunoregulation induced by antigenic stimulation. Therefore, it would seem likely that the mechanism of the suppression of responsiveness of B cells in aged mice is also based on an idio-type-specific mechanism. In this case, since the aged mice were not specifically immunized to the haptenic determinant in question, the antiidiotype immunoregulatory events would appear to have been spontaneously acquired. Since, as noted above, the antibody-specific immunoregulatory phenomenon observed after antigenic stimulation is quite long lived, it is, perhaps, not surprising that animals aged in a conventional environment and presumably subjected to a variety of antigenic stimuli would, over time, accumulate a substantial amount of antiidiotypic regulation. Thus, it would appear that antiidiotypic regulatory phenomena can be a part of the normal physiology of the B cell response, however, this phenomenon seems to play little role in healthy young individuals.

The finding of increased antiidiotypic regulation in aged individuals is consistent with findings in other laboratories that have come to similar conclusions using very different systems (Szewczuk and Campbell, 1980; Goidl *et al.*, 1980, 1984; Siskind *et al.*, 1982; Flood *et al.*, 1981). The presumptive existence of a pan-specific antiidiotypic regulation present at high levels in aged individuals provides an opportunity to determine whether the existence of such regulatory

effects is capable of altering the expressed B cell repertoire. As discussed above, aged mice generate normal levels of B cells responsive to most antigens and increased levels of B cells responsive to some antigens. Nonetheless, the proportion of newly generated B cells that ultimately mature to become functional within the spleen is disproportionately low in aged animals (Zharhary and Klinman, 1983, 1984, 1987). Although there may be differences in the biology of B cells generated in aged mice as compared to young mice these findings seem most likely to be consistent with the interpretation that B cells, newly generated in the bone marrow of aged animals, have a shorter functional half-life than those generated in younger animals. Given the existence of a pan-specific idiotype-specific suppression in aged animals, it is tempting to speculate that the shortened half-life of many of the B cells in aged mice is due to the existence of a down-regulation via antiidiotypic recognition. Consistent with this notion, it has been found that the bone marrow B cells developing in young animals that have received high numbers of T cells from aged versus young mice also display a shorter functional half-life (Zharhary, 1985). Additionally, aged nude mice do not show a shortened functional half-life of newly generated B cells (Zharhary, 1985). If a pan-specific idiotype regulation is responsible for the relatively low frequency of functional splenic B cells in aged mice, there seems to be no discernible idiotype selection within this process. That is, the diminished frequency of splenic B cells relative to newly generated bone marrow precursor B cells holds for all tested predominant clonotypes. In addition, the depression in frequency of B cells of predominant clonotypes is approximately the same as the depression in frequency for the vast majority of all B cells.

The second phenomenon that might be attributable to a naturally occurring antiidiotypic regulation has been observed in a detailed analysis of the response to dextran by sIg⁻ precursor cells as compared with splenic B cells in various murine strains using DEX coupled to Hy as the stimulating antigen. The most dramatic of these findings concerns the response to the α ,1-3 dextran determinant. As discussed above, the phenotype of the response to this determinant is highly polymorphic among murine strains. In particular, mice expressing the Igh^a heavy chain haplotype have a response to α ,1-3 dextran which typically has relatively little κ -bearing antibodies and a vigorous λ -bearing antibody response (Riblet *et al.*, 1975a). Murine strains bearing other Igh haplotypes appear lacking in the λ -bearing antibody component of this response and, therefore, only exhibit a low level κ -bearing antibody response. This phenomenon is so reproducible

that it has been used as the phenotypic marker to map genetic recombinants within the *Igh* locus (Riblet *et al.*, 1975b). The majority of λ -DEX antibodies share idiotypic determinants and it is possible to generate both private and public antiidiotype antibodies against this family of specificities. An analysis of sIg^- bone marrow precursors compared with mature splenic B cell precursors in the BALB/c mice indicated that, like the T15 clonotype in response to PC, the λ -DEX family of clonotypes was represented by a relatively high frequency of precursors both in the sIg^- pool and in the splenic B cell pool (Table IIA) (Froscher and Klinman, 1985). As mentioned above, this finding is consistent with the conclusion that environmental regulatory mechanisms do not appear to play an important role in the expression of the λ -DEX clonotype family in BALB/c mice. A similar analysis using *Igh^b* mice, however, demonstrated a strikingly different phenotype. It can be seen in Table V that when either splenic or sIg^- bone marrow precursor cells from *Igh^b* mice were transferred to syngeneic carrier-primed *Igh^b* recipients, a very low frequency of λ -DEX-responsive B cells was observed. Indeed, the majority of the response was represented by κ -bearing α ,1-3 dextran-specific B cells. However, when sIg^- precursor cells from *Igh^b* mice were transferred to *Igh* allogeneic

TABLE V
 sIg^- B CELL RESPONSES TO DEXTRAN^{a,b}

Source of cells	Hy-primed recipient	λ -DEX responses per 10^8 cells
Spleen		
BALB/c	BALB/c	18.4
	C.B20	17.7
C.B20	BALB/c	1.0
	C.B20	1.5
sIg^- bone marrow		
BALB/c	BALB/c	1.3
	C.B20	1.8
C.B20	BALB/c	1.8
	C.B20	0.2

^a From Froscher and Klinman (1985).

^b Donor mice were 2–4 months old. Recipient mice were primed with Hy in CFA and boosted with Hy in saline 4 weeks later. Four to six weeks after boosting, they received 1300 rad followed in 1–4 hours by cell transfer. Each recipient mouse received 2.5×10^7 pooled spleen cells or 5.0×10^7 sIg^- bone marrow cells intravenously. Antibody-producing clones were detected by RIA of culture fluids collected from days 9–24 of culture.

carrier-primed BALB/c recipients, the frequency of λ -DEX responses was equivalent to the frequency observed when BALB/c sIg⁻ cells were transferred to either Igh^a or Igh^b carrier-primed recipients. Splenic B cells from Igh^b mice transferred to BALB/c carrier-primed recipients continued to show a low frequency of λ -DEX-responsive cells.

Because of the Igh haplotype specificity of this phenomenon, we have tentatively concluded that the nonresponsiveness of Igh^b sIg⁻ precursor cells in syngeneic Igh^b carrier-primed recipients is due to a naturally occurring idiotype-specific down-regulation. The low frequency of α ,1-3 DEX-specific λ -bearing B cells in the spleen of Igh^b mice assessed in either syngeneic or allogeneic recipients would, therefore, be interpreted as indicating that the idiotype-specific down-regulation demonstrated in Igh^b recipients is also likely to be responsible for the elimination of λ -DEX-specific Igh^b B cells as they emerge from the immature B cell pool. This phenomenon has been judged to be the result of an idiotype-specific down-regulation as opposed to tolerance because of the finding that the responses of these cells can be observed only in Igh allogeneic recipients. In other examples of lower frequencies of responsive splenic versus sIg⁻ precursor cells, the sIg⁻ cells could be shown to be responsive in either carrier-primed Igh syngeneic or Igh allogeneic recipients. In this context, antiidiotypic down-regulation would fit the description of a special case of tolerance induction, identifiable by the combination of both a relatively low frequency of responsive mature splenic B cells as compared to sIg⁻ precursor cells and the capacity to obtain high-frequency responses from sIg⁻ cells only by crossing the Igh and presumably antiidiotypic barrier.

Given the close linkage of the λ -DEX response to Igh^a it was rather surprising that this particular polymorphism in responsiveness among murine strains appeared to be the result of a regulatory polymorphism as opposed to a structural polymorphism. Therefore, in order to confirm the finding of λ -DEX-responsive B cells in the sIg⁻ precursor cell pool of Igh^b mice, hybridomas were constructed from sIg⁻ bone marrow cells obtained from Igh^b mice using the transfer fusion protocol (Froscher and Klinman, 1985; Liu *et al.*, 1980). By this procedure, numerous hybridomas were obtained, most of which bore the λ light chain and were α ,1-3 DEX specific. In addition, it was observed that these Igh^b hybridomas expressed the IdX idiotype characteristic of BALB/c λ -DEX antibodies. Therefore, it is likely that the λ -DEX antibodies obtained from the sIg⁻ pool of Igh^b mice are closely related to the antibodies obtained in BALB/c mice. This indicates that, whatever the antiidiotypic mechanism responsible for

the down-regulation of this clonotype family in Igh^b mice, it is highly specific, discriminating among IdX-positive precursor cells obtained from BALB/c mice, as compared to IdX-positive cells from C.B20 mice. In this context, it is of interest that previous findings with antigen-induced antiidiotypic regulation in the response to PR8-HA had identified a haplotype-specific regulatory phenomenon that seemed dependent, at least in part, on the allotype of the responding B cells implying that recognition might have been more allotype than idiotypic specific (Raychaudhuri and Cancro, 1985). However, a considerable amount of further analyses will be required to determine the exact specificity of naturally occurring antiidiotypic regulatory phenomena with respect to which determinants are dominant in the recognition phenotype.

Since dextran is a ubiquitous environmental antigen, it is perhaps not surprising that spontaneously occurring antiidiotypic regulatory mechanisms are abundant in this particular response. To determine if this might be the case, a parallel analysis was carried out for the κ -bearing antibody responses to both $\alpha,1-3$ and $\alpha,1-6$ dextran determinants (Froscher and Klinman, 1988). In this case, the findings were far more complex and applied not only to Igh^b B cells but also to those obtained from Igh^a donors. In the response of κ -bearing B cells to the $\alpha,1-6$ dextran determinant, it was observed that not only was the frequency of Igh^b sIg⁻ bone marrow precursor cells higher than would have been anticipated from the frequency of splenic precursor cells, but also the frequency of Igh^a sIg⁻ precursor cells was higher. In this case, both populations of sIg⁻ precursor cells could only be revealed by stimulation in irradiated, carrier-primed Igh allogeneic recipients. Thus, in this situation, it would appear that both strains of mice exhibit an ambient antiidiotypic suppression, although not as profound as that found for the λ -DEX response in Igh^b mice.

Finally, in the response of BALB/c precursor cells with the potential to produce κ -bearing $\alpha,1-3$ specific anti-dextran antibodies an even more complex situation occurs (Froscher and Klinman, 1988). In this case, the frequency of splenic B cells was quite low when transferred either to BALB/c or C.B20 carrier-primed recipients. Similar to the Igh^b response to $\alpha,1-3$ dextran the sIg⁻ bone marrow precursor cells of BALB/c mice showed an equivalently low frequency when transferred to syngeneic BALB/c carrier-primed recipients. When BALB/c sIg⁻ bone marrow precursor cells were transferred to C.B20 mice, there was a 3-fold increase in the frequency of responsive cells, thus indicating the existence in BALB/c mice of an ambient antiidiotypic down-regulation of $\kappa,1-3$ dextran-responsive B cells. Surprisingly, when either BALB/c spleen cells or BALB/c sIg⁻

bone marrow cells were transferred to Hy-primed irradiated B10.D2 mice, which differ from BALB/c not only in the Igh locus but also in background loci, an even higher frequency of responsive cells was obtained. In fact, the frequency of sIg⁻ bone marrow precursor cells responsive to α ,1-3 dextran that bear the κ light chain was approximately the same as the frequency of λ -bearing α ,1-3 dextran-responsive B cells. Since no mechanism is known that would map to genes outside of the Igh locus, the mechanism for the suppression of sIg⁻ α ,1-3 dextran-specific bone marrow and spleen cells is not as yet understood.

In summary the current status of our understanding of the role of antiidiotypic regulatory events, although less concrete, is much the same as our current understanding of the role of tolerance to self-antigens in shaping the primary B cell repertoire. In both instances, changes in the primary B cell repertoire can be induced by experimental manipulation, and, in this sense, a physiologically relevant mechanism is likely to be available. In addition, manipulations of the immune system, particularly in neonates, which should lead to changes in the idiotypic regulatory environment of individuals, can have significant and long-lasting effects on the expression of the primary B cell repertoire. In contrast to tolerance induction, however, relatively few examples exist wherein disparities in repertoire between sIg⁻ and splenic precursor cells can be attributed to idiotypic regulatory phenomenon. It is likely that this is due to a lack of information on a sufficiently wide range of antigen responses as well as inadequacies in the parameters used to attribute differences in the repertoire expressed in sIg⁻ versus mature precursor cells to an antiidiotypic recognition process. Thus, it is conceivable that some differences currently attributed to tolerance could better be attributed to an antiidiotypic regulation, but perhaps one that is not Igh haplotype specific. In addition, the identification of non-Igh-linked regulatory phenomena, particularly in the response to determinants on dextran, could imply that idiotypic regulatory events may link to genetic loci whose effects may be indirect, as might be expected if various genetic loci encoded for the expression of self-antigens whose presence may cause a skewing in the idiotypic network.

VI. The Relationship of B Cell Repertoire Expression and B Cell Subpopulations

Driven by advances in cell separation technology as well as the increasing availability of monoclonal antibody reagents for the discrimination of cell surface markers, an emerging recognition that B

cells exist as definable cell subpopulations is beginning to impact on our understanding of B cell repertoire expression. Thus, a decade ago B cells were generally segregated according to responsiveness to various antigen forms such as T-dependent versus T-independent Type 1 or Type 2 antigen-responsive B cells. In recent years, such delineations seem to be more apparent than real and as stimulatory regimens have improved, B cells stimulatable by T-independent versus T-dependent antigens seem to be overlapping sets or representative of different stages in the development of the same cells. In any case, as detailed above, studies carried out to assess differences in repertoire expression among so called T-dependent and T-independent B cells have revealed no substantive differences.

The T-independent Types 1 and 2 subpopulations of B cells were delineated by virtue of disparities in the T-independent responsiveness of normal mice and CBA/N immunologically defective mice in that B cells from the latter could respond only to a subset of T-independent antigens (Scher *et al.*, 1975). Although, as detailed above, CBA/N mice may lack certain elements of the B cell repertoire and their responsiveness to various antigenic forms differs from that of conventional mice, the immunologic defect in CBA/N mice appears to be pleiotropic and it is therefore possible that the B cells of CBA/N mice are not truly representative of any B cell subset in conventional mice (Wortis *et al.*, 1982; Sprent and Bruce, 1984). A second type of segregation of B cells derived by studies of mice bearing the CBA/N defect is the designation of B cells as being either LyB5⁺ or LyB5⁻ wherein the CBA/N mice appear to lack the former population (Huber, 1982; Ahmed *et al.*, 1977). Although considerable effort has been extended toward obtaining hybridoma or polyclonal antisera that could clearly delineate these two subpopulations, to date these efforts have shown only limited success. Thus, it remains a possibility that this marker delineates stages of development of the same B cells in normal mice or possibly stages of activation of those cells. Nonetheless, in the absence of reagents capable of clearly delineating B cells into LyB5⁺ versus LyB5⁻ subpopulations, only preliminary repertoire analyses of these cell populations have thus far been accomplished (Kenny *et al.*, 1983).

As suggested above, the emergence of reagents that can delineate B cells on the basis of cell surface markers and permit their isolation, particularly by fluorescence-activated cell sorting (FACS), has enabled the identification of subpopulations of B cells which appear to be representative of developmentally independent lineages, and which differ significantly in function and potentially in repertoire expression. The remainder of this discussion will therefore focus on a

presentation of the status of B cell subpopulations thus defined and projections as to the potential impact investigations of these cell populations will have on our understanding of repertoire expression.

A. REPERTOIRE EXPRESSION IN Ly1 B CELLS

The prototype of the newly identified B cell subpopulations is a subpopulation that was defined primarily through the use of monoclonal antibodies that recognize the Ly1 marker generally associated with T cells, and the subsequent separation of B cells bearing low levels of this marker from those that bear no detectable levels of this marker using the fluorescence-activated cell sorter (Hayakawa *et al.*, 1983, 1984, 1985, Hardy *et al.*, 1984, 1986; Braun, 1983; Davidson *et al.*, 1984; Lanier *et al.*, 1981; Manohar *et al.*, 1982; Stall *et al.*, 1981). Extensive studies by several laboratories have now identified a multiplicity of interesting characteristics of the Ly1 B cell subpopulation. Recently, many of these findings have been extensively reviewed (Hardy and Hayakawa, 1986; Herzenberg *et al.*, 1986; Braun *et al.*, 1986; Haughton *et al.*, 1986) and, therefore, will not be a subject of this review. Ly1 B cells have been identified by the expression of a low level of Ly1 and Mac 1 on their surface, as well as by disparate representation of other normal B cell markers such as IgD. This population appears to inhabit primarily the peritoneal cavity of certain inbred murine strains and is found only in low frequency in the spleen and rarely in the bone marrow. The overall representation of Ly1 B cells is probably less than 2% of all B cells in the adults of most murine strains. Whereas in some murine strains, particularly those susceptible to autoimmune disease (Stall *et al.*, 1981; Hayakawa *et al.*, 1983), the proportion can be considerably higher, in other strains, such as mice with the CBA/N defect, Ly1 B cells appear to be absent. In mice that do express Ly1 B cells the proportion of B cells of this category is substantially higher in neonatal spleens and it has been postulated that the peripheral B cell population has been seeded by these neonatal splenic B cell progenitors (Herzenberg *et al.*, 1986). Unlike the majority of B cells this lineage is self-renewing within the peritoneal cavity and does not appear to emanate from the bone marrow of adult mice.

The high frequency of Ly1 B cells in autoimmune strains of mice has led to the proposal that Ly1 B cells participate in various autoimmune states. Studies on mice repopulated with Ly1 B cells have shown poor responses to T-dependent antigenic stimulation and a paucity of Ly1 B cell responses to standard haptenic determinants such as NP (Förster and Rajewsky, 1987). On the other hand, these

cells appear to give rise to substantial amounts of anti-PC antibodies and anti-dextran antibodies via T cell-independent stimulation. Since the anti-PC and anti-dextran antibodies derived from these cells appear to express the same predominant clonotypes previously observed for bone marrow and splenic B cells, it is likely that at least some aspects of repertoire expression in Ly1 B cells and normal primary B cells overlap. Indeed, it may be possible that some aspects of the immunologic defect in CBA/N mice could be a reflection of the paucity of Ly1 B cells in these mice. For example, the low level of PC-responsive B cells in CBA/N mice may be due in part to their missing Ly1 B cell compartment.

B. REPERTOIRE EXPRESSION IN THE NEONATAL B CELL SUBPOPULATION

The finding that Ly1 B cells represent a sizable proportion of B cells in the neonatal spleen has spurred renewed interest in analyses of the neonatal splenic B cell population. It has been suggested from analyses of antigen responsiveness (Neil and Klinman, 1987) and by analyses of cell surface markers (Kincade *et al.*, 1981) that B cells present in the fetal liver and early after birth in the spleen of neonatal mice may represent a B cell lineage that is distinct from that which predominates the adult primary B cell pool (Kincade, 1984; Jyonoughi *et al.*, 1981; Kincade *et al.*, 1981). Kincade and co-workers demonstrated that both immature and mature neonatal B cells expressed QA antigens and Lyb2 to a greater level than their counterparts in adult bone marrow. In addition, the determinant recognized by the 14.8 monoclonal antibody was differentially expressed on immature B cells in the developing fetus as compared to the adult bone marrow.

In studies described above, important functional differences were noted between B cells emerging in late fetal and early neonatal development compared with those developing from the bone marrow of adult mice. The major difference concerns the timing of sIg expression and the developmental stages during which B cells are tolerance susceptible (Klinman and Press, 1975b; Metcalf and Klinman, 1976, 1977). The developing neonatal B cell subpopulation appears to be tolerance susceptible for a considerable time after sIg expression, whereas B cells isolated from the adult bone marrow are tolerance susceptible in the fragment culture system only if isolated as sIg⁻ cells (Riley and Klinman, 1986; Klinman *et al.*, 1981). In addition, it has been reported by several laboratories that B cells in the adult bone marrow do not divide extensively after light chain rearrangement and sIg expression (Osmond, 1986; Osmond and Nossal,

1974; Landreth *et al.*, 1981). On the other hand, it appears from studies on clonal development in the BALB/c neonate that clonal doubling of neonatal B cells continues for several days after immunoglobulin expression (Sigal and Klinman, 1978; Klinman and Press, 1975b).

These biological differences have important consequences for consideration of B cell repertoire expression. B cell clones developing with (1) the kinetics, (2) the sIg expression, and (3) the tolerance susceptibility as those applicable to adult bone marrow precursors would be tolerance susceptible only after clonal expansion. In this case, the purging of self-reactive cells by self-antigens would seem wasteful from the standpoint of necessitating the elimination of B cell clones after all clonal expansion had occurred. However, since cell division and somatic mutation in these cells do not occur subsequent to the developmental stage wherein cells are tolerance susceptible, there would be little danger of new, anti-self, specificities entering the primary B cell pool at a time when the cells were resistant to tolerance induction. Such a mechanism would seem ideal to ensure the integrity of the expression, as expanded clones, of a diverse genetically (evolutionarily) dictated primary B cell repertoire.

Since neonatal B cells express their sIg while they continue to divide and exhibit tolerance susceptibility, it would appear that potentially self-reactive clones of neonatal B cells could be inactivated even at a very early stage in their clonal expansion. That these cells remain tolerance susceptible makes the generation of novel specificities which could be anti-self via the somatic mutation mechanism somewhat more acceptable since such B cells could be inactivated as they arise. The overall consequences of the pattern by which neonatal B cells develop would be consistent with a very limited B cell space in neonatal mice wherein clonal integrity might be less important than the availability of as great a variety as possible of specificities emanating from a limited and sequentially expressed variable region gene pool. In adults, the variable region gene pool accessible to the cells developing in the bone marrow presumably represent all functional variable region genes and, thus, considerable repertoire diversity would be available to the adult primary B cell pool in the absence of somatic mutational events.

Because so few neonatal antibodies have been sequenced, insufficient sequence information is available from neonatal hybridomas to conclude that somatic mutations are more likely to accumulate in the neonatal versus the adult primary B cell repertoire. However, it should be noted that one variable region somatic mutant has already

been identified in a neonatal IgM hybridoma (Riley *et al.*, 1986), a finding that is exceedingly rare in adult IgM hybridomas, particularly those obtained from primary B cell responses (see Section IV). It is still possible that this somatic mutant might have been the product of division after antigenic stimulation during the course of generating the hybridoma.

Given the above findings, it must be considered likely, but speculative, that the neonatal B cell population (or populations) may constitute a different B cell lineage than that responsible for the bulk of adult primary B cells. As detailed extensively in Section III, there are substantial differences in the neonatal B cell repertoire as compared to the adult repertoire. Most likely, these differences relate primarily to the fact that the neonatal repertoire displays the consequences of the sequential readout of available variable region gene elements. Nonetheless, it is uncertain whether, even if given unlimited time and space to develop, this B cell subpopulation would show the same repertoire diversity as that observed in cells emanating from the adult bone marrow. Conversely, it is not yet clear that cells developing within the bone marrow pass through precisely the same sequential acquisition of V region specificities as has been observed for fetal liver and neonatal splenic B cells. This issue is greatly compounded by the finding that a large proportion of neonatal splenic B cells may be of the Ly1 B cell lineage. The contribution of these cells to the expressed repertoire in neonates is even more uncertain.

C. REPERTOIRE EXPRESSION IN THE ADULT PRIMARY B CELL SUBPOPULATION

Since the vast majority of studies detailed in Sections II through V of this review concern analyses conducted on the primary B cell repertoire, repertoire expression in this population will not be reviewed here. However, it should be mentioned that within the population of cells categorized as primary B cells are numerous subpopulations that are, as yet, too poorly defined to permit comparative analysis. For example within the primary B cell population are both cells that bear the LyB5 marker as well as cells that do not (Kenny *et al.*, 1983; Huber, 1982; Ahmed *et al.*, 1977). Aside from the aforementioned disparities in responses to environmental antigens such as PC in the sIg⁻ versus sIg⁺ B cell pools of CBA/N mice (which appear to have only LyB5⁻ B cells) considerable repertoire overlap appears to exist between LyB5⁺ versus LyB5⁻ populations (Busto *et al.*, 1987; Woodland and Huber, 1984; Dzierzak *et al.*, 1980; Hiernaux *et al.*, 1983). In addition, a variety of studies have delineated a

subpopulation, comprising 5–20% of primary B cells, that has a relatively long half-life (weeks–months) (Sprent and Basten, 1973) as compared with the remainder of B cells whose half-life appears to be between 1 and 7 days (Strober, 1972; Elson *et al.*, 1976). In this same context, recently, several investigators have observed that a minority of primary splenic B cells are in cycle and the possibility exists that this cell population replenishes itself by remaining in cycle (Holmberg *et al.*, 1986; Coutinho *et al.*, 1987; Holmberg, 1987). It has been speculated that this population of cells expresses a limited subset of the B cell repertoire particularly enriched in the V_h 7183 and V_h Q52 families, and that it is highly subject to idiotypic network events. As these studies are just emerging, extensive information concerning the repertoire as expressed in these cells is not as yet available. However, it is now assumed by these investigators that many of the hybridomas derived from nonstimulated splenic B cells, including many of those that show a high level of anti-self and anti-idiotypic recognition, are the product of this cell subpopulation. It should also be noted that the relationship of this cell subpopulation to the Ly1 B cell subpopulation has not as yet been defined.

D. REPERTOIRE EXPRESSION IN THE SECONDARY B CELL LINEAGE

In contrast to the primary B cell repertoire which appears primarily determined by the subcellular molecular events that lead to V gene segment rearrangement and expression with little impact of environmental influences in the generation of specificities, the secondary B cell repertoire appears to be highly dependent on environmental influences both for its generation and its regulation. Since antigenic stimulation and continued antigenic selection is necessary for the establishment of secondary B cells specific for any given antigen, it would be anticipated that the secondary repertoire among individual mice within an inbred murine strain could vary considerably since the microenvironment, antigen processing, antigen presentation, helper T cell availability, and available elements of the B cell repertoire could vary greatly from individual to individual. An interesting example of the consequences of this can be found in the analysis of the response of DBA/2 mice to tobacco mosaic virus protein (TMVP) (Morrow *et al.*, 1984; Benjamini *et al.*, 1987). Murine strains differ with respect to their capacity to recognize regions of the TMVP and these differences appear to be B cell rather than T cell dependent. However, even within the response of DBA/2 mice, which recognize the terminal decapeptide of TMVP, the fine specificity pattern differs considerably from mouse to mouse. In addition, the recognition

pattern of any given mouse remains relatively stable throughout the course of immunization. Similarly, as will be detailed below, in the response to PR8-HA each individual mouse displays secondary responses that are comprised of the descendants of relatively few progenitors and even within a single strain, the progenitors from which predominant specificities arise differ markedly (Clarke *et al.*, 1985; McKean *et al.*, 1984).

Although it is likely that the secondary repertoire is constrained by the inheritance of the same variable region gene segments and the same sets of rearrangements as primary B cells, as discussed in Section II, secondary specificities appear to be generated largely through the process of accumulation of somatic mutations. Since the degree of diversity possible as a consequence of the accumulation of somatic mutations is likely to be enormous, comparisons of individuals within a strain and comparisons of specificities between strains are unlikely to provide substantial overlaps. Thus, assessing the secondary B cell repertoire in detail is likely to be a preclusively difficult task. In this situation it would seem that the process rather than its specific results is the important aspect of repertoire expression to understand.

Although still in their nascent stages, studies of the contribution of the somatic mutation process to the generation of the secondary B cell repertoire have already begun to establish some of the general rules of this process (see Section II). These include the fact that somatic mutations not only accumulate rapidly during the course of secondary B cell generation, but also that mutations which affect amino acid changes, particularly in the hypervariable regions appear to be favored. These findings have been interpreted as indicating that continued selection upon the products of somatic mutation plays an important role in the ultimate establishment of the secondary B cell repertoire. This is consistent with the finding that, in general, the affinity of somatically mutated antibodies is higher than the affinity of their germline antecedents (Malipiero *et al.*, 1987; Manser *et al.*, 1987; Allen *et al.*, 1987; Berek and Milstein, 1987) and the affinity of secondary antibodies is significantly higher than the affinity of primary antibodies (Eisen and Siskind, 1964; Klinman, 1972). An additional rule, that seems to be generally applicable, is that the generation of secondary B cells is greatly facilitated by the maximization of T cell-B cell collaborative interactions. However, although there are general rules which appear to underlie the generation of secondary B cells, as will be discussed in detail below, each individual response appears to have its own peculiar set of characteristics.

One aspect of the secondary response, wherein the broad spectrum of potential phenotypes can be observed, is in a comparison between determinant recognition and clonotype representation in the primary response to a given antigen versus that observed in the secondary response. In certain instances, it has been observed that the clonotypes or clonotype families which dominate the primary response also dominate the secondary response. Thus, in response to PC, BALB/c mice express predominantly the T15 clonotype as well as a minority of specificities that utilize the V_h S107 but which do not express the T15 idiotype presumably because of the utilization of one of two other light chains, the V_λ 167 or V_λ 603 (Malipiero *et al.*, 1987; Perlmutter *et al.*, 1984; Gearhart *et al.*, 1981; Crews *et al.*, 1981). To date, all sequences observed which include somatic mutations, and are therefore presumably the product of secondary B cells, have been derivatives of the very same heavy chain and set of light chains (Malipiero *et al.*, 1987; Perlmutter *et al.*, 1984; Gearhart *et al.*, 1981; Crews *et al.*, 1981; Selsing and Storb, 1981). Similarly, in the response of BALB/c mice to α ,1-3 dextran, particularly with respect to antibodies that bear the λ light chain, antibodies that bear somatic mutations appear to generally fall within the same V_h J558 gene segment that predominates in the primary response (Clevinger *et al.*, 1981). The response of Igh^b mice to NP has some similarity to these responses in that the λ NP^b clonotype family dominates the primary response and is also represented in secondary NP-responsive B cells, including those that have accumulated variable region somatic mutations (Boersch-Supan *et al.*, 1985; Allen *et al.*, 1987). In the case of this latter response, however, secondary responses are ultimately dominated by other clonotype families, particularly those that are homoclitic for NP and bear κ light chains (Imanishi-Kari and Makela, 1973).

At an intermediate position between total overlap between the variable region gene segments used by primary and secondary antibodies and total nonoverlap lies a set of responses wherein secondary responses can be found to include B cells expressing variable regions that are present or dominate the primary response, but that these constitute a small minority of the secondary response. This is true in the example of the response of PR8-HA as described in Section II (Clarke *et al.*, 1985; McKean *et al.*, 1984) and is also true of the response to ARS in A strain mice. In the latter case, B cells which bear the CRI are relatively poorly represented in the primary repertoire (Sigal, 1982), but are almost always greatly expanded and usually dominate the secondary ARS-specific B cell repertoire (Tung and Nisonoff, 1975). Other clonotypes using different variable region

genes which can often be identified in the primary repertoire, and sometimes have higher affinity than the CRI as primary antibodies, are rarely found in the secondary B cell pool (Manser *et al.*, 1985, 1987). Based on these findings, it has been proposed that accumulation of somatic mutations in the variable region gene elements that comprise the CRI may lead to significant enhancement in antibody affinity (Manser *et al.*, 1987). Therefore, while CRI antibodies may not be prominent in the primary response, given their ability to accumulate somatic mutations and increase their affinity dramatically, the CRI antibodies ultimately dominate the secondary B cell pool, whereas the other available specificities may not have this capability. Some of these same aspects might also be true for the response to phOX in BALB/c mice (Berek and Milstein, 1987). In this case, the primary repertoire is quite limited with the V_h gene segment dominating the response. Although the secondary repertoire can be found to include this V_h , in general it is dominated by any of a variety of other variable region gene complexes, most of which are not observed in primary responses.

There are several responses which might ultimately prove to be even more dramatic than the phOX response in displaying repertoire disparities between the primary response and the secondary response. There are now several instances in which antibodies can be found in secondary response that recognize determinants that apparently are not recognized by the primary B cell repertoire. For example, as discussed in great detail in Sections IV and V, responses of B cells bearing the T15 variable region are not found in the mature primary B cell repertoire of (CBA/N \times BALB/c) F_1 male immunologically defective mice (Klinman and Stone, 1983; Quintans, 1977; Mond *et al.*, 1977). It should be remembered, however, that sIg⁻ B cells in these mice do express that specificity (Klinman and Stone, 1983). Nonetheless, upon extended immunization, secondary responses expressing this variable region gene complex have been identified (Kenny *et al.*, 1983; Clough *et al.*, 1981).

Recent findings have shown that while the primary B cell repertoire can recognize certain of the available antigenic determinants on various complex antigens, only secondary B cells recognize a majority of the available determinants. This appears to be true for the response of mice to horse cytochrome *c* (Jemmerson, 1987b) and of BALB/c mice to the *Salmonella typhimurium* (Duran and Metcalf, 1987). There are several possible explanations for such disparities in the secondary versus the primary B cell repertoires. One possible explanation is that the B cells that give rise to secondary B cells actually

express variable region gene combinations that are not present in primary B cells. An alternative explanation is that the mechanism responsible for the selective stimulation of precursors to secondary B cells (see below) and the continued selection (T cell–B cell collaboration) of newly generated secondary B cells could lead to a vast expansion of selected clonotypes that could be extremely rare in the primary repertoire. Finally, it is possible that continued immunization could permit the elicitation of specificities that are generated within the pre-B cell pool but never enter the mature primary B cell repertoire because of tolerance. It is now known that excessive amounts of T cell help can permit the stimulation of immature B cells at a time in their development when antigen contact would normally induce tolerance (Metcalf and Klinman, 1976). Therefore, it is possible that upon hyperimmunization, sufficient helper T cells enter the bone marrow and are present in the milieu of newly developing B cells to permit otherwise tolerogenic signals to be stimulatory. Such a mechanism would be a likely explanation for the finding of κ homoclitic antibody bearing secondary B cells in Igh^b mice (Riley and Klinman, 1985). As discussed in Section III (see Fig. 3), high-affinity κ homoclitic NP-responsive B cells are present in the sIg^- pool of Igh^b mice but are eliminated during B cell maturation. The fact that B cells of this phenotype are abundant in the secondary NP-responsive B cell pool may indicate that extensive immunization permits the bypassing of the tolerance mechanism applicable to these cells.

This explanation could also hold for the expression of $T15^+$ secondary B cells in $(CBA/N \times BALB/c)F_1$ mice (Klinman and Stone, 1983). As discussed above, $T15^+$ B cells in $(CBA/N \times BALB/c)F_1$ male defective mice are found in high frequency in the sIg^- pool but apparently never mature into the primary B cell repertoire. Since primary B cells of CBA/N defective mice seem excessively susceptible to tolerance induction, even after migrating to the spleen (Metcalf *et al.*, 1980; McKearn and Quintans, 1980), this finding has been interpreted as evidence for tolerance induction during the maturation of PC-specific B cells in mice bearing the CBA/N immunologic defect (Klinman and Stone, 1983). Such tolerance induction may be bypassed by extensive immunization, particularly with PC complexed to highly immunogenic carriers and this could possibly explain the existence of $T15$ memory B cells in CBA/N defective mice (Clough *et al.*, 1981). A similar explanation could also pertain to secondary B cells that recognize determinants on cytochrome *c* and *Salmonella typhimurium* which are not normally present in the primary B cell pool (Jemmerson, 1987b; Duran and Metcalf, 1987).

A final possible explanation for the presence of variable region gene complexes within the secondary B cell pool that apparently are not present within the primary B cell pool could be the result of potential differences in the affinity threshold for stimulation of progenitors to secondary B cells as compared to primary B cells. If lower affinity interactions are adequate for the stimulation of progenitors to secondary B cells than is required for the stimulation of primary B cells, it would be possible that the same sIg V region could permit triggering of secondary B cell progenitors but not primary B cells. Once triggered, somatic mutations accumulated in that V region would be selected upon with continued immunization yielding high-affinity secondary B cells. Similarly, it is possible that secondary B cells generated in response to previous contact with cross-reactive antigens could be recruited into a given response and affinities improved by somatic mutation. Such explanations could be applicable to any of the aforementioned situations. In any case, the affinity threshold for triggering secondary versus primary B cells should now be approachable experimentally (see below) so that a role for any of these mechanisms can be assessed.

The origins of secondary B cells have been a controversial subject for a number of years. In general, it has been assumed that secondary B cells are generated from the same primary B cells that give rise to antibody-forming cell clones either as an alternative final differentiation step or as the product of an unequal division during the generation of a clonal progeny from a stimulated primary B cell (Byers and Sercarz, 1968; Williamson *et al.*, 1976). Alternatively, it is possible that secondary B cells are the product of a separate progenitor lineage from those cells that give rise to antibody-forming cells after primary antigenic stimulation (Klinman *et al.*, 1981). In addition to some of the aforementioned differences in repertoire expression, findings from various types of analyses have supported the latter postulate. The kinetics of memory cell generation differ somewhat from the kinetics of antibody-forming cell generation after primary antigenic stimulation (Williamson *et al.*, 1976; Klinman, 1976). Secondary B cell generation appears to require T cell-dependent stimulation (Braley-Mullen, 1977; Linton and Klinman, 1987; Miller and Sprent, 1971), whereas most primary cells can respond to either T cell-dependent or -independent stimulation (Cebra *et al.*, 1983). Finally, the biological properties of secondary B cells are significantly different in many respects from those of primary B cells. This would necessitate a multitude of distinct differentiative processes if primary B cells were to evolve into secondary B cells. These differences include longevity

(Strober, 1972; Elson *et al.*, 1976), cell recirculation patterns (Strober, 1972), cell surface isotype expression (Black *et al.*, 1978; Teale *et al.*, 1981) (although memory cells like primary cells often express surface IgM, they rarely express surface IgD and often express other isotypes), as well as various parameters of their stimulation (Klinman, 1972; Pierce and Klinman, 1976, 1977; Klinman *et al.*, 1973; Klinman and Press, 1975c).

An additional disparity between primary and secondary B cells is the expression of the cell surface antigen recognized by the J11D monoclonal antibody (Bruce *et al.*, 1981; Symington and Hakamori, 1984). Primary B cells express high levels of this antigen, whereas secondary B cells generally express low or undetectable levels of this antigen. Recently, experiments in this laboratory have indicated that a population of B cells preexists in the spleen of naive mice, which also expresses low levels of the J11D marker (Linton and Klinman, 1986, 1988). As the result of an extensive series of experiments, it now appears likely that the majority of progenitors to secondary B cells within the spleen of nonimmune mice are included within the population of J11D low or negative B cells, whereas the majority of primary B cells that express high levels of J11D respond only primarily to antigenic stimulation by giving rise to antigen-forming cells and do not give rise to secondary B cells (Linton and Klinman, 1988). These findings would imply that the secondary B cell subpopulation is actually the product of a B cell lineage separate from that which is responsible for most primary antibody responses. It should be noted, however, that some of the B cells that express low levels of J11D and give rise to secondary B cells can, upon occasion, give rise to primary antibody-forming cells as well (Linton and Klinman, 1988).

Progenitors to secondary B cells represent between 5 and 15% of splenic B cells and lower proportions of both bone marrow and lymph node B cells; therefore, this population is clearly a minority of all B cells (Linton and Klinman, 1988). The isolation of this population from the majority of B cells has presented the opportunity to fully characterize the cells which are likely to be responsible for the bulk of secondary B cell responses. In the future, the availability of these cells should permit a detailed analysis of the parameters of secondary B cell generation, as well as an analysis of repertoire expression within the secondary B cell lineage. As would be expected from the aforementioned results showing overlap of primary and secondary repertoire in some antigen systems, our findings to date indicate that, at least for the response to NP in Igh^b mice and PC in BALB/c mice, secondary B cell progenitors can express the same variable region

gene complexes as do precursors to primary antibody-forming cells (Gilmore *et al.*, 1987). However, it is clear that far more extensive analyses must be carried out on secondary B cell progenitors in order to resolve the issues concerning repertoire disparities in secondary versus primary B cell responses detailed above.

Several findings concerning the parameters of antigen responsiveness of progenitors to secondary B cells have already been highly informative with respect to the processes responsible for the generation of the secondary B cell repertoire. First, it appears that both the stimulation of splenic progenitors to secondary B cells and the stimulation of their secondary B cell progeny require the presence of carrier-specific T cells (Linton and Klinman, 1988). Second, it appears that newly generated secondary B cells are responsive to antigenic restimulation at least as early as 5 days after the stimulation of their progenitors (Linton and Klinman, 1988). The finding that the generation of antigen-responsive secondary B cells occurs so early after primary stimulation of their splenic progenitors implies that in situations where antigen may persist after primary immunization, much of the later part of a primary response may include responses of cells of the secondary B cell lineage as well. Thus, it would be anticipated that responses to different antigens could differ in their proportionate representation of primary versus secondary precursor cells within the response, even after a single antigenic stimulation. For example, antigens which are rapidly eliminated from the system or antigens which stimulate T cells poorly and thus engender poor T cell-B cell collaboration, would favor the stimulation of primary B cells as opposed to the progenitors of secondary B cells or secondary B cells. Alternatively, responses to antigens which persist for a long time and engender copious amounts of T cell help might be only evanescently characterized by the antibody products of primary B cells and may be dominated ultimately by the antibody products of cells of the secondary B cell lineage even after a single immunization.

Examination of this balance with respect to given antigenic stimuli will help unravel the variable phenomenology associated with immune responses to different antigens. In addition, to the extent that secondary B cells participate in the latter part of primary responses to certain antigens, the finding of somatic mutations toward the latter stages of primary responses (Manser *et al.*, 1987; Berek and Milstein, 1987; Gearhart, 1987) could be attributed to the participation of newly generated and stimulated secondary B cells within these responses. Finally, it appears that while the progeny of stimulated primary B cells do display multiple isotype production (Klinman, 1972; Teale *et*

al., 1981), isotype switching appears to be a major and integral part of the generation and responsiveness of secondary B cells (Linton and Klinman, 1987; Teale *et al.*, 1981). Indeed, most responses of secondary B cell clones do not include any IgM, and those that do include IgM as a minority component (Linton and Klinman, 1988). Since somatic mutation appears to be primarily associated with the generation of secondary B cells, this finding could account for the disproportionately high representation of somatic mutations in isotypes other than IgM.

In the discussion above, concerning the development of neonatal versus adult bone marrow B cells and the relative stages during which they are susceptible to tolerance induction, an interesting symmetry was described. Although these two populations have been shown to differ considerably with respect to their biological properties and division potential at various stages of development, both had in common a developmental stage-dependent tolerance susceptibility. In the case of B cells developing within the bone marrow, the stage of tolerance susceptibility follows clonal expansion and V gene segment rearrangement so that the newly developed B cell pool can be purged of expressed specificities that might be high affinity anti-self. Importantly, cell division does not normally occur after this stage. Since cell division would presumably be necessary for the accumulation of somatic mutations, new specificities would presumably not enter the primary B cell pool after the tolerance susceptible stage of developing B cell clones and consequently after the repertoire had been purged of potentially harmful specificities.

Although developing neonates follow a different set of rules in that B cells continue to divide after sIg expression, these cells remain tolerance susceptible throughout this divisional phase. Thus, in the case of neonatal B cells, as well, it would appear that the repertoire would have been purged of harmful specificities prior to the expression of mature nontolerizable cells. Thus, in both instances, tolerance susceptibility appears to correlate with those stages in cell development wherein novel specificities (potentially anti-self) are generated and once maturation proceeds beyond the tolerance susceptible phase, no new specificities can be generated.

If this is true for developing neonatal and bone marrow B cell populations, should it not also be true for newly developing secondary precursor cells which also represent a population which is both dividing and diversifying at a very rapid rate? Given the availability of isolated secondary B cell progenitors in the form of J11D low or negative splenic B cells, it has been possible to test this hypothesis by

assessing tolerance susceptibility during the course of *in vitro* generation of secondary B cells in fragment cultures (Gilmore *et al.*, 1987). The findings from these studies indicate that although neither the splenic progenitor cells to secondary B cells nor mature secondary B cells are tolerance susceptible, newly generated secondary B cells appear to be exquisitely tolerance susceptible. Indeed, preliminary experiments indicated that these cells can be more readily tolerized, particularly by cross-reacting antigens, than appears to be the case for immature bone marrow precursor cells. Importantly, however, as with developing immature neonatal or bone marrow precursors cells, the tolerance induction of newly generated secondary B cells can be bypassed by the presence of helper T cells which recognize the antigen in culture. Thus, all three developing B cell populations, neonatal, adult bone marrow, and newly generating secondary B cells, have in common a tolerance susceptible developmental stage which accompanies or follows all stages of cell development where repertoire diversification is taking place. Additionally, in all three instances, tolerance induction can be bypassed by the presence of antigen-specific helper T cells.

What then are the consequences of the findings that (1) secondary B cells emanate from a separate cell subpopulation than the one responsible for primary antibody responses, and (2) following the stimulation of secondary B cell progenitors, during the course of secondary B cell proliferation, the newly generated secondary B cells display tolerance susceptibility. The finding that newly generated secondary B cells pass through a tolerance susceptible phase makes the process of somatic mutation considerably more palatable, particularly in lessening the danger from newly generated potential anti-self specificities that could arise via the accumulation of somatic mutations. Indeed, since it would be anticipated that the stimulatory antigen concentration would progressively diminish during the course of a secondary response, it is likely that only secondary B cells with the very highest affinity for antigen would be able to capture antigen and T cell help and other progeny of stimulated progenitor cells would be selectively disfavored. Furthermore, it is implicit in the finding that the affinity for tolerance induction of newly generating bone marrow B cells is higher than required for their stimulation as mature B cells (see Section V), that a significant proportion of mature B cells would have some self-reactivity. Thus, it is likely that in the course of secondary B cell generation, the newly acquired tolerance susceptibility of the progeny of stimulated secondary progenitor cells could enable the elimination of many cells expressing

low-affinity anti-self specificities that might have escaped tolerance induction during their initial development. Thus, in the course of the generation of secondary B cells it might be anticipated that a selective advantage would accrue to B cells expressing variable regions that included somatic mutations that yielded a higher affinity for the stimulating antigen whereas the progeny of progenitor cells whose sIg bound a self-constituent would be selectively disfavored. Thus, the selective process could be a two-edged sword selecting for high-affinity antigen reactivity and against low-affinity anti-self reactivity.

A considerable amount of the above discussion has been directed toward differences in the primary and secondary B cell repertoire. Several examples were presented wherein the secondary B cell repertoire appeared to include specificities not generally observed in primary responses and potential mechanisms for this phenomenon were described. Presumably because a sufficiently extensive analysis of the secondary B cell repertoire has not yet been carried out, relatively little information is available that indicates the inverse, that specificities present in the primary B cell repertoire might not be present in the secondary B cell repertoire. Even if this were the case, however, given the extraordinary tolerance susceptibility of newly generating secondary B cells and the need for extensive T cell help in the generation of secondary B cells, it might be anticipated that many clonotypes may not be found as secondary B cells even if these clonotypes were present in the pool of progenitors to secondary B cells. Thus, for example, secondary B cells would be rarely observed for clonotypes specific for determinants generally presented as T cell-independent antigens.

An interesting example of specificities that are present in the primary repertoire but sparse in the secondary repertoire are B cells that recognize foreign antigens presented only in the context of MHC alloantigens on the cell surface. Such antibodies have been described in several murine strains and have been observed in primary B cell recognition of influenza-infected cells (Wylie and Klinman, 1981b; Wylie *et al.*, 1982; Sherman *et al.*, 1983; Klinman *et al.*, 1987) and SV40-transformed cells (Froscher and Klinman, 1986). Although B cells displaying this recognition phenotype are fairly plentiful within the primary B cell repertoire, they are exceedingly difficult to find in the secondary B cell repertoire (Klinman *et al.*, 1987; Pestolozzi *et al.*, 1987; Tamminera *et al.*, 1987). Since T cell receptors appear to be dedicated to the recognition of foreign antigens in the context of MHC alloantigens, the existence within the primary B cell repertoire of antibodies that can exhibit similar recognition implies that T cell

receptor specificities are included within the spectrum of B cell receptor specificities. The paucity of secondary B cells that recognize foreign antigens in the context of MHC alloantigens may reflect either inadequate helper T cell function for such responses, T cell suppression, or tolerance of newly generated secondary B cells perhaps because of their low-affinity interactions with self-MHC determinants.

Another major avenue of investigation that has been opened up by the availability of progenitors to secondary B cells is the comparative analysis of progenitors of secondary B cells versus precursors to primary antibody-forming cells and their ability to generate somatic mutations after primary stimulation. As mentioned above, the process of somatic mutation has been attributed to the cell population that is in the process of generating secondary B cells. Thus, for purposes of understanding the role and mechanism of somatic mutation it is critical to evaluate the rate at which newly generated secondary B cells proliferate and the time period during which somatic mutations are being accumulated in these cells. It will also be of particular interest to determine whether precursors to primary antibody-forming cells also somatically mutate. Thus, the establishment of hybridomas from these two separate cell populations becomes an important experiment that will enable the comparison of two B cell populations present in naive mice, one of which might somatically mutate and the other of which might not.

VII. Conclusions

At the time of our review of murine B cell repertoire expression a decade ago (Sigal and Klinman, 1978), the issues that had been resolved with respect to B cell repertoire expression included (1) that the repertoire of B cell specificities is clonally distributed among the vast population of B cells, (2) that each B cell expresses a single potential immunoglobulin product, (3) that each stimulated primary murine B cell gives rise to a clone of an antibody-producing cells whose antibody product replicates with considerable fidelity the variable region clonotype of the sIg receptor of the original stimulated B cell, (4) that the primary B cell clonotype repertoire is exceedingly diverse consisting of more than 10^7 specificities, (5) that, with respect to all tested specificities and particularly predominant clonotypes, individuals of the same murine strain tend to express similar repertoires, and (6) that the B cell clonotype repertoire of individual mice and the strain as a whole is highly restricted during late fetal and early

neonatal development and the diverse clonotype repertoire characteristic of adult mice is acquired in a highly patterned and reproducible fashion in all mice of a given strain.

Much of the research of the past 10 years has served to reaffirm each of these points and most importantly has established a molecular basis for (1) the diversity of B cell repertoire expression, (2) the mechanisms by which each B cell obtains a single specificity, and (3) the mechanisms that underly the gradual acquisition of the B cell repertoire during neonatal development. Indeed, acquiring a wealth of information with respect to variable region gene inheritance, the molecular mechanisms of variable region gene rearrangement, and expression and the interrelationship of these processes to B cell development has been the major triumph of research on the B cell repertoire during the last decade. Less dramatic, but equally important, has been the progress during the last decade in delineating B cell subpopulations and their importance to B cell responsiveness in general, and developing an understanding of the mechanisms by which the environment impacts on B cell repertoire expression. Finally, a new understanding is beginning to emerge concerning the generation of secondary B cells, the unique participation of somatic hypermutation in this process, and the power of positive and negative antigen selection in deriving a secondary B cell population which is highly specialized and extremely efficient in responding to a second challenge with antigen.

In part as a result of this dramatic increase in the level of our understanding of the molecular and cellular mechanisms responsible for B cell repertoire diversity and expression, it has been possible to define a large set of fundamental issues that remain to be resolved. Most fundamental of all is the extent to which the primary B cell repertoire is the product of evolutionarily selected, genetically determined, and reproducible events as opposed to stochastic events that would lead to a different repertoire in each member of an inbred murine strain. Although this issue has lost much of the philosophic fervor that characterized it in years past, the issue still remains unresolved and no less important than it was 10 years ago when adherents to germline versus somatic mutation hypotheses battled furiously at every opportunity. It is of interest that the major tenants of each postulate as originally stated, as well as the major arguments against each postulate no longer hold true. For example, it was argued that the germline hypothesis could not be correct since it was impossible to have multiple variable region genes in the face of variable region allotype conservation (Cohn, 1971; Cohn 1974). The

issue of variable region gene allotype conservation (in rabbits) is still unresolved (Mage, 1986), however, it is obvious that, since there are hundreds of variable region genes, any hypothesis that ruled out a multiplicity of variable region genes was incorrect. Similarly, both germline and somatic mutation hypotheses as originally constituted could account for repertoire of 10^4 – 10^6 specificities (Cohn, 1971; Cohn *et al.*, 1974; Hood *et al.*, 1974). Clearly, a primary repertoire of 10^7 – 10^8 was well beyond the upper limits of either theory.

Given the set of findings available several years ago that (1) the B cell repertoire was generated from multiple gene rearrangements that, at face value, appeared to represent a random assortment of large sets of variable region gene elements, (2) junctional diversity was abundant, and, most importantly, (3) a wealth of evidence became available for a contribution of somatic mutations within rearranged variable gene elements, the issue of the basis of repertoire expression appeared to have been resolved in favor of theories espousing random and stochastic events. However, as detailed in Section IV of this review, as events have continued to unfold, it has become less and less certain how much of primary B cell repertoire expression is, in fact, the product of random or stochastic events. Indeed, if it holds true that somatic mutations, per se, do not contribute to primary B cell repertoire diversity and that variable region gene selection and rearrangement are selective rather than random, then the likelihood of genetic determination of repertoire expression becomes more and more a reality.

Several years ago, when it became apparent that repertoire diversity was too great to be accounted for by classical germline theories yet repertoire expression in genetically identical individuals seemed highly reproducible, we proposed that primary B cell repertoire expression might best be thought of as the product of evolutionarily "predetermined permutations" of a large set of genetically inherited variable region genes (Klinman *et al.*, 1976b, 1977). This theory proposed that whether the requisite permutations of V gene segments included recombination or insertional events, this set of permutations was sufficiently predetermined, that each genetically identical individual would express at least a large proportion of the potential repertoire. Furthermore, it was predicted that the strain as a whole would reproducibly express all potential specificities with a periodicity of recurrence sufficient to ensure that evolutionary selection would be put to bear, not only on all available germline genes but also on all potential permutes of those germline variable gene elements. The importance of this theory was not in its anticipation of the precise

molecular events that would be necessary to generate a vast repertoire from a relatively limited set of genetic information, but rather the notion that even a vast repertoire constructed out of extremely complex molecular permutative processes could reflect the strong influence of the evolutionary history of the species.

Although not anticipated at the time, the gradual and reproducible acquisition of the neonatal B cell specificity repertoire could serve as a prime example of the consequences of an evolutionarily predetermined repertoire acquisition. In this context, it is of considerable interest that theories which predict important biological selective forces for a highly selected expression of neonatal specificities are now being tested (see Section VI). It should be anticipated that in years to come, as more and more sequence information becomes available, particularly with respect to primary B cell repertoire expression, examples of nonrandomness in primary B cell repertoire expression will become the rule rather than the exception. At that time, perhaps, attention will finally be given to an understanding of the types of evolutionary selective forces that could account for the reproducible expression of so vast a primary B cell repertoire as well as the evolutionary mechanisms that might be responsible for maintaining (1) diversity, (2) the invariant expression of important clonotypes, and (3) polymorphism in variable region gene elements among strains and species.

The second aspect of B cell repertoire expression that might be anticipated to show considerable progress over the next few years will be in the understanding of the impact of environmental influences on B cell repertoire expression. It is clear that the use of transgenic mice both as expressers of selectable immunoglobulin genes (*Grosschedl et al.*, 1984; *Ritchie et al.*, 1984; *Rusconi and Kohler*, 1985) and as expressers of defined "pseudo-self-antigens" (*Chisari et al.*, 1985; *Adams et al.*, 1987) will serve more and more to enable clear-cut definitions of selective phenomena as applied to sizable populations of B cells. In addition, it is unlikely that the general terms "tolerance induction" and "antiidiotypic immunoregulation" will be sufficient to describe the plethora of phenomena that will probably be found to affect, in subtle ways, primary B cell repertoire expression.

Perhaps the aspect of B cell repertoire expression that will advance furthest during the next decade will involve our understanding of B cell subpopulations and repertoire expression within these subpopulations. As detailed in Section VI, identification of the Ly1 B cell subpopulation has already led to important gains in our understanding of the means by which specialized subpopulations might diverge and interact with the environment. Indeed, examples of further rear-

rangements of already rearranged variable region genes have been demonstrated for the Ly1 B cell population (Kleinfield *et al.*, 1986). Similarly, one might anticipate a considerable amount of impact on our understanding of bioregulatory events, in general, of the subpopulation of primary B cells that appears to be continuously activated (Holmberg *et al.*, 1986). It has been proposed that the idiotypes and anti-idiotypes which appear to interact within this population might have important roles in interfacing as regulators in other biological systems (Holmberg *et al.*, 1986). In addition, it is likely that as more cell surface markers are identified and sophisticated cell separation techniques are applied to B cell populations using these markers, more and more functionally relevant B cell subpopulations will be delineated within the so-called "primary B cell" population.

Among the B cells subpopulations, the one that will receive the majority of attention with respect to its origins, stimulation, and variable region gene expression will likely be the cells of the secondary B cell lineage. It is already clear that this subpopulation functions via many of the rules that were thought to pertain to a repertoire generated primarily through somatic mutation (Cohn, 1971). For example, antigen selection serves as a powerful selective force on this population and shifts in entire populations occur with extraordinary rapidity. Because any given secondary response occupies a very small proportion of the B cell repertoire, sufficient division cycles are permissible within the space occupied by these responses to enable multiple divisions and time to favor the accumulation of multiple somatic mutations as well as the selection, positively or negatively, of cells expressing antibody derivatives of these mutations. Furthermore, this population, at least during its generation, is subject to "one hit-two hit" (Bretcher and Cohn, 1970) types of stimulatory versus tolerogenic influences in that contact with antigen alone is tolerogenic while contact with antigen in the presence of T cell help is stimulatory. It has been argued that such a selective mode would be necessary to appropriately account for a repertoire largely derived by somatic mutational events. To the extent that strong selective forces, applied to mutations occurring within short time periods, is akin to an evolutionary phenomenon per se, the study of secondary B cell generation could be considered the study of evolution in a microcosm.

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The Molecular Genetics of the Arsonate Idiotypic System of A/J Mice

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I. Introduction

A primary function of immunoglobulins is to specifically bind to nonself or foreign molecules, termed antigens, and then to effect the deactivation and/or subsequent removal of these antigens from the organism. The ability of the humoral arm of the immune response to accomplish this task is predicated on a capacity to generate a seemingly endless repertoire of specificities from a limited amount of information that is stored in the genome. The interaction of this germline-encoded information with specific somatic processes results in the production of structurally similar antibody molecules that are distinct on an individual basis.

A comparison of several variable region amino acid sequences early on revealed that the variable region could be subdivided into portions that were postulated to make contact with the antigen and those that maintained the molecule's proper structural integrity (Kabat, 1968). This was based on the recognition that within the variable regions there existed defined regions that appeared particularly prone to variability (Milstein, 1967; Kabat, 1968; Capra, 1971; Capra and Kehoe, 1975). These hypervariable (HVR) or complementarity determining (CDR) regions (Wu and Kabat, 1970) have been associated with antigen contact in X-ray crystallographic studies (Poljak *et al.*, 1974), and are embedded in relatively invariant regions termed framework regions (FW).

Variable region diversity is now known to result from a highly orchestrated, interdependent association between a limited number of germline-encoded elements and the somatic processes that specifically act upon these elements (reviewed in Tonegawa, 1983, Kindt and Capra, 1984; Yancopoulos and Alt, 1986). The functional expres-

sion of antibody variable regions depends upon the successful (or productive) rearrangement of several gene segments: in heavy chains, V_H , D_H , and J_H are united and in light chains, V_K and J_K or V_λ and J_λ are recombined. The recombination of these variable region elements provides a foundation of diversity that is amplified by somatic mechanisms such as imprecision in gene segment joining (Max *et al.*, 1979; Jeske *et al.*, 1984), somatic mutation, and gene replacement (Kleinfield *et al.*, 1986; Reth *et al.*, 1986a). Together with the pairing of heavy and light chain polypeptides, all of these mechanisms contribute to the ultimate generation of the repertoire of antibody specificities.

An approach that we and several other laboratories have taken in studying the structural basis for antibody diversity has been to analyze the response of certain inbred murine strains to immunization with haptens coupled to carrier proteins. A number of these strain-specific systems have been allotypically linked to the heavy chain (*Igh*) locus on chromosome 12 by several criteria. These *Igh*-associated antibody responses have been observed to contain a predominant component within which a limited number of heritable variable region gene elements are utilized repeatedly. Since the same D_H and J_H are commonly used in unrelated responses in different murine strains, attention has focused on the V_H gene segment as the key heritable element that dictates the strain specificity of these responses. The strain-specific system that shall be considered in detail in this review is the predominant response of A/J mice to immunization with the hapten *p*-azophenylarsonate coupled to keyhole limpet hemocyanin (Ars-KLH).

Since Ars-KLH is an artificial immunogen it can be viewed as a random probe into the variable region repertoire. As such, it has provided the means with which to study specific mechanisms that contribute to the acquisition of both germline and expressed diversity in both V_H and V_L genes. To address these mechanisms a variety of techniques have been employed including serological studies, splenic focus assays, amino acid sequence, and nucleic acid analyses. The studies have generated information concerning the regulation and expression of a defined antibody system.

II. The Arsonate System

The utilization of the hapten *p*-azophenylarsonate and its derivatives has a rich history in immunology. Landsteiner (1945), through the use of diazonium compounds, demonstrated the striking specific-

ity of the humoral immune response to the particular haptenic configuration that was utilized to induce the response. Chemical modification of antibodies against *p*-azobenzoate or *p*-azophenylarsonate implicated arginines (Grossberg and Pressman, 1968), lysines (Freedman *et al.*, 1968), and tyrosines (Pressman and Sternberger, 1951; Pressman and Roholt, 1961) as amino acids that were associated with antiarsonate combining sites. However, more definitive studies concerning the structure, function, and genetics of this system awaited the development (largely through the elegant studies of Nisonoff and his colleagues) of the strain-specific murine response to immunization with *p*-azophenylarsonate coupled to keyhole limpet hemocyanin.

When immunized with Ars-KLH, A/J mice produce an antibody response in which 20–70% of the antibodies bear a cross-reactive (intrastrain) idio type (CRI) as defined by rabbit antisera to the anti-Ars antibodies absorbed with preimmune A/J sera (Kuettner *et al.*, 1972). The CRI is heritable within A strain mice and has been linked to the heavy chain locus (Pawlak *et al.*, 1973), but requires an appropriate V_K chain as well (Laskin *et al.*, 1977; Brown *et al.*, 1980). This idio type is not produced in BALB/c mice (Kuettner *et al.*, 1972) except under unusual circumstances (see below) and is not suppressed in (BALB/c \times A/J) or C57BL/6 \times A/J)F₁ hybrids (Pawlak *et al.*, 1973) arguing for the strain-specific presence or absence of a particular V_H gene (Kuettner *et al.*, 1972; Hart *et al.*, 1973; Pawlak *et al.*, 1973). Heavy chain allotype congenic A \times BALB/c (C.AL-20) mice immunized with Ars-KLH express the CRI, indicating that the gene that controls idio type expression is linked to the *Igh* locus (Pawlak *et al.*, 1973).

The majority of CRI binding by rabbit anti-CRI sera is hapten inhibitable suggesting a close association between CRI determinants and the antigen binding site (Brient and Nisonoff, 1970; Ju *et al.*, 1977). Biochemical analyses utilizing isoelectric focusing as well as amino acid sequencing of both V_H and V_L correlates the CRI with the CDRs of both heavy and light chains (Capra and Nisonoff, 1979; Margolies *et al.*, 1981). Prior to molecular genetic studies it was widely believed (correctly it turns out) that a single or relatively few germline genes underwent extensive somatic mutation to give rise to these antibodies (Pawlak *et al.*, 1973; Capra *et al.*, 1976a).

Like most areas of immunology, hybridoma technology (Kohler and Milstein, 1975) had a major impact on the raw material available for detailed analysis in the arsonate system (Estess *et al.*, 1979, 1980; Lamoyi *et al.*, 1980a,b; Marshak-Rothstein *et al.*, 1980; Alkan *et al.*, 1980; Wysocki and Sato, 1981). CRI-positive hybridoma antibodies

exhibited microheterogeneity in their N-terminal sequences and exhibited a gradation in CRI reactivity, from strongly CRI-positive to CRI-negative molecules. When the N-terminal V_H sequences of antiarsonate CRI-positive and -negative molecules were examined (Fig. 1), they fell into three distinct antibody families: Ars A, Ars B, and Ars C (Milner and Capra, 1982). The Ars A antibody family is A/J specific and the Ars-A monoclonal antibodies that have been examined contain CRI-positive as well as some CRI-negative molecules (Estess *et al.*, 1979; Wysocki and Sato, 1981; Milner and Capra, 1982). A serological marker derived from one of these CRI-negative antibody molecules, 91A3, recognizes all Ars A molecules and has been shown to be heritable in a Mendelian fashion in strain A \times BALB/c recombinant inbreds (Milner and Capra, 1982). Chain recombination experiments localized both the Ars A and the CRI serological markers to the V_H region, however, CRI expression requires an appropriate

	10	20	30	91A3	CRI
93G7	EVQLQQSGA	ELVRAGSSVK	MSCASGYTFTSYGIN	+	+
R16.7	_____	_____	_____	+	+
123E6	_____T_____	T_____	T_____	+	+
124E1	_____	T_____	_____D_____	+	+
3D5-2	_____	_____	_____	+	+
36-65	_____	_____	_____	+	+
31-62	_____	P_____	_____	+	+
91A3	_____	T_____	_____S_____	+	-
45-49	_____	M_P_____	T_____A I_____L_____	+	-
96B8*	EVQLQQSGPE	LVKPGASVK	ISCKTSGYTF TAYTMH	-	-
4AC7*	_____	M_____	A_____	-	-
1AD10*	_____	M_____	A_____	-	-
44-1-3	<_____	R_____	A_____T_G_____	-	-
31-41	<_V_____	D_____	A_____DH_I_____	-	-
45-112	<_____	D_____	A_____DH_I_____	-	-
45-165	< I_____	V_____K_____	ET_____A_____D_R_N_____	-	-
92D5	EVQLQESG	PSLVKPSQ	TLSLTCSVTGDSITADYWN	-	-
94B10	_____	_____	_____	-	-
36-60	_____	_____	_____	-	-
31-64	_____	_____	_____N_____	-	-

*BALB/c

FIG. 1. Antiarsonate heavy chain families. Antiarsonate hybridomas from A/J and BALB/c mice were selected solely on the basis of binding to the arsonate hapten. Amino acid sequence analysis allowed the heavy chains to be grouped into three families. All CRI-positive molecules were in the first (Ars A) family. From Milner and Capra (1982).

light chain in order to be expressed (Laskin *et al.*, 1977; Milner and Capra, 1983).

The Ars B family of antiarsonate molecules is heterogeneous and is present in both A/J and BALB/c. Also present in A/J and BALB/c is the Ars C family (Milner and Capra, 1982; Near *et al.*, 1984). This antibody family constitutes a minor CRI in A/J (Gill-Pazaris *et al.*, 1981; Marshak-Rothstein *et al.*, 1981) but is the major BALB/c CRI in response to immunization of the latter strain with Ars-KLH (Brown *et al.*, 1981; Milner and Capra, 1982). The Ars A and Ars B sequences derive from the J558 V_H family (Milner and Capra, 1982; Sims *et al.*, 1982) and the Ars C sequences derive from the 3660 V_H family (Marshak-Rothstein *et al.*, 1981; Milner and Capra, 1982; Near *et al.*, 1984). The idiotypes associated with each family have been termed CRI_A, CRI_B, and CRI_C. CRI_D, a recently described idiotypic, is found in molecules bearing Ars A light chains and Ars C heavy chains (Robbins *et al.*, 1986).

III. Molecular Genetics of the Ars A Heavy Chain

A. A SINGLE V_H GENE SEGMENT IS REPEATEDLY UTILIZED IN THE Ars A RESPONSE

Detailed amino acid sequence analyses of Ars A heavy chains provided strong support to the idea that Ars A V_H sequences derive from a single V_H gene segment and that the slightly different sequences seen in independently derived Ars A monoclonal antibodies resulted from somatic mutation (Slaughter and Capra, 1983) (Fig. 2). Ars A V_H sequences seem to derive from only three gene segments: the Ars A V_H gene segment (a member of the J558 family), an A/J counterpart to the BALB/c *DFL16.1* D_H gene segment, and J_H2 . Early molecular genetics experiments revealed that a single J_H hybridizing fragment, identical in size in each hybrid, that correlated with the productive *VDJ* rearrangement in each hybrid, helped to confirm this conclusion (Estess *et al.*, 1982). However, formal proof required molecular cloning of each relevant gene segment.

Siekevitz *et al.*, (1982), utilizing a DNA fragment containing a portion of the V_H gene of an Ars A hybridoma, identified a 6.4-kb *EcoRI* fragment in A/J kidney DNA under stringent hybridization conditions that was absent in BALB/c DNA. A restricted probe was necessary because of the complexity of the J558 family. Additionally, the presence of this 6.4-kb hybridizing fragment correlated perfectly with A strain \times BALB/c recombinant inbreds that expressed Ars A

	CRI	ARS	10	20	30	40	50	60						
HP 93G7	+++	+	EVQLQ	SGAELV	RAGSSV	KMSC	CKASGY	TFTSYGINWV	KRPPGQ	GLEWIGY	INPGXG	YINY		
HP R16.7	+++	+	_____						L	NV	TK	_____		
HP 101F11	+++	±	_____						X		TK	_____		
HP 123E6	+++	+	T	T	T	_____						(_____	
HP 124E1	+	+	_____						D	S	X	T	_____	
HP 91A3	-	+	_____						S	H	K	H	_____	

	CRI	ARS	70	80	90	100	110	120							
HP 93G7	+++	+	NEKFKG	KTTTL	TVDKSS	SSTAYM	QLRSLT	SEDSAVY	FCARSH	.YYGG	SYDFDY	WGQG	TPLTV	SS	
HP R16.7	+++	+	_____						G	N	.	S	T	_____	
HP 101F11	+++	±	_____						()	N	.	S	.	M	SV
HP 123E6	+++	+	_____)	VR	D	Y	S	T	G
HP 124E1	+	+	_____						N	D	.	F	Y	N	T
HP 91A3	-	+	_____						R	.	.	S	M	S	ST

FIG. 2. Antiarsonate hybridoma heavy chains. Comparison of amino acid sequences of heavy chain V regions with that of HP93G7. (—), Identical residues; (●), gap introduced to maximize homology; [(), region in which no assignments are available. Numbering is sequential. Complementarity-determining regions are outlined. X, Secondly modified Asn residue. The expression of the CRI and the level of arsonate binding are noted for each molecule. Modified from Slaughter and Capra (1983).

antibodies and was absent in those that could not. Thus, the Ars A V_H gene segment was shown to display a heritable basis in Southern filter hybridizations that correlated with serology. Siekevitz *et al.*, (1983) then screened a genomic library of size fractionated DNA (6.4 kb) from A/J kidney with the restricted probe and isolated 24 clones containing hybridizing *EcoRI* fragments of 6.4 kb. The clones fell into three groups. Two of these groups contained V_H gene segments that exhibited identical nucleotide sequences but different flanking fragments when compared to each other. Both encoded amino acids that had not been observed in expressed Ars A proteins. The third, IdCR11, contained a nucleotide sequence that was identical to that of the expressed V_H sequence of an Ars A CRI-positive hybridoma.

Using quite different approaches our laboratory identified the Ars A V_H gene segment as well as several closely related V_H gene segments. We chose to screen a partial *EcoRI* A/J liver DNA library initially with a fragment (V_HA) that encompassed amino acid positions -4 to 92 in the Ars A cDNA clone 93G7 (Sims *et al.*, 1982; refer to Fig. 3). This

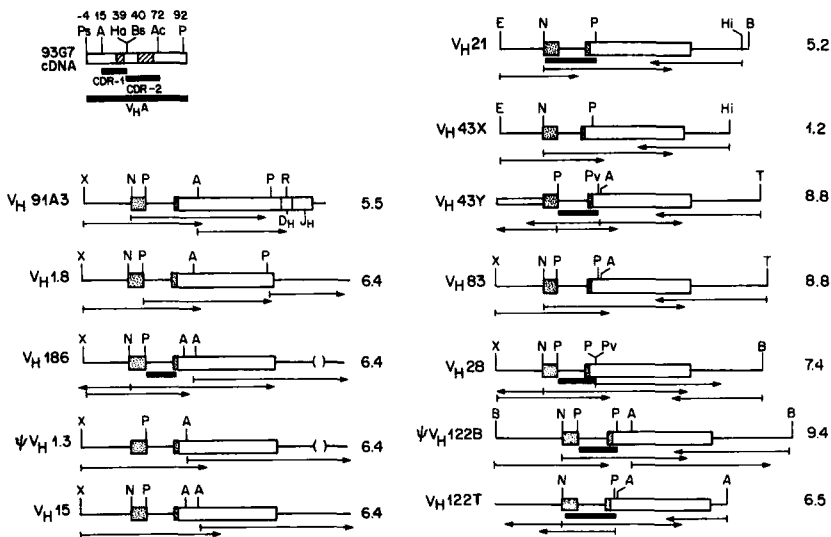


FIG. 3. Partial restriction maps, probes, and sequencing strategies. Ac, A, Ba, Bs, E, Ha, Hi, N, P, Pv, Rs, T, and X represent *AccI*, *AvaII*, *BamHI*, *BstNI*, *EcoRI*, *HaeIII*, *HindIII*, *NcoI*, *PstI*, *PvuII*, *RsaI*, *TaqI*, and *XbaI*, respectively. Diagonals represent CDR-1 and CDR-2 or 93G7, stipples indicate leader sequences, and the open rectangle in the 5' flank of V_H43Y represents a highly repetitive sequence. Bars indicate specific probes used in subsequent experiments. The number to the right of each V_H gene is the size of the *EcoRI* fragment in kbp on which that V_H gene resides. Arrows show sequencing strategies. From Rathbun *et al.* (submitted).

fragment detects the highly represented *J558 V_H* family. Approximately 100 clones were analyzed for hybridization to specific fragments derived from the CDR-1 and CDR-2 regions of the cDNA 93G7 (Fig. 3) and five of the clones that varied in hybridization to these restricted *V_H* fragments were chosen for further analysis. The phage library was also screened with a CDR-1-specific oligomer that encompassed amino acid positions 30–36 of IdCR11 to isolate additional *J558 V_H* gene segments that were closely related to Ars A *V_H* sequences. In contrast to the results obtained with the *V_HA* fragment, only 14 phage clones hybridized to the CDR-1 oligomer, one of which (phage clone 1.8) contained the *V_H* gene segment recruited in the Ars A response (see below). Finally, a partial *Mbo*I library of A/J liver DNA, cloned into a cosmid vector, was screened with a CDR-2 oligomer that encompassed amino acid positions 50–62. Three *V_H* gene segments from two cosmid clones were isolated and analyzed. Partial restriction maps of the 11 A/J *J558 V_H* gene segments are shown in Fig. 3.

In addition to the *J558 V_H* gene segments described above, the productively rearranged *V_H* gene from the Ars A hybridoma 91A3 was isolated. It was considered useful to examine the nucleotide sequence of *V_H91A3* since this hybrid contained the most different Ars A *V_H* sequence and could be compared to the sequence of 3665, the hybridoma studied by Siekevitz *et al.*, (1983) which appeared to be an unmutated version of the germline *V_H* gene segment.

Figure 4 shows the nucleotide sequences of the 5' flanking, coding regions and 3' regions, respectively, of 11 A/J *J558 V_H* gene segments; the translated amino acid sequences are shown in Fig. 5 (Rathbun *et al.*, 1988). The 5' flanking region of *V_H91A3* contains two nucleotide differences from *V_HL.8* while the coding region contains 12 nucleotide substitutions (Fig. 4A). The most closely related *V_H* gene segments to *V_HL.8* both with respect to restriction maps (Fig. 3) and coding region nucleotide sequences (Fig. 4B) (*V_HI86*, *V_HI5*, and ψ *V_HL.3*) vary in their 5' flanking regions from *V_H91A3* by a minimum of 20 nucleotide substitutions. When compared to the published sequences of *IdCR11* and 3665, *V_H91A3* and *V_HL.8* contained 4 identical differences in the L-IVS (leader-intervening sequence) from the former two. The expressed Ars A *V_H* gene segment, 3665, was resequenced within this specific region and the results showed that 3665 was identical to *V_HL.8* and *V_H91A3* (data not shown). Collectively, these data formally document that one *V_H* gene segment encodes the *V_H* utilized in the Ars A response, since the rearranged *V_H* gene from the most similar (3665) and most different (91A3) Ars A

A

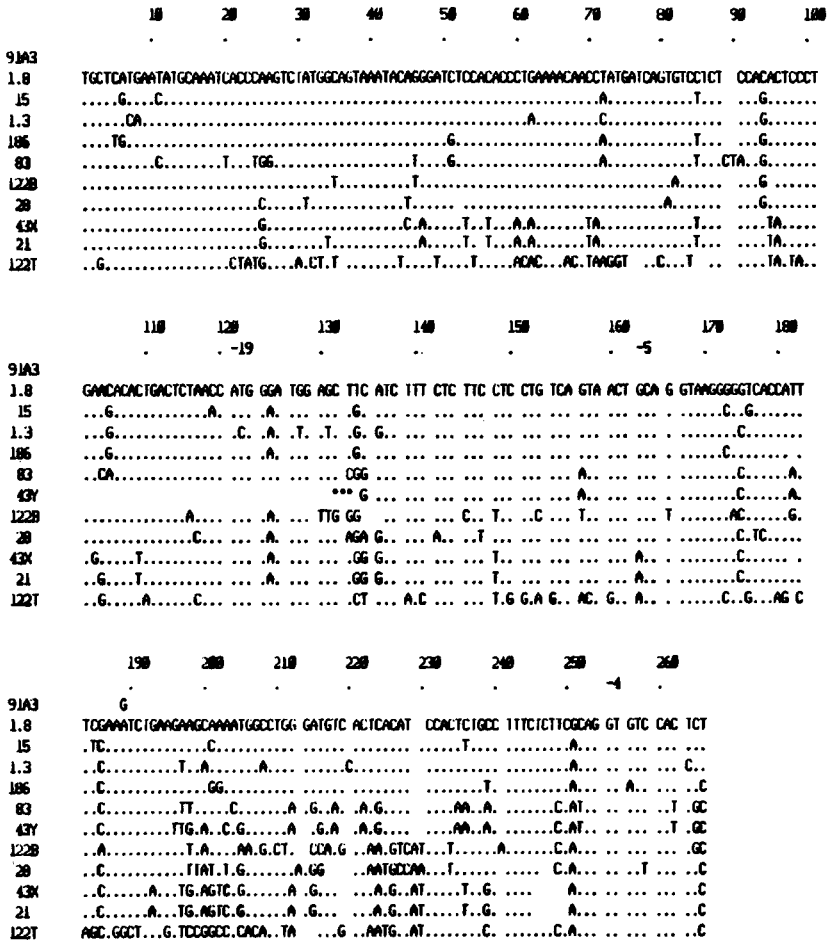


FIG. 4. Nucleotide sequences of 10 A/J J558 V_H gene segments and the V_H gene of 91A3 compared to V_H1.8. The dots under the V_H1.8 sequence show nucleotide identity. (A) 5' flanking region sequences. Nucleotide differences in the sequence of V_H91A3 are indicated by the presence of the nucleotide on top of the V_H1.8 sequence. The leader sequences have been split into codons. Three asterisks in the 5' flank of V_H43Y indicate a region that contains a highly repetitive, GC-rich sequence. (B) Coding region sequences. The IdCR7 sequence is from Siekevitz *et al.* (1983) and Wysocki *et al.* (1986a). A W in this sequence indicates a T or an A. The CDR-1 and CDR-2 are boxed. NT, Nucleotide position; AA, codon (amino acid) position. (C) 3' flanking sequences of seven A/J J558 genes. Heptameric and nonameric recombination recognition sequences are bracketed. From Rathbun *et al.* (1988).

C

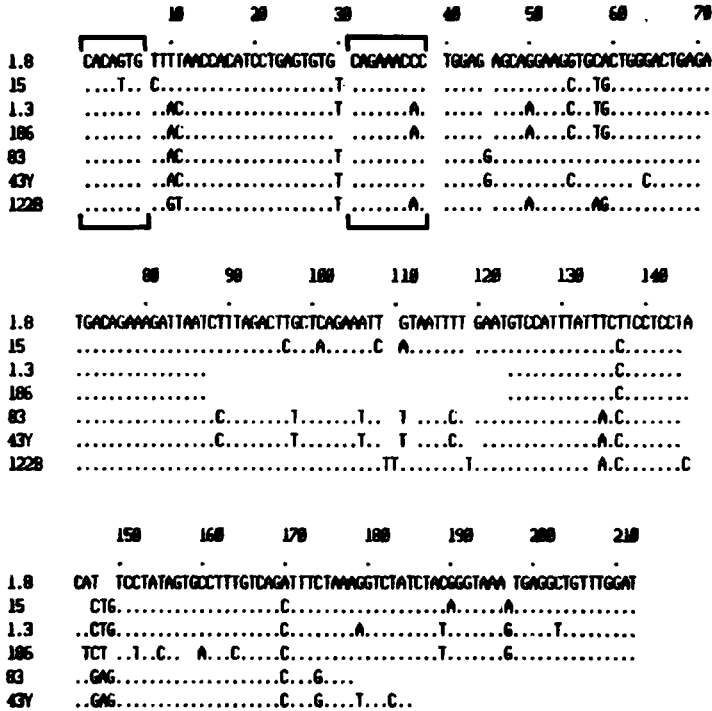


FIG. 4. (Continued)

antibodies to *V_HL.8* (or *IdCr11*) originates from the same *V_H* gene segment.

B. Ars A ANTIBODIES UTILIZE THE *DFL16.1* GENE SEGMENT

The amino acid (Slaughter and Capra, 1983; Smith and Margolies, 1984) and nucleic acid sequences (Sims *et al.*, 1982; Siekevitz *et al.*, 1982; Gridley *et al.*, 1985; Wysocki *et al.*, 1986a) of Ars A *V_H* regions suggested that the Ars A *D_H* gene strongly resembled the BALB/c *FL16.1 D_H* gene segment (Kurosawa and Tonegawa, 1982). Therefore, the partial *EcoRI* A/J liver DNA phage library was screened with a 5' flanking fragment of the BALB/c *FL16.1 D_H* gene segment and the A/J equivalent (*DFL16.1'*) was isolated (Landolfi *et al.*, 1986). The nucleotide sequence of the coding and flanking regions of the A/J *FL16.1'*

A/J J_H2 amino acid or nucleotide sequence when compared to BALB/c, although our preliminary studies suggest that limited restriction fragment polymorphism exists within the A/J J_H cluster. Interestingly, although J_H2 is required for hapten binding, it is not necessary for CRI expression. Ars A antibodies that utilize J_H4 exhibit greatly diminished binding to the arsonate hapten but may still express the CRI (Slaughter *et al.*, 1984; Smith and Margolies, 1984). Thus, while serological studies have clearly shown an intimate association between the CRI and the binding site of antiarsonate antibodies, the utilization of different J_H segments in Ars A antibodies has permitted a clear distinction between idiotypic and binding site determinants.

D. MOLECULAR CHARACTERIZATION OF THE V_H IN ANTIARSONATE RESPONSE

1. Kinetics of the Ars A Response

The response of certain murine strains, primarily A/J and BALB/c, to immunization with Ars-KLH has been studied in detail. These analyses have served to illuminate several features with respect to mechanisms that result in the diversification of the expressed antibody response to a defined antigen. Thus, we and others have utilized the arsonate system to explore such issues as somatic mutation, junctional diversity, selection, and regulation in the antiarsonate response.

The Ars A family of antibodies is the predominant response to Ars-KLH in A/J mice. A number of studies that measure the affinities of arsonate antibodies to hapten but using different techniques have been performed with conflicting results (Kapsalis *et al.*, 1976; Rothstein and Gefter, 1983; Kresina *et al.*, 1982; Near *et al.*, 1985). Gefter and colleagues concluded that unmutated Ars A antibodies exhibit higher affinity for hapten than unmutated Ars C antibodies with respect to the V_H gene segment (Near *et al.*, 1985). This conflicts with the results of earlier studies (Kapsalis *et al.*, 1976; Rothstein and Gefter, 1983) which suggested that affinity was not the driving force for the predominance of the Ars A family. Most studies agreed, however, that Ars A antibodies appearing to exhibit somatic mutation bound hapten with higher affinity.

Gefter and colleagues focused on the molecular characterization of the Ars A response as it pertained to its predominance in A/J mice. By probing mRNA lysates of hybridomas derived from A/J spleen cells expanded by the use of phytohemagglutinin (PHA), dextran sulfate, and goat anti-mouse Ig with a probe restricted for the CDR-2 in 3665

and a 3660 V_H probe, they showed that 3660 and *IdCRI1* ($V_H1.8$) were expressed in roughly equal frequencies in the adult preimmune repertoire, that is, approximately 1 in 300 (Manser *et al.*, 1984; Wysocki *et al.*, 1986a). Antigen apparently selects the combination of V_H and V_L elements during immunization, since, in the primary antiarsonate response (at which time little or no somatic mutation was observed), the Ars A *VDJ* in conjunction with the proper V_L , exhibited a higher affinity for the hapten than the Ars C antibody family (Manser *et al.*, 1984; Near *et al.*, 1985; Wysocki *et al.*, 1986a). This conclusion is in line with Burnet's clonal selection theory (1957) which states that antigen binding to the surface immunoglobulin of a B cell clone preferentially and selectively expands that clone for its immune response participation. Thus, the initial affinity of surface immunoglobulin for antigen (in this case, the arsonate hapten) is considered one of the key elements that determines the eventual dominance of that clone.

However, it was noted that in the primary response, Ars A antibodies constituted only a minor fraction of the response (Wysocki *et al.*, 1986a; only in the secondary response did the Ars A antibodies predominate. This result was consistent with earlier studies obtained from splenic focus assays showing that only 6.8×10^{-4} B cells in the A/J mouse could bind arsonate and only 1.2×10^{-6} expressed the predominant CRI in the preimmune repertoire (Sigal, 1982). This argued that antigenic selection was a potent force capable of focusing the response to arsonate in A/J mice by underscoring the rarity of Ars A B cell clones in the preimmune repertoire. Sigal (1982) also noted that the frequency of CRI-positive, arsonate binding antibodies increased 100-fold to 1.7×10^{-4} reactive B cell clones in the primary response. Collectively, the data from the Geffer laboratory and those generated by Sigal argue that Ars A antibody-producing B cells are preferentially expanded due to efficient competition for antigen. During the secondary response, this affinity rises and outstrips all other arsonate binding families of antibodies probably due to somatic mutation having occurred in key regions of Ars A V_H and V_L . Alternatively, the predominance of the Ars A responses in A/J mice may result from immunoregulatory networks (Jerne, 1974; Sigal, 1982; Froscher and Klinman, 1985).

2. CRI-Positive Molecules That Derive from Different V_H Genes

Sigal (1982) noted that BALB/c produced CRI-positive molecules at a very low frequency. This observation was not easily reconciled since CRI-positive molecules were (1) associated with higher Ars-

KLH affinity, and (2) the CRI positivity was associated with the presence of a strain-specific V_H gene segment. Moreover, reports were surfacing that BALB/c could be induced to express the Ars A CRI through idiotypic manipulations (Moser *et al.*, 1983) (see below) and, in one case, directly through conventional Ars-KLH immunization (Leo *et al.*, 1985). While these antibodies were CRI positive, they were negative for the Ars A serologic marker (Leo *et al.*, 1985). Amino acid sequence analysis of these BALB/c antibodies (Meek *et al.*, 1984) revealed that the V_H gene segments derived, in retrospect, from different members of the VGAM3-8 V_H family (Winter *et al.*, 1985) instead of the J558 V_H family. These antibodies utilized J_H1 and J_H4 elements. They had in common with the Ars A V_H sequences, the identical serine at position 99, the *FL16.1* D_H gene segment, and the same light chain. This argued strongly that given the proper tertiary structure, CRI expression was likely due to the D_H gene segment. Since these antibodies exhibited weaker hapten binding than Ars A antibodies, this explained why the latter predominated in the A/J response. It is not known whether the BALB/c CRI-positive molecules exhibit weaker binding to arsonate than Ars C (the BALB/c major CRI), but the prediction would be that these molecules do, unless immunoregulatory networks are involved. These studies also emphasize that an idio type may not be expressed due to a single V_H gene segment, rather that an idio type can be expressed in conjunction with V_H gene segments from different V_H families.

3. Repetitive Substitutions in Ars A V_H Sequences

A characteristic of some of the expressed Ars A V_H sequences is the presence of repetitive amino acid interchanges. This might suggest that certain sequences are donated by other highly related V_H gene segments. A close examination of these repetitive amino acid interchanges reveals no consistent linkage in their appearance in the expressed sequences (Slaughter and Capra, 1983). This weakens but does not formally exclude arguments that gene conversion plays a role in the generation of these repetitive substitutions. An examination of the 11 A/J germline V_H sequences (Figs. 4B and 5) supports this contention since not even the highly related V_H gene segments (greater than 92% relatedness to $V_H1.8$) contain nucleotides that can account for the amino acid interchanges in the expressed sequences. This is suggestive that in certain instances, somatic mutation has operated in a seemingly nonrandom fashion. An alternative explanation is that a patchy gene conversion mechanism involving potentially quite different V_H gene segments has generated the observed

changes (Reynaud *et al.*, 1985, 1987). Perhaps V_H gene replacement may play a role; only the 3'-most end of replaced V_H genes has been characterized and it will be interesting to see the results when the complete sequences of replaced V_H genes become available.

The Ars A response is characterized by microheterogeneity in both heavy and light chain amino acid sequences (Estess *et al.*, 1979, 1980; Alkan *et al.*, 1980; Margolies *et al.*, 1981; Marshak-Rothstein *et al.*, 1981; Siegelman and Capra, 1981; Slaughter and Capra, 1983; Smith and Margolies, 1984; Meek *et al.*, 1987). Somatic mutation in the V_H sequences is primarily localized to the CDR-2 and CDR-3 (D_H) regions of the assembled V_H (see Fig. 2). The presence of several different amino acids at certain positions in the CDR-2 region in the expressed sequences suggests that these positions are released from antigen selection due to their relatively minor role in antigen binding. Alternatively, the variability exhibited by the codons in CDR-2 may reflect a key accommodation point for replacement mutations that occur elsewhere in Ars A V_H sequences. Nevertheless, Ars A V_H sequences are strikingly similar, suggesting that while differences from the germline sequences can be tolerated to some degree in Ars A antibodies, loss of arsonate binding likely results in Ars A antibodies that contain differences greater than 8%. No Ars A antibodies have been identified that contain over 8% differences in their V_H or V_L sequences, however, it should be noted that the majority of such antibodies have been isolated utilizing antiidiotypic reagents. Thus, the saturation limits for changes in Ars A sequences with respect to hapten binding have not yet been determined. Since the Ars A response is so predominant in the secondary response, the prediction would be that Ars A antibodies can tolerate a significant amount of mutation from germline. On the other hand, from the limited sequence analysis, the Ars C sequences appear to exhibit less heterogeneity than the Ars A sequences (Milner and Capra, 1982; Near *et al.*, 1984), suggesting that the 3660 V_H gene may be more sensitive to somatic mutation with respect to loss of hapten binding than Ars A sequences (Near *et al.*, 1985).

4. The Invariant Serine at the V_H - D_H J $_H$ Junction of Ars A Molecules

At amino acid position 99, a serine that is always encoded by TCX (where X represents any nucleotide) appears in all Ars A V_H sequences at the V_H - D_H junction (Slaughter *et al.*, 1982; Gridley *et al.*, 1985; Milner *et al.*, 1986; Wysocki *et al.*, 1986a). This codon cannot be generated by nucleotides present immediately 3' to V_H 1.8 (Fig. 4C) or

IdCR11 nor by nucleotides immediately 5' to the A/J *DFL16.1** (Fig. 6). Wysocki *et al.* (1986b) noted that an *MboI* site (GATC) is always present within Arg-Ser codons at amino acid positions 98 and 99, but failed to identify a V_H gene segment containing this restriction site that was closely related to $V_H1.8$ or *IdCR11* in Southern filter hybridization experiments. Sharon *et al.* (1986) showed that this serine residue can be replaced by threonine without noticeable effect on the binding ability of the antibody molecule to Ars-KLH. Yet neither threonine nor other codons for serine have been found at this position. At position 100 in the expressed Ars A V_H sequences, marked variation suggestive of a typical N segment (Alt and Baltimore, 1982) is exhibited by the expressed molecules, indicative of $V_H-D_HJ_H$ assembly at this position (Slaughter and Capra, 1983; Wysocki *et al.*, 1986a). We concluded, therefore, that a novel mechanism may have generated the serine codon at position 99 in Ars A antibodies (Milner *et al.*, 1986).

IV. The Ars A V_H Gene Segment as a Probe Into the J558 V_H Gene

A. THE J558 V_H GENE FAMILY

The Ars A V_H gene segment $V_H1.8$ (or *IdCR11*) is a member of the largest V_H family, J558. To date over 50 different germline V_H gene segments have been isolated and sequenced from this family primarily from the BALB/c and C57BL/6 strains of mice (Givol *et al.*, 1981; Bothwell *et al.*, 1981; Cohen *et al.*, 1982; Loh *et al.*, 1983; Cohen and Givol, 1983; Bothwell, 1984; Schiff *et al.*, 1985, 1986). DNA-DNA liquid hybridization studies have been interpreted to suggest that in BALB/c, the J558 V_H family contains 1000 V_H gene segments (Livant *et al.*, 1986). This number is in conflict with earlier estimates of 60 members which was based on Southern filter hybridization experiments (Brodeur and Riblet, 1984).

Detailed examinations of the nucleotide sequences of J558 V_H gene segments have produced insights with respect to the pattern of variation and evolution of this V_H family. Givol and colleagues first suggested that the size of the intron that splits the leader sequences (L-IVS) in V_H gene segments can predict their V_H subgroup relatedness (Givol *et al.*, 1981). J558 V_H gene segments contain L-IVS sequences of about 83 bp in contrast to the S107 V_H family which vary from about 120 to 150 bp in mice (Kim *et al.*, 1981; Perlmutter *et al.*, 1985a). A number of subsequent studies of J558 V_H gene segments have confirmed that J558 V_H gene segments exhibit only small

variations in L-IVS sizes (Bothwell *et al.*, 1981; Loh *et al.*, 1983; Cohen *et al.*, 1982; Cohen and Givol, 1983; Schiff *et al.*, 1985).

The second important observation in the study of Givol *et al.* (1981) was that similar to the expressed V_H sequences, *J558* V_H gene segments exhibit significant variation in the CDR regions, while the variation in the framework regions was more limited; amino acid replacement substitutions in CDR regions were significantly more prevalent than in the FW regions (Givol *et al.*, 1981; Bothwell *et al.*, 1981; Loh *et al.*, 1983). This argued that diversity in the CDR regions was present in the germline and arose during the evolution of these V_H gene segments.

V_H pseudogenes were also identified by Givol *et al.* (1981); later studies have suggested that pseudogenes may constitute 30–40% of the entire V_H repertoire (Bothwell *et al.*, 1981; Loh *et al.*, 1983; Rechavi *et al.*, 1983; Cohen and Givol, 1983). However, with only two exceptions, one in the *S107* V_H family (Huang *et al.*, 1981; Perlmutter *et al.*, 1985b) and the other in the *J558* V_H family (Cohen and Givol, 1983) mutations rendering V_H genes pseudogenes were minimal, usually involving point mutations and small deletions and insertions. Cohen and Givol (1983) and Schiff *et al.* (1985) speculated that given the minimal nature of the mutations, correcting mechanisms may operate to stabilize the V_H gene segments of the *J558* V_H family within the population rather than eliminate them, thus rendering such genes potentially active, contributing members to the evolution of the V_H *J558* repertoire.

It has been well known that the murine *J558* V_H family was highly represented when compared to other murine V_H gene families. Cohen *et al.* (1982) have identified simple repetitive elements in the 5' flanking region of *J558* V_H gene segments (Fig. 7) that were postulated to function in generating frequent gene interactions such as gene conversion, unequal crossing-over, and homologous recombinations.

A highly conserved octameric sequence (Parslow *et al.*, 1984; Falkner and Zachau, 1984) is present in the 5' flanks of V_H and V_L genes and is required for efficient transcription (Falkner and Zachau, 1984; Bergman *et al.*, 1984; Mason *et al.*, 1985). This octamer was first identified in murine *J558* V_H gene segments (Rechavi *et al.*, 1982).

Several studies have pointed out that similar structural patterns exist with respect to the diversity exhibited by germline and expressed *J558* V_H sequences (Givol *et al.*, 1981; Bothwell *et al.*, 1981; Rechavi *et al.*, 1982; Cohen and Givol, 1983; Schiff *et al.*, 1985). For example, extensive CDR-2 diversity is observed in unrearranged *J558* germline elements as well as in expressed *J558* V_H genes, the latter of

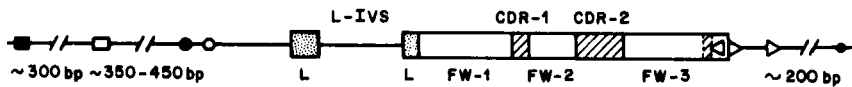


FIG. 7. An unrearranged *J558 V_H* gene segment. Closed square represents the CA/TA repetitive sequences approximately 300 bp upstream of a series of TCA–TCC repeats (open square) (Cohen *et al.*, 1982). The latter are 350–450 bp 5' of a highly conserved octamer important for transcription (closed circle) which is 16–17 bp 5' of the TAAATA box promoter element (open circle). Approximately 75 bp 3' to the TAAATA box is an ATG translation initiation codon in the leader sequences (designated by L and represented by stippled boxes). The leader sequences are split by a small intron (L-IVS), the size of which is *V_H* family specific. The small "HVR" region at the 3' end of FW-3 (marked by vertical dashes) is a focus of variation observed in the A/J *J558 V_H* gene segments in our study. The inverted triangle just 3' of the "HVR" region is a 5' heptameric sequence (i.e., typical of the heptamer 5' of *D_H* and *J_H*) embedded in FW-3 that may mediate *V_H* gene replacement (see text). *J558 V_H* gene segment coding regions are usually 294 bp in length. Directly adjoined 3' to FW-3 are the heptamer and nonamer recombination recognition sequences, separated by a 22–23 bp spacer, that mediate *V_H* recombination to *D_HJ_H*. Poly(A) addition sites have been identified in some *J558 V_H* gene segments about 200 bp 3' to FW-3 (small closed circle) (Yancopoulos and Alt, 1985; Rathbun *et al.*, submitted).

which is exemplified by the Ars A response (Fig. 2) (Slaughter and Capra, 1983). Thus, a relationship may exist between some of the processes that modified the *J558 V_H* family repertoire during the evolution of this *V_H* family and those that modulate the generation of diversity in expressed *J558 V_H* genes.

We reasoned that a detailed structural analysis of closely related *V_H* gene segments in the *J558 V_H* gene family together with that of a defined, highly characterized response should yield additional information with respect to the mechanisms that generate germline and expressed diversity within this *V_H* gene family. The Ars A response is ideal for such analyses.

The results of our study of the A/J *J558 V_H* germline gene segments has provided a detailed molecular characterization of the A/J *J558 V_H* gene family in four respects. First, nucleotide sequence analyses of 11 A/J *J558 V_H* gene segments have provided insights into the organization and pattern of variation of *V_H* gene segments within the *J558 V_H* gene family. Second, deletion mapping has determined the relative position of the *V_H1.8 J558 V_H* gene segment within the *J558 V_H* family. Third, the relative position of this *V_H* gene family within the *V_H* complex has been established by deletion mapping in not only A/J, but also BALB/c and C57BL/6. Fourth, the nucleotide sequence of an

expressed *J558 V_H* gene has provided information with respect to somatically generated diversity. Each of these aspects will be considered separately.

B. STRUCTURAL FEATURES OF 11A/J *J558 V_H* GENE SEGMENTS

An examination of the nucleotide sequences of the 5' flanking and coding regions (Fig. 4A and B) as well as the 3' flanks (Fig. 4C) of 11 A/J *J558 V_H* gene segments reveals several common structural features. Ten of these *V_H* gene segments contain the highly conserved octameric sequences (Parslow *et al.*, 1984; Falkner and Zachau, 1984) approximately 250 bp 5' to the coding region (Fig. 4A). These 10 *V_H* gene segments likewise contain TAAATA box promoter elements 16–17 bp 3' of the octamers.

Two pseudogenes are evident within the leader sequences of 11 *J558 V_H* gene segments. $\psi V_{H1.3}$ contains a T→C (Met/Thr) interchange in the ATG translation initiation codon while ψV_{H122B} has a 2-bp deletion at nucleotide positions 135–136. This deletion shifts the reading frame and generates a nonfunctional protein. In all the *V_H* gene segments, the leader sequences are interrupted by small introns that vary from 78 to 84 bp in length. The sizes of the leader introns are thus consistent with the observations of Givol *et al.* (1981), who noted similar sizes in BALB/c *J558 V_H* gene segments. ψV_{H122B} contains a transcription stop codon at position 115 in the coding region and a single bp deletion at position 135 (Fig. 4B). With the exception of ψV_{H122B} , the gene segments are 294 bp in length. Similar to other studies of *J558 V_H* gene segments (Givol *et al.*, 1981; Bothwell *et al.*, 1981; Loh *et al.*, 1983; Schiff *et al.*, 1985), variation is pronounced within the CDR regions of the 11 A/J *J558 V_H* gene segments. The size of CDR-2 is somewhat smaller in this group of genes with the majority of differences localized within codons for amino acid 50–60 (Fig. 4B) rather than the usual 50–75. Seven of the gene segments contain core (NNCTGTG, when N is any nucleotide) heptameric recombination recognition sequences (Rathbun and Tucker, 1987) at nucleotide positions 283–289 in FW-3 that could potentially function in *V_H* gene replacement (Kleinfeld *et al.*, 1986; Reth *et al.*, 1986b). Interestingly, immediately 5' of these heptamers is a small hypervariable region in the *J558 V_H* gene segments. Another heptamer is embedded within FW-3 at nucleotide positions 204–210 just 3' of CDR-2 in the *J558 V_H* gene segments. *V_{H21}* contains two addition "spacer" nucleotides at the 3' end of the *V_H* gene segment but is otherwise identical in sequence in its coding region to *V_{H43X}*. While two and possibly three

of the A/J *J558 V_H* gene segments are pseudogenes, all exhibit structural features characteristic of the *J558 V_H* family with respect to their pattern of variation and architecture.

Each of the A/J *J558 V_H* gene segments contains functional recombination recognition sequences immediately 3' of the coding sequences (Fig. 4C). These are separated by a spacer region that varies in length from 22 to 23 bp. These spacer sequences are surprisingly conserved within *J558 V_H* gene segments that can vary 20% within their coding regions and up to 44% in their 5' flanks (Table I; see also Givol *et al.*, 1981; Bothwell *et al.*, 1981). A closer examination of these spacer sequences reveals an apparent substructure containing 5'-CACA/TGTG 3' inverted repeats separated by a small spacer within which TCCT is the predominant sequence. Within the *7183 V_H* gene family, the *81X V_H* gene segment contains a CA as well as the TGTG

TABLE I
PERCENTAGE RELATEDNESS OF THE L-IVS AND CODING REGIONS
OF THE 11 A/J *J558 V_H* GENE SEGMENTS^a

Region	15	186	ψ1.3	83	43Y	ψ122B	28	43X	21	122T
L-IVS										
1.8	91.6	91.6	91.6	78.0	75.3	75.0	71.6	72.8	70.3	55.6
15		90.4	86.9	76.8	74.3	72.6	72.8	72.0	73.1	62.2
186			86.7	78.0	77.1	72.3	71.6	75.3	75.3	61.7
ψ1.3				79.8	78.6	69.9	77.1	77.3	77.1	62.7
83					90.2	76.2	75.3	78.0	79.3	60.5
43Y						76.2	75.3	82.7	84.0	60.5
ψ122B							71.8	73.8	75.0	57.1
28								72.5	75.0	58.0
43X									98.7	65.4
21										66.6
Coding										
1.8	93.4	93.2	92.9	90.1	90.1	87.5	86.7	86.4	86.4	85.0
15		95.9	94.6	87.8	87.1	85.0	84.0	83.7	83.7	83.7
186			94.6	87.2	86.7	84.4	84.4	84.1	84.1	83.7
ψ1.3				89.5	88.8	85.4	85.0	83.7	83.7	86.1
83					98.3	88.1	87.4	86.7	86.7	86.7
43Y						88.1	87.1	87.4	87.4	86.1
ψ122B							87.8	87.8	87.8	88.4
28								84.8	84.4	86.4
43X									100.0	80.3
21										80.3

^a Sequences were aligned by the method of Wilbur and Lipman (1983). Deletions and insertions were counted as single events.

sequence within its spacer (Yancopoulos *et al.*, 1984). *S107 V_H* gene segments contain the TGTG sequence 5' of their nonamers, lack the CA dinucleotide sequence, and contain AGGA (the complement of TCCT) 3' of their heptamers. *D_H* segments lack the CA/TG sequences. It is not readily apparent what role, if any, such sequences play in the recombination process since the spacer length rather than spacer sequence is rigidly preserved (Tonegawa, 1983).

A small focus of variation is present in the 11 A/J *J558 V_H* gene segments beginning at nucleotide position 260 and extending to position 291 (codons 86–97) (Fig. 4B). This region is particularly variable at positions 275–279, just one codon upstream of the 5' heptameric sequence embedded in FW-3. In some human *V_H* sequences, a 5' heptameric sequence is present in this part of FW-3 as well (Rechavi *et al.*, 1982; Kodaira *et al.*, 1986) and a third hyper-variable region has been identified at amino acid positions 86–90 (Capra and Kehoe, 1974). A triplet of 3' heptameric sequences is also present in human *V_{HII}* and *V_{HIII}* sequences at codons 57–59 and 61–63 within the CDR-2 as well as codons 77–79 (Rechavi *et al.*, 1982; Kodaira *et al.*, 1986); within the A/J *J558 V_H* sequences there are 3' heptamers present in codons 29–31 5' of CDR-1 in seven A/J *J558 V_H* gene segments, as well as in codons 68–70 just 3' to CDR-2 in 11 of the *V_H* gene segments. Wu and Kabat (1982) and Rechavi *et al.* (1983) have identified a region within the CDR-2 of human *V_{HIII}* and *V_{HII}* genes that bears a striking homology to a human *D_H* segment.

C. THE *J558 V_H* GENE FAMILY IS ORGANIZED INTO DISTINCT *J558 V_H* SUBFAMILIES

The 11 A/J *J558 V_H* gene segments can be grouped into discrete *J558 V_H* subfamilies based on the degree of relatedness exhibited by a specific nucleotide stretch that includes the L-IVS, FW-1, and CDR-1 regions of the *V_H* gene segments. Comparisons of the 11 *V_H* gene segments in their L-IVS regions (Table I) subdivide them into six *J558 V_H* subfamilies that are consistent with those reflected by the coding regions. The first subfamily (designated the *V_{H1.8 J558}* subfamily, Fig. 8) is represented by *V_{H1.8}*, $\psi V_{H1.3}$, *V_{H186}*, and *V_{H15}*. These *V_H* gene segments are 87–92% related in their L-IVS regions and exhibit multiple differences when compared to the other *V_H* gene segments. The *V_{H21 J558}* subfamily contains two members, *V_{H43X}* and *V_{H21}*; these vary in their L-IVS by a single deletion or insertion at nucleotide position 102. *V_{H28}*, ψV_{H122B} , and *V_{H122T}* are considered the sole representatives of their respective *J558 V_H* subfamilies since the maximum similarities exhibited by these *V_H* gene segments to each

other as well as the remainder are less than 77%. Generally, two *J558 V_H* gene segments that are 86–87% related in their L-IVS are highly related throughout. Thus, the similarities exhibited by *J558 V_H* gene segments within their L-IVS are considered to be a criterion that may be utilized to designate subfamilial relatedness.

A second criterion that applies to the definition of *J558 V_H* subfamilies is reflected in the pattern of similarities in the FW-1 of *J558 V_H* gene segments. Within the first 60 nucleotides of FW-1, a series of linked nucleotide similarities define member *V_H* gene segments within three *J558 V_H* subfamilies (Fig. 4B). The *V_H1.8 J558* subfamily is immediately identified by the first nucleotide in FW-1, where a C→G transversion (Gln/Glu interchange) (Fig. 5) distinguishes this *J558 V_H* subfamily from all others. This difference is accompanied by nucleotides only characteristic of the *V_H1.8 J558* subfamily that occur at nucleotide positions 1, 12, 46–47, 78, 81, and 84 (Fig. 4B). Maximal variation of the member *V_H* gene segments of the *V_H1.8 J558* subfamily in their coding regions is about 7% (Table I). Two other *V_H* gene subfamilies whose members clearly contain linked nucleotide similarities are the *V_H83* and *V_H21 J558* subfamilies. Consistent with the variation exhibited by their L-IVS regions, *V_H28*, *ψV_H122B*, and *V_H122T* represent three additional *J558 V_H* subfamilies since these *V_H* gene segments contain multiple (greater than nine differences) and distinctive variations within FW-1 and throughout their entire coding regions when compared to the other *V_H* gene segments (Fig. 4B, Table I).

The CDR-1 nucleotide sequence predicts (with a single exception; see below) the subfamilial relatedness of a given *J558 V_H* gene segment. The member *V_H* gene segments within the *V_H1.8 J558* subfamily vary in CDR-1 by a single silent substitution and the respective *V_H83* and *V_H21 J558* subfamily members are identical within CDR-1 (Fig. 4). By contrast, the *V_H83 J558* subfamily members and *V_H122T* all differ by 3–4 nucleotide substitutions in CDR-1 from any given *V_H1.8* subfamily member in CDR-1 (Fig. 4). Although the *V_H83 J558* subfamily is 90% similar to the *V_H1.8* gene segment (Table I), the pattern of variation exhibited by member *V_H* gene segments in the former clearly defines it as a distinct *J558 V_H* subfamily from *V_H1.8*. *V_H122T* varies from any single member of the *V_H1.8 J558* subfamily by 14–16% in the coding region (Table I). The complexity of the *J558 V_H* gene family, the members of which can vary by 26% (Cohen and Givol, 1983) in their nucleotide sequences, can therefore be reduced into distinctive *J558 V_H* gene subfamilies on the basis of four criteria. Members of a given *J558 V_H* gene subfamily are at least

92% related within their coding regions and demonstrate a characteristically high degree of similarity in their L-IVS, FW-1, and CDR-1 regions. This relatedness suggests that the *J558 V_H* gene family is comprised of specific sets of recently duplicated or considerably corrected *J558 V_H* gene segments.

D. INTERSUBFAMILIAL COMPARISONS REVEAL THAT ψV_H122B MAY HAVE UNDERGONE GENE CONVERSION

Inconsistent patterns of similarities with the other *J558 V_H* subfamilies that are suggestive of nonreciprocal genetic exchange are present in ψV_H122B . ψV_H122B contains 7 differences from *V_H43Y* in FW-1 and is identical to the latter in the first 37 nucleotides (Fig. 4B). The identity exhibited by ψV_H122B to *V_H43Y*, however, undergoes a striking shift at CDR-1, a region of high variation in intersubfamilial comparisons and nearly complete identity in intrasubfamilial comparisons. Within CDR-1, ψV_H122B varies by 5 nucleotides from *V_H43Y* but contains a single nucleotide difference from *V_H122T*, the *V_H* gene segment with which it is physically linked and from which ψV_H122B varies by 22% overall (12% within the coding region). Significant variation is observed in the two *V_H* gene segments in three of the first five codons of CDR-2; ψV_H122B and *V_H122T* vary by 7 of 9 nucleotides in codons 50, 52, and 54. Beyond nucleotide position 162, ψV_H122B and *V_H122T* regain their high degree of similarity; the two vary by 3 nucleotides throughout the remainder of CDR-2 and over half of FW-2. Beyond nucleotide position 234 in FW-3, ψV_H122B varies least from the *V_H1.8* gene segment and the *V_H83 J558* subfamily. Thus, the sequence similarities exhibited by ψV_H122B to other *J558 V_H* subfamily members suggest that ψV_H122B has undergone gene conversion.

E. THE SIX *J558 V_H* SUBFAMILIES ARE POLYMORPHIC IN A/J AND BALB/c MICE

Since the subfamilial relationships of *V_H* gene segments are reflected in their L-IVS subregions as well as within their coding regions, fragments containing the former (Fig. 3) were utilized as probes to assess the presence of the six *J558 V_H* subfamilies in two strains of mice. Under the hybridization stringency conditions used in these analyses, L-IVS probes do not cross-hybridize to phage clones containing other *J558 V_H* subfamilies. The results of the *J558 V_H* subfamily analyses in A/J and BALB/c are shown in Fig. 9 (lanes 1 and 2). Each *J558 V_H* subfamily is detected in BALB/c as well as A/J but varies in the number of hybridizing fragments from three in the *V_H1.8*

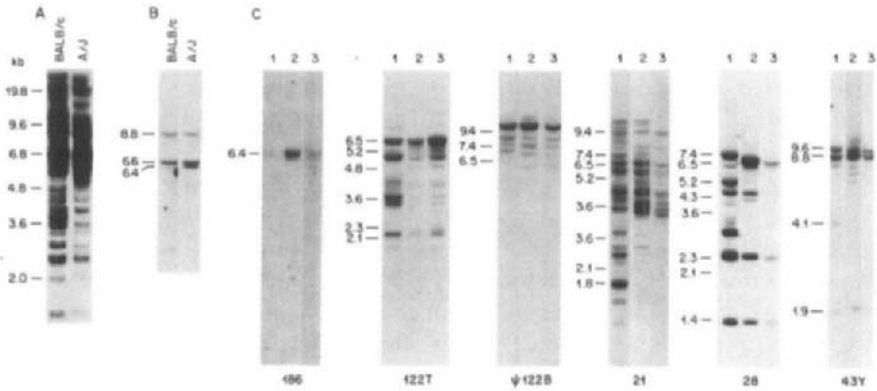


FIG. 9. Southern filter hybridizations of BALB/c liver (lane 1), A/J liver (lane 2), and 91A3 (lane 3) DNA digested with *Eco*RI and probed with (A) the V_HA fragment from 93G7; (B) the CDR-2 restricted V_H fragment from 93G7; (C) the L-IVS probes for the J558 subfamily analyses. (See FIG. 3 for probe descriptions.) Molecular weights in kb are indicated to the left of each hybridization.

J558 subfamily in A/J to approximately 30 in the V_H21 J558 subfamily in both strains. While all of the V_HJ558 subfamily members are represented in both strains in roughly equivalent numbers with respect to hybridizing fragments, striking differences are evident with respect to the number of major hybridizing fragments of most of the V_H subfamilies in each strain. The $V_H1.8$ J558 subfamily contains a single major *Eco*RI hybridizing fragment at 6.4 kb while BALB/c contains one minor hybridizing fragment (Fig. 9). It is important to note, however, that within this size range, A/J mice contain a minimum of six different $V_H1.8$ J558 subfamily-related V_H gene segments on essentially identically sized *Eco*RI fragments (Fig. 3 and Siekevitz *et al.*, 1983). Therefore, each strongly hybridizing fragment may represent several copies of highly similar V_H gene segments present on *Eco*RI fragments that are nearly identical in size.

In contrast to A/J, BALB/c contains a greater number of strongly hybridizing fragments in the V_H122T and V_H28 J558 subfamilies and the A/J V_H43X L-IVS probe hybridizes more strongly in BALB/c at 1.8 kb than any fragment in A/J. These results suggest that both strains contain several highly related copies of most of the A/J J558 V_H gene segments in their J558 V_H gene subfamilies. These J558 V_H gene segments are differentially represented in each strain as polymorphic J558 V_H subfamilies, both with respect to *Eco*RI restriction fragment sizes and number.

F. J558 V_H SUBFAMILIES ARE INTERDIGITATED

In contrast to the general organization of the murine V_H families within the V_H complex (Kemp *et al.*, 1981; Ben-Neriah *et al.*, 1981; Rechavi *et al.*, 1982; Brodeur and Riblet, 1984; Rathbun *et al.*, 1987), J558 V_H gene subfamilies are interdigitated. Evidence of J558 subfamily interdigitation is suggested in the J558 V_H family in deletion mapping experiments that position $V_H1.8$ proximally in the J558 V_H gene family with respect to the D_HJ_H cluster. The results show that a few members of at least five A/J J558 V_H gene subfamilies were deleted (Fig. 9). This interpretation requires caution, however, since V_H gene replacement could have deleted some of the V_H gene segments upstream or downstream of the expressed $V_H1.8$ gene. The presence of two different J558 subfamily representatives in each of two clones in this study as well as the physical linkage of J558 V_H gene segments in other investigations clearly show that different J558 V_H gene subfamily members are linked (Givol *et al.*, 1981; Cohen *et al.*, 1982; Cohen and Givol, 1983). In another study, two C57BL/6 NP^b-related V_H gene segments (186-2 and 186-1) that are members of the same J558 V_H gene family were present in the same phage clone (Bothwell *et al.*, 1981). The results of these latter studies as well as those in our own suggest that the duplication units are variable in size and that this characteristic may contribute to generate the interdigitation by unequal cross-over events. In this respect, Pohlenz *et al.* (1987) have reported that the units of duplication in V_K gene segments are estimated to vary from 35 to 50 kb.

G. SEQUENCES IN THE 5' FLANKING REGION AND FW-1 SUGGEST EVOLUTIONARY RELATIONSHIPS OF THE SIX A/J J558 V_H SUBFAMILIES

The sequences 5' of the translation initiation codons, with the exception of V_H122T , are highly conserved among the A/J J558 V_H gene segments (Fig. 4A). However, the V_H21 J558 subfamily contains several substitutions in common with the V_H122T J558 subfamily representative between nucleotide positions 58 and 97, a region that exhibits a high degree of variation in V_H122T when compared to the other V_H gene segments (Fig. 4A). Within the L-IVS, V_H43Y varies by only two nucleotides from the V_H21 J558 subfamily between positions 196 and 206. Intersubfamilial relationships between V_H28 , ψV_H122B , V_H122T , and V_H21 J558 subfamilies are suggested by clusters of shared nucleotides. The scattered sequence similarities exhibited by the V_H gene segments in their 5' flanking regions could have resulted

from coincidental mutations or patchy gene conversion. The other obvious alternative is that some of these similarities may reflect residual evolutionary relationships between these V_H gene segments.

The similarities present in the 5' flanking regions and FW-1 can be used to construct a hypothetical pathway for the evolution of the 11 *J558* V_H gene segments (Fig. 10). Under this scheme, the designated *J558* V_H gene subfamilies in this study have duplicated from a common ancestor (consensus P) early in their history. In constructing a pathway for the evolution of the $V_H1.8$ *J558* subfamily, only the 3' flanks of the member V_H gene segments gave clues to their relatedness since there was no consistent pattern to the substitutions in both the 5' flanking and coding regions. $\psi V_H1.3$ and V_H186 contain identical 32-bp deletions, indicating a close evolutionary relationship between the two. Thus, the acquisition of mutations during their evolutionary history obscures relationships of the V_H gene segments suggesting that a variety of mechanisms may have contributed to the diversity in the *J558* V_H gene family.

H. THE EVOLUTION OF THE *J558* V_H FAMILY

It seems clear from an examination of the nucleotide sequences of the A/J *J558* V_H gene family that the evolutionary strategies in this as well as other V_H families appear to be predisposed toward selecting for diversification in defined regions within the genes. Thus, the extensive representation of the *J558* V_H family may function to provide a library of highly related V_H gene segments that balances, by redundancy, forces that operate to diversify them by gene interaction, mutation, and selection. A second strategy that applies to the V_H complex in general is the expression of multiple antibody families in response to a given antigenic stimulus as is evident, for example, in the arsonate (Milner and Capra, 1982) and phenylloxazolone (Berek *et al.*, 1985) systems. Thus, even the loss of an entire V_H family due to some catastrophic event may not appreciably alter an organism's ability to survive. It is, therefore, not surprising that the *J558* V_H family, as an example, may be subject to a multiplicity of processes (see below) that operate to diversify it, even with the possibility that such mechanisms will introduce mutations that preclude the expression (although not necessarily the contribution in an evolutionary sense) of given *J558* V_H gene segments to the expressed repertoire. Gene duplication in the *J558* V_H family may therefore provide not only new functions with respect to antibody specificity, but equally, if not more importantly, also provide a means to overcome considerable evolutionary diversification pressures. In these respects, gene dupli-

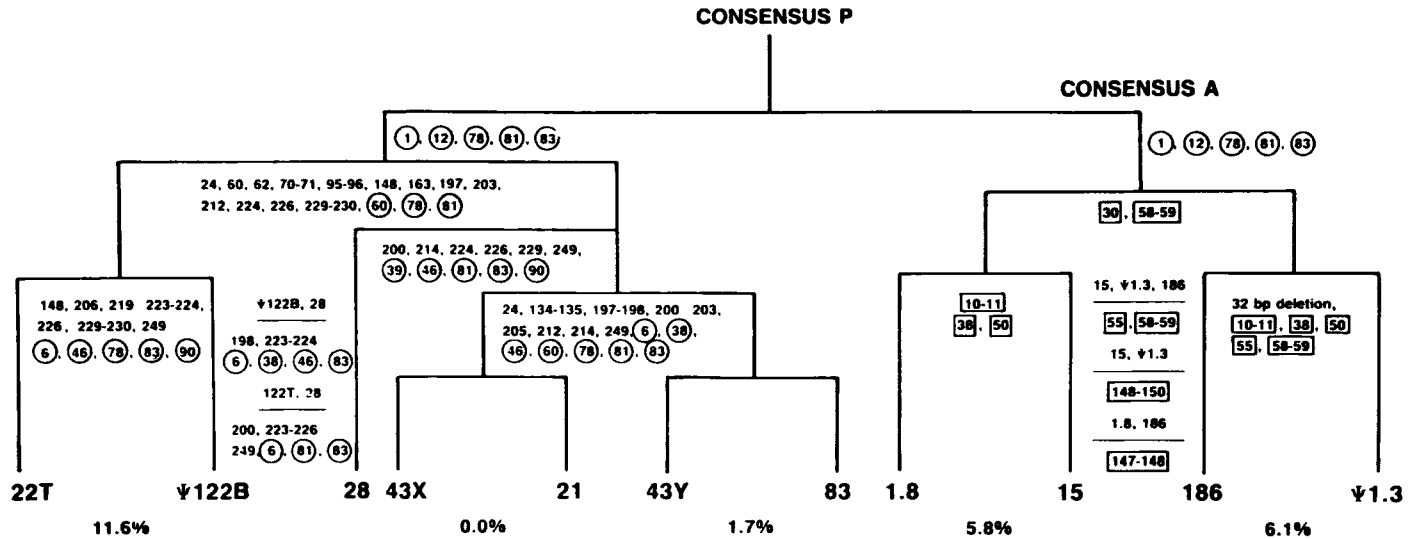


FIG. 10. Hypothetical geneological tree for the 11 A/J *J558 V_H* gene segments. Plain, boxed, and circled numbers represent the positions of nucleotide similarities used to derive the relationships of the *V_H* gene segments in the 5' and 3' flanking regions and coding regions, respectively. The similarities between *J558 V_H* gene subfamilies and subfamily representatives are indicated by the nucleotides in common between each branch. Percentages in between two branches indicate the variation in the coding region of the two *V_H* gene segments. Consensus sequences P and A were generated by deriving a common sequence from nucleotides that appeared most often in a given position in each A/J *J558 V_H* gene subfamily representative and the members of the *V_HL.8 J558* subfamily, respectively.

cation may serve to introduce an evolutionary homeostasis with respect to a gene family that must diversify in order to provide maximum protection against an organism's antigenic universe, the latter of which is obviously also evolving.

To examine the pattern of variation in the $V_H1.8$ *J558* V_H gene subfamily, a consensus sequence (Consensus A) for both the coding (Fig. 8) and 5' flanking sequences (not shown) was generated under the assumption that the member V_H gene segments in the $V_H1.8$ *J558* subfamily arose from a single common precursor. The members of the $V_H1.8$ subfamily vary from the Consensus A sequence by 2.8%. Variation occurs in approximately equal frequencies in the 5' flanking and coding regions (3.0 and 2.7%, respectively) and appears, at first glance, to be randomly distributed throughout these regions. Within the coding regions, however, there is a marked predisposition for transitions to occur in positions 2 and 3 of the codons (Table II), although transversions should occur twice as frequently. This bias is consistent with that noted in an interstrain comparison of the *S107* V_H gene segments (Perlmutter *et al.*, 1985a).

An examination of the types of transversions present in the $V_H1.8$ *J558* subfamily reveals an additional bias. R-type transversions are three and four times more prevalent in codon positions two and three, respectively, although R and Q transversions would be expected to occur equally (Table II). When a consensus sequence is derived utilizing a single representative from each of the six A/J *J558* V_H subfamilies (Consensus P) and compared to the 11 A/J V_H gene segments in this study, R-type transversions appear to predominate as well (Table II).

The pattern of variation in the $V_H1.8$ *J558* subfamily suggests a steady accumulation of changes during the evolution of the member V_H gene segments. This is in striking contrast to the pattern exhibited by the C57BL/6 NP^b-related *J558* V_H gene segments (Bothwell *et al.*, 1981). Five of the six C57BL/6 *J558* V_H gene segments vary by a maximum of 8% when compared to 186-2, the V_H gene segment recruited in response to NP. However, these six V_H gene segments clearly fall into at least two groups as noted by Bothwell *et al.* (1981) (or two *J558* subfamilies). If it is assumed that these V_H gene segments arose from a common precursor, then these V_H gene segments may have accumulated differences in an episodic fashion during their evolution. While it is possible that in the latter study V_H gene segments that reflect transition states were not isolated, this latter pattern of variation together with that exhibited by the $V_H1.8$ *J558* subfamily may indicate that mutations can be fixed in either an erratic or incremental fashion.

TABLE II
TRANSITION AND TRANSVERSION FREQUENCIES IN THE CODING REGIONS
OF THE 11 A/J J558 V_H GENE SEGMENTS

	Consensus ^a A ^b codon position			Consensus P ^c codon position		
	1	2	3	1	2	3
P ^d (%)	40	64	64	60	59	55
Q + R (%)	60	36	36	40	41	45
Q (%)	55	25	19	35	61	36
R (%)	45	75	81	65	39	64

^a The nucleotide that appeared most frequently at a given position was considered consensus; positions where different nucleotides appeared in equal frequencies or that contained deletions were not included in the derivations.

^b All members of the V_HI.8 J558 subfamily were used to derive Consensus A.

^c V_H83, V_H43X, V_H122T, ψ V_H122B, V_H28, and Consensus A sequences were used to derive Consensus P.

^d P, Q, and R categories are as described in Kimura (1981). P represents transition categories: G→A, A→G, T→C, C→T. Q and R are transversion categories. Q: T→A, A→T, G→C, C→G; R: A→C, C→A, G→T, T→G.

The pattern of variation in J558 subfamilies suggests that a variety of steps were required to attain the observed diversity in the member V_H segments. There is evidence that mutations accumulate at random without regard to any particular subgenic region or position of the codons. In the V_HI.8 subfamily, however, a bias appears to exist for the 3' half of the V_H gene segments' coding regions to fix changes when compared to Consensus A (Fig. 8); overall, the 3' halves of the V_H gene segments exhibit nearly three times more substitutions than the 5' halves, possibly as a result of selective structural constraints and/or gene conversion. Even when variation may have been acquired in an episodic fashion, the FW-1 and CDR-1 regions were spared, as is the case of the C57BL/6 NP^b-related V_H gene segments (Bothwell *et al.*, 1981). During the evolution of a J558 V_H gene segment, positive selective pressures permit the CDR-1 and CDR-2 regions to accumulate more mutations than the framework regions. Selection appears to operate differentially on the CDR-2 region, resulting in the preservation of key codons perhaps to maintain CDR-2 structure and, at the same time, releasing others to provide maximum flexibility to fix changes. This would permit V_H gene segments, through forces of evolution, to accumulate mutations in key areas in order to randomly follow antigenic drift. The CDR-1 in J558 V_H gene segments also appears to be important in this regard; although the CDR-1 appears to be under negative selective con-

straints in a set of recently duplicated *J558 V_H* gene segments, this region also appears susceptible to changes in intersubfamilial comparisons. Collectively, the results suggest that the architecture of a *J558 V_H* gene segment may differentially influence its modification by either positive and negative selective forces, gene conversion, unequal cross-overs, or any combination of these mechanisms.

The size and location of the *J558 V_H* gene family within the *V_H* complex may bring to bear additional crucial elements that generate the pattern of diversity in *J558 V_H* gene segments. Since the *J558 V_H* gene cluster has been mapped as one of the more distal *V_H* families with respect to *D_H* in certain murine strains (see below), this *V_H* family, through its size and overall relatedness of its member *V_H* gene segments, may act as a focal point in bringing together the *V_H* locus during meiosis. A possible consequence is a greatly enhanced predisposition for various types of genetic recombinations to take place in the *J558 V_H* gene family that may be mediated, in part, through the unique repetitive elements upstream of *J558 V_H* gene segments (Cohen *et al.*, 1982).

The amplified propensity of the *J558 V_H* family members to interact may result in expansion and contraction of the *J558 V_H* gene family through saltatory (Hood *et al.*, 1975; Rechavi *et al.*, 1982) or overreplicative (Schimke *et al.*, 1986) processes and unequal crossing-over and may play as great a role as selection and random mutation in generating the pattern of variation within the *J558 V_H* gene family. The evolution of diversity in the *J558 V_H* gene family can thus be divided into three stages that are not necessarily temporally related or exclusive. These are (1) the incremental and, at times, episodic accumulation of point mutations, (2) overreplication of *V_H* segments or sets of *V_H* gene segments, and (3) an increased susceptibility for genetic recombinations due to the fluctuations that may result from the organization of the *J558 V_H* gene family within the *V_H* complex, the structure of *J558 V_H* gene segments, the interdigitation of the *J558 V_H* gene subfamilies, and the large number of *V_H* gene segments within this *V_H* gene family. These stages appear to be modified, in part, by positive and negative selective forces.

I. EXPRESSED AND GERMLINE SEQUENCES SUGGEST A COMMON MECHANISM MAY GENERATE DIVERSITY IN BOTH

The pattern of variation in *V_H9IA3* reflects that seen in the germline *J558 V_H* sequences. In CDR-2, 75% of the substitutions result in amino acid replacement in *V_H9IA3* compared to 100% of the mutations present in the *V_H1.8J558* subfamily. Transitions account for 50% of the mutations in *V_H9IA3* and 66% of the transversions are of the R

category. Third base changes account for 50% of the substitutions. This pattern is not limited to V_H91A3 . When the substitutions in the $V_{\kappa}167$ genes of four PC-specific hybridomas (Gearheart and Bogenhagen, 1983) are categorized in P, Q, and R frequencies, 57% of the substitutions are transitions while 64% of the transversions are of the R type. Taken together, this indicates that similar to the variation exhibited by unrearranged V_H sequences, somatic mutation is not focused with respect to nucleotide position within a codon and introduces an element of randomness as well as a bias to the changes that appear as a result of somatic mutations.

The simplest interpretation for the mirrored variation in the germline and expressed V_H sequences is that the same DNA repair system(s) that generate the variation in the former may operate in a potentiated or deregulated form with respect to its editing function in the latter, termed somatic mutation. Intertwined with this system is intense structural and antigenic selection. Structural constraints both with respect to protein folding as well as requirements for antigen binding would explain the observation that certain codons within CDR-2 that exhibit marked variation in the germline sequences are rigidly conserved in the expressed sequences.

Similar to the pattern of variation exhibited by the rearranged $V_{\kappa}167$ genes in the PC system, the substitutions in V_H91A3 are clustered. Eight of the 12 differences in V_H91A3 occur between nucleotide positions 153–188 and the majority of these substitutions are localized between nucleotide positions 170 and 181 (Fig. 4B). Several investigators have shown that somatic mutations is diminished in the 5' flanking regions of expressed V genes (Kim *et al.*, 1981; Pech *et al.*, 1981; Gearheart and Bogenhagen, 1983; Gorski *et al.*, 1983) and the results with V_H91A3 agree with this observation. One possibility for the clustering of somatic point mutations in the coding regions is that accessibility to putative error-prone repair enzymes (Lederberg, 1959; Brenner and Milstein, 1966; Gearheart and Bogenhagen, 1983) due to the configuration of the rearranged, transcriptionally active variable region focuses somatic mutation. In this regard, the clustered nature of the substitutions in the CDR-2 that appear randomly scattered 5' and 3' of this region in V_H91A3 appears to resemble a contact point.

V. Organization of the Murine V_H Complex

In their analyses of antiarsonate hybridomas, Estess *et al.* (1982) noted that two appeared to contain only the productive VDJ rear-

rangement. This suggested to us that these hybrids might prove useful in deletion mapping experiments to position the $V_H1.8$ $J558$ V_H gene segment within the A/J $J558$ V_H family and then to position the $J558$ V_H family within the V_H complex. The latter experiments resulted in a different V_H map than that generally accepted. It is therefore of interest to review some of the information that generated the accepted map with the hope that the two V_H maps may reflect characteristics that may be unique to the V_H complex; such characteristics may result in additional clues with respect to the generation of diversity in both the expressed as well as the germline V_H repertoire.

A. THE IDIOTYPIC V_H MAP

The relative positions of V_H gene families in the V_H complex were initially based on the recombination frequencies of defined idiotypic specificities within a limited number of IgC allotypes in various inbred, F_1 hybrids, and recombinant inbred mice (Riblet, 1977; Weigert and Potter, 1977). In several of these analyses, multiple strain-specific idiotypic specificities were tested for expression in the recombinants that exhibited idio/allo cross-overs. Since it was recognized that an idio/allo could be associated with different V_H genes, precise mapping data were difficult to obtain (Weigert and Potter, 1977). Additionally, several of the idiotypes were "strain-predominant" in induced responses, providing another potential complication in that the strain of mouse may be important with respect to the recombinational frequency of its specific idio/allo (Weigert and Potter, 1977). In order to provide a preliminary map, the working assumption was that the V_H complex was similarly organized in the various mice exhibiting cross-overs (Weigert and Potter, 1977). While the V_H map was recognized to be imprecise in some respects, many interesting observations resulted from these studies.

There were a number of puzzling features revealed in the analysis of recombinant mice. Of great interest was the observation that there appeared to be a high rate of multiple recombinations between IgC and prealbumin (Riblet, 1977), a marker linked to the IgC allotype (Taylor *et al.*, 1975). Recently, IgC-prealbumin linkage has been questioned by Erikson *et al.* (1986), who identified V_H complex-prealbumin linkage in a specific chromosomal translocation. Since the recombination frequencies (given the calculated distance between IgC and prealbumin) were significantly higher than expected, this suggested to Riblet (1977) that there was unusual structures within the *Igh* locus that might exhibit a predilection for cross-overs.

The predicted "gene" order based on the heritable patterns of idiotypic specificities combined from several inbred, F_1 hybrid and

recombinant inbred mice (Weigert and Potter, 1977) was (*ESE*, *A5A*)–(*Ars*, *InuIDX*)–(*S117*, *Dex*, *T15*)–*Nase*–*IgC*. The idiotypic specificities in parentheses could not be ordered. Referring to Riblet and Brodeur (1986), the V_H map order translates to (*7183*, *J606*)–(*J558*, *J606*)–(*X24*, *J558*, *S107*)–*7183*–*IgC*. Later studies placed *Dex* and *Ars* (both members of the *J558* V_H gene family) together and downstream of *J606* (the *A5A* idio type) with respect to D_H (Riblet, 1977). It should be noted that *Dex* is a complex idio type (Riblet, 1977; Weigert and Potter, 1977), hence the confusion of its position with respect to other idiotypes. Makela *et al.* (1984) ordered the V_H map, *J558*–*S107*–*Q52*–*IgC*, and Riblet described recombination points that had occurred within the *S107* and *Q52* V_H families in certain recombinant inbreds. Collectively, some of the problems in the interpretation of the above data become readily apparent. First, the data are combined from different allotypic systems and assume that the V_H order in all the inbred strains is the same. Second, idiotypic definitions are sometimes imprecise, thereby obscuring a retrospective definition of V_H family from which the idiotypes are derived. Third, this method was incomplete with respect to measuring a large number of idiotypic specificities on several occasions. Nevertheless, with some notable exceptions (see below), the idiotypic V_H map was surprisingly correct given the uncertainties involved in its delineation.

B. SOUTHERN FILTER HYBRIDIZATION ANALYSIS OF THE V_H COMPLEX

Ordering the V_H complex continued to be an interest of Riblet and colleagues who switched to mapping strain-specific V_H RFLPs in recombinant inbreds, primarily in those generated by a cross between *A/J* and *BALB/c*. These investigators generated a generally accepted ordering of the V_H complex by Southern filter analysis of strain-specific V_H family polymorphisms (Brodeur *et al.*, 1984; Riblet and Brodeur, 1986; Riblet *et al.*, 1987): (*3609*, *J606*, *X24*, *3660*)–*J558*–*S107*–*Q52*–*7183*– D_H – J_H –*IgC*. The V_H families in parentheses were not mapped relative to each other. An additional V_H family, *VGAM3-8* (Winter *et al.*, 1985), has not been mapped.

The organization of the V_H complex has important implications with respect to understanding the selection, regulation, and expression of V_H gene segments from the germline encoded array in V_H – D_H – J_H rearrangements. This is due to the recognition that there is an ordered appearance of V_H gene segment utilization during ontogeny. Additionally, delineation of V_H map order can result in insights with respect to the mechanism of the rearrangement process.

C. PREFERENTIAL UTILIZATION OF D_H - J_H PROXIMAL V_H GENE SEGMENTS IN MICE

1. *In Vivo* Analyses

In 1963, Silverstein *et al.* observed that fetal lambs exhibited an age-related, hierarchal immune response to various antigens. Thus, lambs *in utero* were unable to generate an antibody response to diphtheria toxin, *Salmonella*, and ovalbumin, but could respond to the bacteriophage ϕ X, ferritin, and ovalbumin in descending order with respect to gestational age (Silverstein *et al.*, 1963). These observations have since been extended in detail, primarily in murine systems. In the latter, the immune response of neonatal mice to haptens such as 2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP) (Press and Klinman, 1974; Sigal, 1977; Riley *et al.*, 1986), phosphorylcholine (PC) (Sigal, 1977), influenza hemagglutinin virus antigens (Cancro *et al.*, 1979), Ars (Sigal, 1977, 1982; Slaoui *et al.*, 1984), and α 1,3-dextran (Dex) (Stohrer and Kearney, 1984; Froscher and Klinman, 1985) in several inbred strains exhibits a reproducible, restricted, and age-related hierarchy in the frequency of antigen-responsive B cells in splenic focus assays. The restricted nature of most of these responses diversifies considerably as the animal matures. In these systems, which have been examined primarily in BALB/c, a restricted response to DNP and TNP occurred in the first 4–5 days after birth after which time these responses began to diversify. The response to neonatal BALB/c mice to immunization with phosphorylcholine, as defined by the T15 idio type, was expressed at an extremely low frequency when compared to frequencies of the DNP and TNP responses in the first 4–5 days of life. At day 6, however, the PC response was expressed in comparable frequencies with the DNP and TNP responses (Sigal *et al.*, 1976). The Dex response arises at day 4 and is expressed at a low value until after day 30, when the major idiotypes expressed in the Dex response (e.g., J558 and MOPC104E) increase in frequency (Stohrer and Kearney, 1984). These data suggest that the maturation of the immune response appears to follow an ordered program.

2. *In Vitro* Analyses

Studies in A-MULV-transformed B cell lineages (Yancopoulos *et al.*, 1984; Reth *et al.*, 1986b) as well as in fetal liver hybridomas and hybridomas derived from neonatal mice (Yancopoulos *et al.*, 1984; Perlmutter *et al.*, 1985b) suggest that there is a preferential utilization of V_H gene segments in rearrangement to the recombined D_H - J_H

during B cell differentiation. In these investigations, there was a marked bias for the use of the 7183 V_H family, specifically the V_H81X gene segment. This bias begins to disappear during the first 2 days of life in neonatal mice (Perlmutter *et al.*, 1985b), although these data are limited with respect to the number of hybridomas examined; in mice that were 2 days old, there was an equal distribution of 7183, Q52, and J558 V_H gene segment usage. S107 and J606 V_H gene utilization was evident as well but with much less frequency. The frequencies at which other V_H families appeared were not measured. The conclusion from these studies was that, based on the accepted V_H map (Brodeur *et al.*, 1984), preferential usage of V_H gene segments generally reflected their proximity to the D_H - J_H gene segment clusters.

The functional significance of the utilization of D_H - J_H proximal V_H gene segments is not clear. Perlmutter *et al.* (1985b) hypothesized that limited utilization of V_H gene segments *in utero* might minimize the possibility of fetal responses to maternal or self-antigens prior to the development of regulatory mechanisms that prevent a response to the latter. As an alternative, we suggest that preferential utilization of V_H segments may also reflect an evolutionary strategy whereby the V_H gene segments that exhibit the highest utility to antigens with which the neonate first comes into contact are ordered and proximal to D_H and thus are most likely to be expressed. As the neonate and its immune system mature, its antigenic universe expands together with its ability to bring into play immunoregulatory controls (see Froscher and Klinman, 1985) that may aid in the expression of more 5' distant and relevant V_H gene segments with the result of randomizing V_H gene expression. Yancopoulos, *et al.* (1984) suggest that the programmed rearrangement of V_H gene segments reflects the possibility that the recombinase that mediates rearrangements operates in an associative (Wood and Tonegawa, 1983) or one-dimensional tracking model.

Discrepancies in V_H gene utilization frequencies and the V_H map soon became apparent. In the study of Riley *et al.* (1986), the response to DNP in neonates is dominated by a V_H gene segment that derives from the 3660 V_H family. Since the 3660 V_H family had been mapped as one of the more distal V_H families with respect to the D_H - J_H clusters (Brodeur *et al.*, 1984) and since the utilization frequency of this V_H family decreased significantly during adulthood, Riley *et al.* (1986) interpreted their results to indicate that proximity to D_H - J_H did not always determine the preferential usage of a given V_H gene segment.

Wu and Paige (1986) utilizing RNA colony blot hybridizations from

colony-forming B cells (CFU-B) derived from spleen and fetal liver showed a frequency of V_H utilization that was inconsistent with the accepted V_H map for many of the V_H families; in some cases, V_H usage frequencies exhibited a strain-specific pattern. In this regard, it is interesting that BALB/c (Igh^a) and AKR (Igh^d) mice exhibit a markedly different neonatal response to myoglobin with BALB/c able to respond to myoglobin after 2 weeks of life, while AKR are not able to respond until a month after birth (Sherwin and Rowlands, 1975). This was also consistent with the results in the NIH Swiss outbred mouse-derived bone marrow A-MULV transformant in which a Q52 V_H gene was preferentially utilized (Reth *et al.*, 1986a). Since the 7183 and Q52 V_H families were shown to be interdigitated, there is the possibility that in NIH Swiss, a Q52 V_H gene segment is more proximal to D_H than 7183 in BALB/c (Reth *et al.*, 1986a).

Thus, the major questions arising from the above studies are 3-fold. First, are there immunoregulatory mechanisms in place that can supersede an ordered rearrangement process, even in neonatal mice? Second, how does the V_H repertoire become more balanced or more "accessible" during the maturation of the organism? Third, is the V_H map the same in all strains? Clearly, an understanding of the organization of the V_H complex is a key aspect with respect to the answers to these questions.

D. DELETION MAPPING ANALYSES POSITIONS THE J558 V_H FAMILY AS ONE OF THE MOST 5' IN THE V_H COMPLEX

We have examined the V_H family order in the A/J, BALB/c, and C57BL/6 strains by deletion mapping analyses (Rathbun *et al.*, 1987). In order to be utilized for deletion mapping experiments, the B cell lines had to meet two criteria. These were that the B cell lines contained a single VDJ rearrangement and that the VDJ rearrangement had resulted in the deletion of intervening DNA between V_H , D_H , and J_H . Each of the B cell lines used in these analyses met these two criteria. 91A3 DNA was then digested with *EcoRI* and probed with the V_H A fragment (Fig. 3) from the cDNA 93G7, which contains the rearranged V_H 1.8 J558 gene. Figure 11A shows that 91A3 has retained most of the J558 V_H family defined by this probe but has deleted at least eight hybridizing fragments (arrows to the right, Fig. 11A). The latter result is further support of V_H - D_H rearrangement resulting in deletion of intervening DNA and places V_H 1.8 at the 3' end of the A/J J558 V_H family. When *EcoRI* digested DNA of the Ars C A/J hybridoma 94B10, which utilizes a 3660 V_H gene, was probed with the V_H A fragment, the entire J558 V_H family was present (Fig.

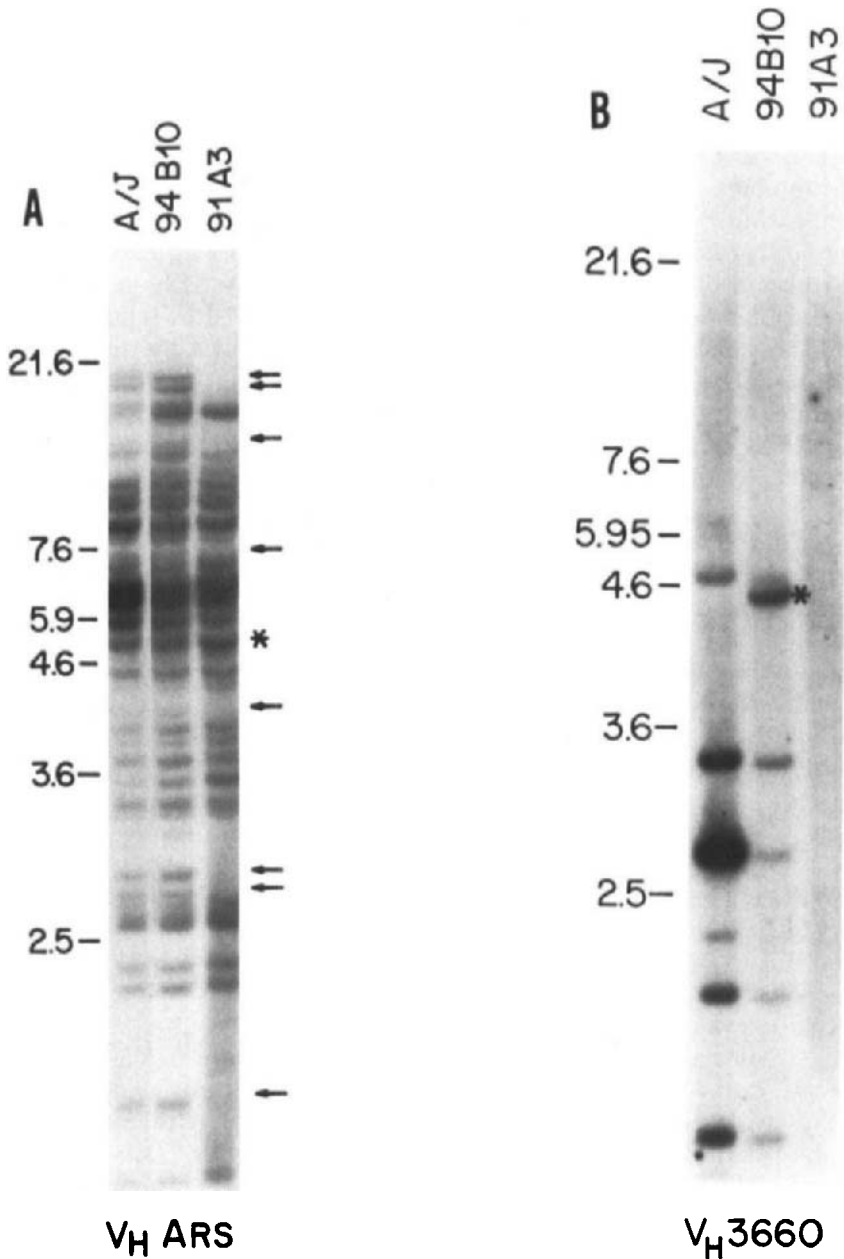


FIG. 11. The *J558* V_H family is positioned 5' of the *3660* V_H family with respect to the D_H - J_H clusters in A/J mice. Deletion mapping was carried out on *EcoRI*-digested DNA of A/J liver and hybridomas 91A3 and 94B10 (which utilized a *J558* and a *3660* V_H gene segment, respectively). Asterisks denote the rearranged V_H genes in each V_H family. Probes are derived from (A) 93G7, which recognizes the *J558* V_H family and (B) M460, which hybridizes to the *3660* V_H family. From Rathbun *et al.* (1987).

11A). This result was in conflict with the accepted map, the latter of which places 3660 5' of *J558*; a hybridoma using a 3660 V_H gene would have been expected to delete the entire *J558* V_H family. The reciprocal experiment was then performed; 91A3 and 94B10 DNA was probed with a 3660 V_H fragment. The results in Fig. 11B clearly show that the 3660 V_H family was absent in 91A3 and present in 94B10, placing the rearranged 3660 V_H at the 3' end of the 3660 V_H family in 94B10 and the 3660 V_H family 3' of *J558*. Thus, the positioning of the 3660 V_H family 3' of *J558* instead of 5' was verified in two independently derived A/J hybridomas. The two A/J hybrids were then probed with V_H fragments representative of each of the remaining seven V_H families. The results, summarized in Fig. 12 and Table III, show significant differences to the accepted V_H map by generating a V_H map order of 5'-3609-*J558*-(*VGAM3-8*, *J606*, *S107*)-3660-(*X24*, *Q52*, *7183*)-3'-*D_H*. The V_H families in parenthesis could not be mapped relative to each other. Importantly, Table III also shows that BALB/c and C57BL/6 B cell lines gave similar results as the 91A3 hybridoma except that the S43.10 C57BL/6 hybrid, which has rearranged a *J558* V_H gene segment, had deleted most of the 3609 V_H family. This suggested that the majority of the comparatively small C57BL/6 3609 V_H family resided 3' of *J558* (Rathbun *et al.*, 1987). Collectively, the results show that in three independent Igh haplotypes, *J558* is one of the most 5' V_H families in the V_H complex. Moreover, the results provide for the first time a relative V_H map of all nine murine V_H families described to date with respect to two V_H families in A/J and specific rearranged *J558* genes in BALB/c and C57BL/6.

E. THE V_H MAP GENERATED BY DELETION MAPPING IS CONSISTENT WITH THE PROXIMAL USAGE MODEL OF V_H GENE SEGMENTS DURING REARRANGEMENT

The 3660 v_H gene family has been mapped toward the distal end of the V_H complex in the accepted V_H map (Riblet and Brodeur, 1986). The present study places it significantly more D_H proximal in A/J and 3' of *J558* in BALB/c and C57BL/6. There is evidence for preferential expression of a 3660 V_H gene in the BALB/c neonatal antibody repertoire (Riley *et al.*, 1986) and preferential utilization of 3660 V_H

5'-3609-*J558*-(*VGAM3-8*, *J606*, *S107*)-3660-
(*X24*, *Q52*, *7183*)-3'- D_H

FIG. 12. The murine V_H map. Some of the V_H families are interdigitated (see text).

TABLE III
ANALYSIS OF DNA OF VARIOUS B CELL LINES CONTAINING A SINGLE VDJ
REARRANGEMENT WITH DNA PROBES FOR NINE V_H GENE FAMILIES

B Cell Line	V _H Family										
	V _H ^a	Igh	3609	J558	J606	VGAM3-8	S107	3660	X24	Q52	7183
91A3	(J558)	e ^b	± ^c	± ^d	-	-	-	-	-	-	-
94B10	(3660)	e	± ^c	+	+	+	+	+	-	-	-
BCI ₁	(J558)	a	± ^e	± ^f	-	-	-	-	-	-	-
5G8	(J558)	a	± ^e	+	-	-	-	-	-	-	-
S43.10	(J558)	b	± ^f	± ^d	-	-	-	-	-	- ^g	ND ^h

^a Productively rearranged heavy chain variable region gene family.

^b V_H haplotype designations are e, A/J; a, BALB/c; b, C57BL/6.

^c At least one 3609 V_H gene is absent.

^d Presence and absence of family members of the productively rearranged V_H gene.

^e Four 3609 V_H genes are absent.

^f Three 3609 V_H genes are present 5' of 186-2.

^g One Q52 V_H gene present.

^h ND, Not done.

genes in B cell lineages derived from fetal liver (Wu and Paige, 1986). Biased expression of the small X24 V_H family as well as the J606 V_H gene families is likewise observed in RNA colony blot assays in colony-forming pre-B cells (CFU-B) from BALB/c and BDF₁ fetal liver (Wu and Paige, 1986); mRNA hybridizing to the 3609 V_H gene family is the most infrequently expressed V_H gene family in these two strains. However, the 3609 V_H gene family is more frequently utilized in C57BL/6 (Wu and Paige, 1986). Our results show that the majority of the 3609 V_H gene family in C57BL/6 is 3' of its J558 rearranged V_H gene. The more precise mapping of 3609 interspersions in A/J places a 3609 V_H gene segment 3' of the 3660 V_H family. Thus, one or more of the interspersed C57BL/6 3609 V_H gene segments may be significantly D_H proximal. If preferential rearrangement of V_H gene segments in relatively "unselected" or immature systems reflects their proximity to the D_H-J_H clusters (Yancopoulos *et al.*, 1984; Perlmutter *et al.*, 1985b; Riley *et al.*, 1986; Wu and Paige, 1986), then the above results are more consistent with the V_H maps generated by deletion mapping than the V_H gene family organization generated previously.

Since details of the experimental design and most of the primary data utilized to generate the accepted V_H map are still unavailable, it is difficult to critically evaluate the discrepancies in the two V_H maps.

However, one possibility for these differences is suggested in some early as well as recent observations concerning the V_H complex. Riblet (1977) noted that the frequency of double recombinations within the V_H complex was higher than expected, particularly with respect to the prealbumin marker. Since a recent study has reoriented this marker in association with the V_H complex instead of IgC (Erikson *et al.*, 1986), the recombination frequency between V_H complex and prealbumin may reflect hotspots of recombination within the V_H complex. In analyzing strain-specific RFLPs in the A/J \times BALB/c recombinant inbreds, hotspots of recombination within the V_H complex may have resulted in multiple recombinations and thus obscured the V_H family organization. Additionally, certain strain-specific RFLPs may, in fact, be interspersed (see below). Thus, mapping the V_H complex by this method may contain further complications.

F. THE V_H FAMILIES ARE INTERSPERSED IN THE INBRED STRAINS

Previous work suggested that related V_H genes in the inbred strains are organized into nonoverlapping clusters (Cory *et al.*, 1980; Givol *et al.*, 1981; Kemp *et al.*, 1981; Crews *et al.*, 1981; Rechavi *et al.*, 1983; Perlmutter *et al.*, 1984). That some V_H gene families can be characterized in this manner is generally supported by our studies, however, our data suggest that at least some V_H families are organized into irregular clusters (Rathbun *et al.*, 1987). The former observation is supported by the observation that some V_H gene segments in A/J, BALB/c, and C57BL/6 are interspersed from other related V_H genes, implying the potential for other V_H family members to be similarly interdigitated. Indeed, Kemp *et al.* (1981) showed a V_H gene segment that in retrospect appears to hybridize in a pattern uncharacteristic of the J606 V_H family in *EcoRI*-digested BALB/c DNA was linked about 14 kb 5' of a V_H gene whose sequence places it in the J606 V_H gene family. More recent studies show that the Q52 and 7183 V_H gene families are interdigitated in the NFS/N inbred strain and NIH Swiss outbred mice (Kleinfield *et al.*, 1986; Reth *et al.*, 1986b). We have shown that at least one 3609 V_H gene in A/J, three in C57BL/6, and four in BALB/c do not reside within their respective V_H families. In addition to the interspersion of the 3609 V_H family in C57BL/6, a Q52 V_H gene is interdigitated 5' of, or within, the J558 V_H family in this strain (Table III). Taken together, data from four independent V_H family haplotypes as well as from NIH Swiss outbreds indicate that some V_H family interdigitation may be a general characteristic of the murine V_H loci.

VI. Molecular Genetics of the *Ars A* Light Chain

A. THE MURINE κ LOCUS

The κ locus, located in the mouse on chromosome 6 (Swan *et al.*, 1979), differs in several aspects from the heavy chain locus. Even though the exact number of V_{κ} genes and their grouping in subfamilies is not completely known, an estimation of 200–400 V_{κ} genes divided in 30–40 families is probably an accurate one (Potter, 1978). Therefore, compared to V_H gene segments, there are fewer V_{κ} gene segments but more V_{κ} families usually formed by groups of 1–10 members. The number of pseudogenes found in different studies is 25–30% of the genes sequenced (Joho *et al.*, 1984). Taking into account that N segments have not been described for the L chains the number of V_{κ} regions that can be formed in a primary immune response (before somatic mutation) is estimated to be 2000–4000. Clearly, combinatorial diversity in the light chain is more limited than in the heavy chain in which at least 500,000 different structures can be formed with the germline elements (Berek *et al.*, 1985).

Another important aspect of the κ locus is that it is much less polymorphic than the heavy chain locus at least with respect to inbred strains of mice. In fact, the vast majority of inbred strains are almost identical to each other at the κ locus when studies by either peptide mapping, isoelectric focusing, or Southern filter hybridization, (Edelman and Gottlieb, 1970; Gibson, 1976, Laskin *et al.*, 1977, Gottlieb *et al.*, 1981); only the strains RF/J, AKR/J, C58/PL/J, and SJL are clearly polymorphic with SJL being the most different at least in some V_{κ} families such as $V_{\kappa}2I$. In the past, this modest polymorphism created some controversy as to the location of the genetic structure(s) responsible for expression of cross-reactive idiotypes: the heavy chain, the light chain, or both (Huppi *et al.*, 1985). Even though studies to locate idiotypic determinants may be obscured by the fact that antiidiotypic reagents are generally raised against the heavy–light chain complex, the majority of idiotypes are formed by a combination of heavy *and* light chain. However, when the “nonpolymorphic” strains were used for genetic studies of idiomorph expression using backcrosses of F_1 (idiomorph positive \times idiomorph negative) mice to the idiomorph-negative parental strain, the results indicated that the expression of the idiomorph was not linked to the light chain. As we will see below, studies using the known polymorphic light chains strains (i.e., RF/J, AKR/J, SJL, C58/PL/J) and the idiomorph-negative parental strain have shown that an appropriate light chain is essential as well.

B. LIGHT CHAINS OF THE Ars A HYBRIDOMAS AND THEIR ROLE IN ANTIGEN BINDING AND CRI EXPRESSION

A total of 10 different Ars A hybridomas from A/J mice and one from a C.AL-20 mouse, all with similar affinities for the immunizing hapten, *p*-azophenylarsonate, but with different levels of expression of the CRI (including the CRI-negative molecule 91A3) have been generated and their light chains sequenced at the protein or mRNA level in three different laboratories (reviewed in Meek *et al.*, 1987). These antibodies also differ in their expression of private idiotypes, in their reactivity with monoclonal antiidiotypic antibodies, and in their fine specificity for antigen. The striking feature that becomes apparent when these sequences are compared is their near identity as they vary from one another only by from one to seven amino acids with less than 3% overall variation (Fig. 13). All the sequences belong to the $V_{\kappa}10$ subgroup (Siegelman and Capra, 1981). This high degree of similarity is consistent with the observation that any one of these light chains will substitute for any other in serological analyses of the CRI and also shows that BALB/c mice can contribute CRI-positive light chains as the C.AL-20 mice carry the light chain locus of the BALB/c strain (Lamoyi *et al.*, 1980a). The sequences show that some molecules bear repeated substitutions at positions 30, 92, and 93. There are two alternative explanations for this finding; either parallel but independent somatic mutations account for these substitutions or at least two very similar but separate germline V_{κ} genes operate in the Ars A family (Siegelman and Capra, 1981).

The variability at positions 92 and 93 is striking. At these two positions three different amino acids have been observed and at the nucleotide level two of these interchanges require two base change substitutions. This variability could be produced by a DNA repair mechanism possibly brought about by the putative recombinase system that mediates the V - J recombination at position 96 (Siegelman and Capra, 1981). The obvious alternative mechanism is somatic hypermutation taking place at position 92 and 93 followed by selection. When one tries to correlate structural data with serological studies it becomes evident that the more amino acid differences the light chain has from the strongly CRI-positive molecule 93C7 (or from a consensus—presumably germline sequence) the weaker their expression of the CRI.

The 91A3 light chain shows (as in the heavy chain) the highest number of substitutions from germline. Early studies with the C.AL-20 strain suggested that the CRI was linked to the heavy chain

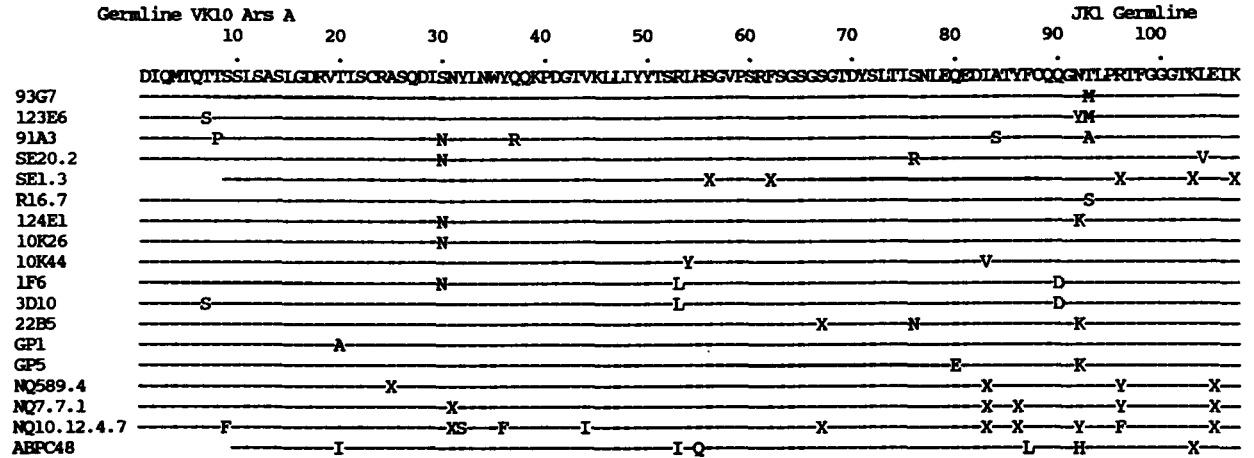


FIG. 13. Comparison of amino acid sequences of light chain variable regions with the deduced germline V κ -10-Ars A and J κ 1 amino acid sequences. (—), identical residues, (X) region in which no amino acid assignment is available. The sequences of the V κ -10-Ars A germline gene and J κ germline gene are from Sanz and Capra (1987). The sequences of 93G7, 91A3, 123E6, R16.7, and 124E1 are from Siegelman and Capra (1981). The sequences of SE20.2 and SE1.3 are from Haba *et al.* (1986). The sequences of 10K26, 10K44, are from Ball *et al.* (1983). The sequence of GP5 is from Meek *et al.* (1987). The sequences of NQ589.4, NQ7.7.1, and NQ10.12.4.7 are from Kaartinen *et al.* (1983). The sequences of 1F6 and 3D10 are from Smith and Margolies (1984). The sequence of ABPC48 is from Legrain and Buttin (1985). From Meek *et al.* (1987).

exclusively. The C.AL-20 strain, a congenic strain in which the heavy chain genes derive from the A strain and the light chain genes derive from BALB/c (which in turn is CRI negative), is CRI positive. These results suggested linkage of CRI expression to the heavy chain locus. The alternative explanation for these findings is that BALB/c and A/J mice are nonpolymorphic at the κ locus (at least $V_{\kappa}10$ -Ars A), and, therefore, both are able to provide appropriate CRI-positive light chains. The latter was proven by performing backcrosses between the A/J strain as the CRI-positive strain and the light chain polymorphic PL/J as the CRI-negative strain (Laskin *et al.*, 1977). Only the progeny that inherited both the heavy and light chain genes from the A/J strain expressed the idiootype upon immunization. These experiments confirmed previous results indicating that at the chemical level, the light chain was necessary for idiootype expression as well.

These studies were extended to include chain recombination experiments in which hybrid molecules were constructed from isolated heavy and light chains of Ars A hybridomas which differ in their expression of the CRI. In these studies, the 91A3 light chain (CRI⁻) was able to restore the CRI expression to the heavy chain of 93G7 (CRI⁺). Neither heavy or light chains alone expressed the CRI. However, light chains of both CRI⁺ and CRI⁻ Ars A hybridomas were able to restore CRI expression to an appropriate heavy chain, whereas light chains from Ars B or Ars C antibodies were not (Milner and Capra, 1983). Collectively, these studies argue that even though structural variations in the heavy chain account for different levels of expression of the A/J arsonate CRI, the idiootype depends on the combination of the appropriate heavy and light chain. These findings are in agreement with other idiotypic systems in which the heavy-light chain pairs are strongly conserved.

C. THE CRUCIAL ROLE OF LIGHT CHAIN Arg 96

Provided that the appropriate heavy and light chain are combined to form an Ars A antibody, the presence of an arginine at the V-J junctional position 96 in the light chain has been shown to be essential for Ars binding (Jeske *et al.*, 1984). All the light chains of Ars A antibodies so far sequenced have an arginine at that position. Furthermore, when the light chain of NQ5, an antiphenyloxazolone antibody which is essentially identical to the 91A3 light chain except for having a tyrosine at position 96, is recombined with the 93G7 heavy chain, the resulting molecule is unable to bind arsonate (Jeske *et al.*, 1984). The reciprocal experiment in which the antioxazolone heavy chain is recombined with the anti-Ars light chain results in a

molecule that has lost its ability to bind oxazolone. These experiments were the first to formally document that the V-J junctional position is crucial (at least in some systems) for antigen binding. This finding was important because although recombinational events leading to junctional diversity have long been thought to be an important mechanism for the somatic diversification of germline elements, it has been difficult to demonstrate both the creation of an amino acid by somatic recombination and the essential role of that amino acid in antigen binding since each of the germline gene segments and their recombination product has not been analyzed in a system in which the junctional residue was essential to antibody function.

The following lines of evidence support the idea that in the Ars system the essential arginine at the position 96 is generated by intracodonic recombination between germline V_{κ} and J_{κ} segment:

1. An arginine codon has never been found at the 3' end of any V_{κ} gene segment sequenced.
2. It was known, by amino acid and mRNA sequence studies, that the Ars A family uses the A/J allelic counterpart of the BALB/c mouse $J_{\kappa}1$ gene segment and this latter segment does not have an arginine codon at its 5' end.
3. Two independently derived Ars A hybridomas from the C.AL-20 strain contained an arginine at position 96, arguing that this residue could not be explained by allelic polymorphism in $J_{\kappa}1$ between the A/J and BALB/c strains.

The mechanism for the formation of arginine 96 was formally proven when the rearranged light chain of 9IA3 was isolated from a genomic library and the 0.4-kb fragment containing the V_{κ} coding region was used as a probe to screen an A/J liver DNA library to obtain the V_{κ} germline equivalent (Sanz and Capra, 1987). From the same A/J library, the J_{κ} locus was isolated by using a J_{κ} probe from BALB/c DNA. All three clones were mapped with restriction enzymes and sequenced. The results showed that 9IA3 V_{κ} and A/J V_{κ} germline segment differed by 9 nucleotides in the coding region and only by 1 nucleotide of the 450 sequenced in the 5' flanking region arguing that the gene isolated was most likely the one that is rearranged to $J_{\kappa}1$ to produce the 9IA3 light chain gene (Fig. 14). Neither by restriction enzyme mapping nor by sequencing 214 nucleotides containing $J_{\kappa}1$ segment was allelic polymorphism detected between the A/J and the BALB/c sequence. The sequence data conclusively showed that arginine 96 in 9IA3, encoded by a CGG

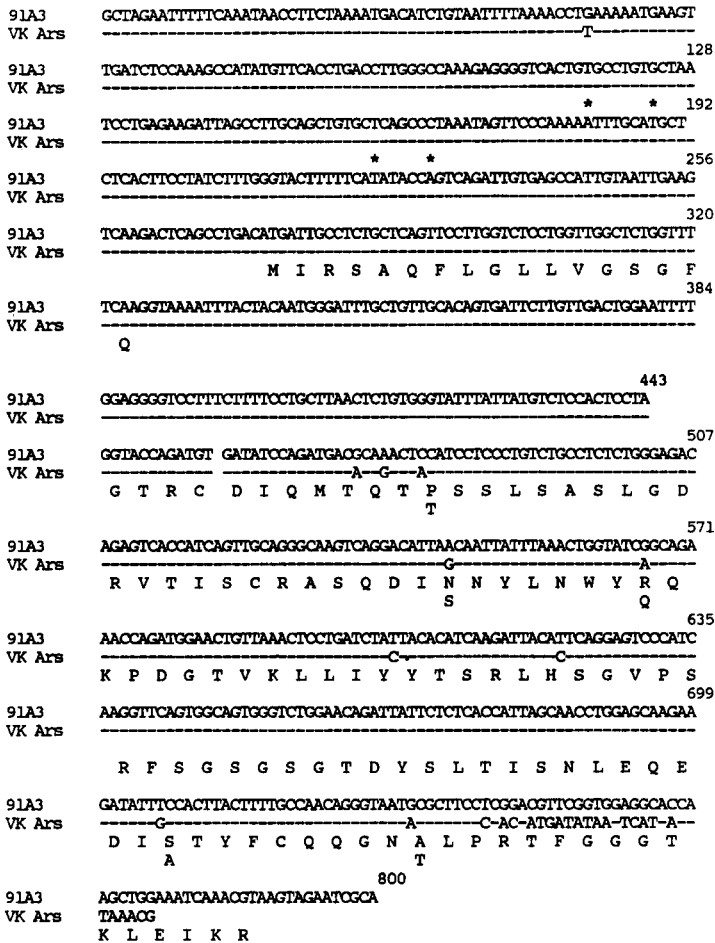


FIG. 14. Complete nucleotide sequence of the productive allele in the hybridoma 91A3 and the $V_{\kappa}10$ -Ars A light chain gene segment. Boxed sequences are the recombination recognition sequences of V_{κ} Ars. From Sanz and Capra, 1987.

codon, was not present in either the 5' end of $J_{\kappa}1$ nor the 3' end of the V_{κ} germline gene (Fig. 15). However, the appropriate nucleotides were present at the end of both gene segments, such that the CGG codon could be generated by recombination between $V_{\kappa}10$ -Ars A and $J_{\kappa}1$. To further elucidate the role of position 96 in arsonate binding,

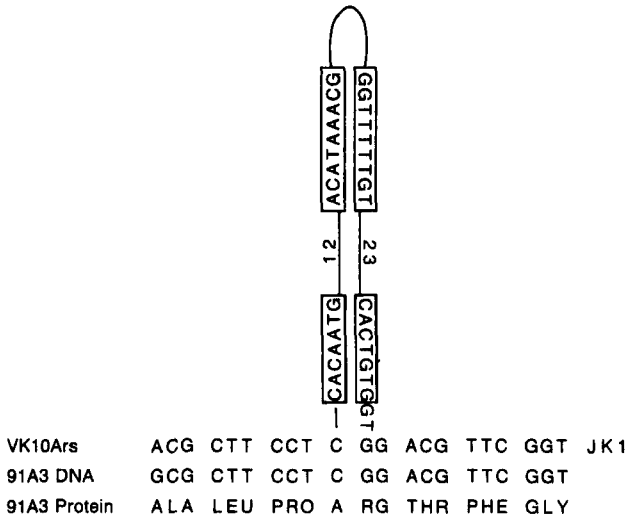


FIG. 15. Recombination model for V-J joining illustrating the creation of the CCG codon at position 96 in 91A3 light chains. From Sanz and Capra (1987).

different substitutions have been introduced by oligonucleotide-directed mutagenesis and the ability of the Ars A antibodies bearing different residues at the junction position is being tested for binding to the arsonate hapten.

D. THE $V_{\kappa}10$ FAMILY OF GERMLINE GENES

Southern filter hybridization experiments in which A/J or BALB/c liver DNA was digested with different enzymes and probed with the V_{κ} coding region from the 91A3 light chain gene revealed three hybridizing fragments, one of which had at least double intensity in A/J DNA and severalfold higher intensity in BALB/c DNA. Other enzymes, such as *Bam*HI, revealed an extra fragment presumably containing several genes in BALB/c that are not present in A/J DNA. These data suggest that the $V_{\kappa}10$ family contains a relatively small number of genes that varies from approximately 4 genes in A/J mice to probably 8–10 genes in BALB/c. Of the 4 genes present in A/J, 2 genes from the 5.2-kb *Eco*RI fragment and 1 from the 3.7-kb *Eco*RI fragment have been cloned, and two of these have been sequenced. We believe that one of the $V_{\kappa}10$ gene segments contained in the 5.2-kb *Eco*RI fragment represents the primordial gene of the $V_{\kappa}10$ family and is the only one that rearranges to produce Ars A antibodies based upon the following lines of evidence:

1. The sequence of this A/J $V_{\kappa}10$ gene segment is consistent with the consensus generated for the Ars A family of light chains (Fig. 14).

2. The $V_{\kappa}10$ -Ars A gene segment exhibits nearly complete identity in its 5' flank of the 91A3 light chain gene (Fig. 14). Additionally, a 5' flanking fragment derived from this gene hybridizes more strongly to the 91A3 V_{κ} 5' flank than the 5' flanking region of any of the other genes. It follows, therefore, that all other Ars A light chains, which in general are more similar to a consensus sequence than is 91A3, are similarly derived from the same germline gene segment.

3. When Ars A hybridomas are analyzed by Southern filter hybridization with either a 5' $V_{\kappa}10$ -Ars A, the $V_{\kappa}10$ -Ars A coding region or J_{κ} probes, the rearranged fragment exhibits an identical size in all hybridomas with the three different probes arguing that the same $V_{\kappa}10$ segment is always rearranged (Fig. 16).

4. The restriction map of the second gene contained within the 5.2-kb *EcoRI* hybridizing fragment is different enough using the same enzymes used for the Southern analysis to rule out the possibility that this gene could be used in some hybridomas (I. Sanz and J. D. Capra, unpublished observations).

5. The sequence of the gene contained in the 3.7-kb *EcoRI* fragment is more distant from the consensus sequence and the 91A3 gene in both its coding region and its 5' flank than the $V_{\kappa}10$ gene segment contained within the 5.2-kb *EcoRI* fragment (I. Sanz and J. D. Capra, unpublished observations). This allows us to conclude that this gene is not rearranged in the Ars A hybridomas.

The reasons why the other members of the $V_{\kappa}10$ family are not used in the immune response to Ars are unclear. One obvious reason could be that the structural features of the other genes prevent them from producing a protein able to bind Ars either due to the structure of the hypervariable regions or due to their inability to generate an arginine at position 96. In this regard it is important to point out that the 3.7-kb gene has a very distinct third hypervariable region (I. Sanz and J. D. Capra, unpublished observations) and also cannot generate (at least by the recombinational mechanism previously postulated) a junctional arginine. Recently, Wysocki *et al.* (1987) have independently confirmed these observations.

E. IDENTITY OF THE $V_{\kappa}10$ -Ars A GENE SEGMENTS OF THE A/J AND BALB/C STRAINS

Figure 13 shows the amino acid sequences of the V_L regions of 18 hybridomas: 11 Ars A antibodies from the A/J strain (R16.7, 93G7,

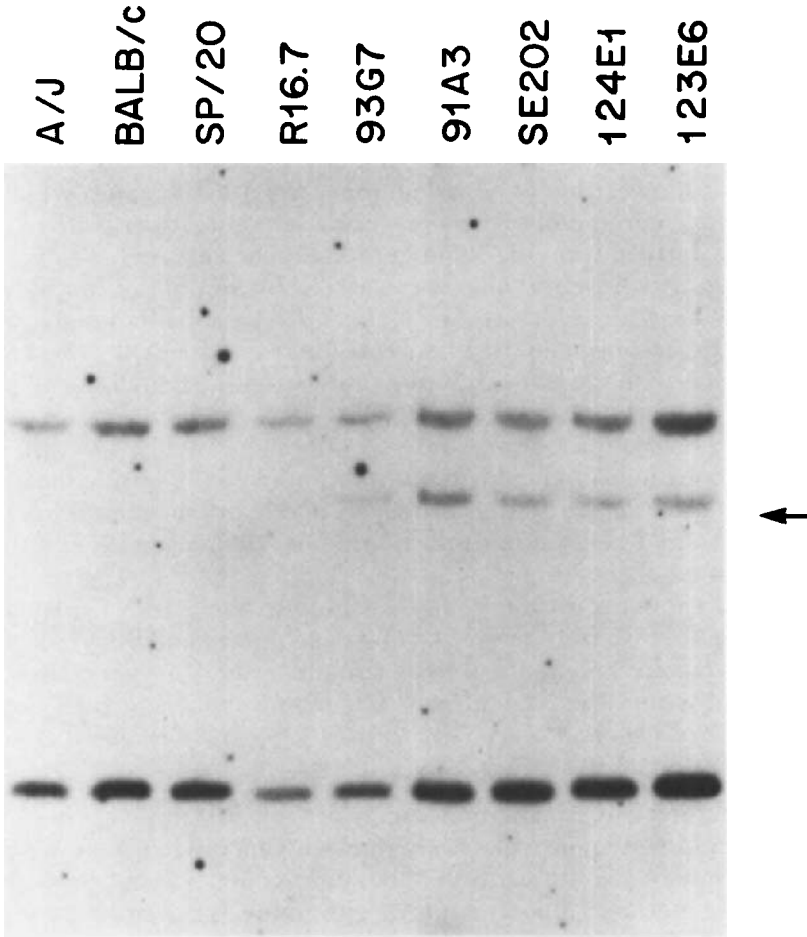


FIG. 16. DNA from A/J and BALB/c liver, the SP2/o fusion partner, and several Ars A hybridomas digested with *Sac*I and hybridized with the $V_{\kappa}10$ -Ars A coding-region probe. The filter was stripped and reprobred with a J_{κ} probe. The same restriction fragment is identified with both probes. From Sanz and Capra (1987).

123E6, 124E1, 91A3, SE20.2, SE1.3, 10K26, 10K44, 1F6, 3D10), 1 anti-Ars antibody from the BALB/c strain (22B5), 2 Ars A antibodies from the C.AL-20 strain (GP1, GP5), 3 antiphenyloxazolone antibodies from the BALB/c strain (NQ589.4, NQ10.12.4.7, NQ7.7.1), and 1 BALB/c antilevan antibody (ABPC48). These sequences are compared in Fig. 13 to the deduced amino acid sequence of the germline

$V_{\kappa}10$ -Ars A gene from the A/J strain and to the germline $J_{\kappa}1$ sequence derived from the A/J strain. As can be seen the amount of variation at the amino acid level is fairly limited. There are only six positions that contain repeat substitutions—positions 7 (Thr-Ser), 30 (Ser-Asn), 53 (Arg-Leu), 91 (Gly-Asp), 92 (Asn-Lys), and 93 (Thr-Met). We had previously shown that the hybridoma products with substitutions at positions 30 and 93 most likely derive from the same germline gene. The studies with J_{κ} and V_{κ} probes shown in Fig. 16 argue that the Lys-Asn substitution at position 92 is also somatically generated. With the exception of lysine at position 92 in GP5, none of the alternative amino acids was seen in the BALB/c or C.AL-20 proteins.

Figure 17 shows the nucleotide sequences of 10 of the above V_L regions, which are compared to the nucleotide sequence of the A/J $V_{\kappa}10$ -Ars A and $J_{\kappa}1$ germline genes. The similarity among these molecules is quite clear. None varies by more than 9 bp (91A3) from the germline V_{κ} sequence. Two (SE1.3 and NQ589.4) express exactly the germline encoded sequence (with two to four uncertainties in their nucleotide sequences).

The most striking feature of these data is the extraordinary homology between the sequences of V_{κ} regions of the BALB/c light chains and that encoded by the A/J $V_{\kappa}10$ -Ars A germline gene. Of the five anti-Ars and antiphenyloxazolone molecules of BALB/c origin (22B5, NQ589.4, NQ7.7.1, NQ10.12.4.7, and GP1), none is more closely related to another than they are to the A/J $V_{\kappa}10$ germline gene. A BALB/c consensus sequence, derived from the expressed sequences of these five molecules, is 100% homologous to that encoded by the A/J $V_{\kappa}10$ -Ars A germline gene.

The lack of even third base pair changes between the BALB/c and the A/J $V_{\kappa}10$ -Ars A gene segments is striking. A similar finding was reported by Kaartinen and Makela (1987), who studied antiphenyloxazolone antibodies derived from the $V_{\kappa}OX1$ (H3) germline gene. mRNA sequences of light chains from six strains (BALB/c, C57BL-Igh⁺, DBA/2, A/J, RF, CE) were virtually identical. In addition, Brown and Fried (1987) have reported the amino acid sequences on a group of BALB/c anti-Ars antibodies of the Ars C family. In the amino terminal 40 amino acids they are structurally identical to Ars C light chains from the A/J strain. However, data from other systems suggest that some allelic differences in the coding region of κ genes may exist. For example, a single amino acid framework region difference, up to amino acid position 42, was observed between light chains from C57BL/6 and BALB/c (T15) antiphosphocholine antibodies (Clarke *et al.*, 1983).

Vk10 Ars A Germline

	10	20	30	40	50	60	70	80	90	100	110	
VK-GL	GATATCCAGATCACACAGACTACATCTCTCCCTCTCTGGGAGACAGAGTCAOCCATCAGTTGCCAGGGCAAGTCAGGACATTAGCAATTATTAACTGGTATCA											
91A3		G	A	C						A		G
123E6			G			X						
SE20.2					M			C		A		
SE1.3												
22B5					G							
GP1						G		C	X		T	
NQ589.4												
NQ7.7.1					X					X		
NQ10.12.4.7			T							X	C	T
ABPC48							T				T	

	120	130	140	150	160	170	180	190	200	210	220	
VK-GL	GCAGAAACCAGATGGAACTGTTAAACTCTTGATCTACTACATCAAGATTACTACTCAGAGTCCCATCAAGGTTTCAGTGGCAGTGGGCTCTGGACAGATTATTCTCTCA											
91A3					T		T					
123E6								X				
SE20.2												
SE1.3						X		XX				
22B5				T						X		
GP1		G							X			
NQ589.4												
NQ7.7.1						G-X				XX-X		
NQ10.12.4.7		A						T		X		
ABPC48					T	G						

JK1 Germline

	230	240	250	260	270	280	290	300	310	320			
VK-GL	CCATTAGCAAACTGGAGCAAGAAGATATTGCCACTTACTTTTGGCCAAACAGGGTAATAAGCTTCTCTCGGAAGTGTGGTGGAGGCCAACAAGCTGGAAATCAA												
91A3					T		G						
123E6	X		X			T	T		X		G		
SE20.2		G								G			
SE1.3								X		X	X		
22B5	A					A			Y				
GP1	X		X			A			X	T			
NQ589.4			XX				TAC		A	G	G	X	A
NQ7.7.1			X	X				XX		X			
NQ10.12.4.7			XX	X		T	GTTC		A	G	G	X	A
ABPC48					A		C				X		

VII. Serologic and Structural Analyses of Ab2 Antibodies in the Arsonate System

A. INTRODUCTION

In 1974, Jerne proposed that regulation of immunoglobulin expression could be mediated by idiotypic interactions at the protein level (Jerne, 1974) via the unique antigenic determinants of immunoglobulin variable regions (idiotypes) described earlier by Kunkel (Kunkel *et al.*, 1963) and Oudin (Oudin and Michel, 1963). The idiotypic determinants of each antibody molecule would be complemented by those of another creating an idiotypic network through which immunoglobulin expression might be controlled. Experiments followed in which manipulation of the immune response was achieved through the use of antiidiotypic reagents, thus, strengthening his hypothesis. Later, the idiootype network hypothesis was extended and Ab2s were grouped into two classes: those that recognize determinants present in the variable region, and those that represent internal images of the original antigen (Jerne *et al.*, 1982). It was suggested that the second group of antibodies might function in establishing the B cell repertoire (operating as endogenous antigen) without requiring exogenous antigen. Thus, the idiotypic network might not be functionally important only in the regulation of immune responses, but may also play a role in the establishment of the immune repertoire.

Antiidiotypic antibodies of both types have been described by several groups in several systems (Dildrop *et al.*, 1984; Legrain *et al.*, 1985). However, the immune repertoire is nearly complete in its capability to respond to protein antigens. The existence, then, of antiidiotypic antibodies does not necessarily infer their relevance. Thus, the physiologic significance of the idiotypic network continues to be a source of debate.

It seems logical (and indeed Jerne proposed) that the network should be germline encoded. If antiidiotypic antibodies are functionally crucial, their generation should not be random, so one might predict that the structures of these antibodies should reflect germline

FIG. 17. Nucleotide sequences of light chain variable region sequences compared to the germline $V_{\kappa}10$ -Ars A gene and the $J_{\kappa}1$ gene from the A/J strain. (—), identical nucleotide; A, adenosine; C, cytosine; G, guanosine; T, thymidine, M, cytosine or adenosine; X, nucleotide undetermined. The sequences of the $V_{\kappa}10$ -Ars A germline gene, $J_{\kappa}1$ germline gene, and 9IA3 are from Sanz and Capra (1987). The sequences of SE20.2 and SE1.3 are from Haba *et al.* (1986). The sequences of NQ589.4, NQ7.7.1, and NQ10.12.4.7 are from Kaartinen *et al.* (1983). The sequence of ABPC 48 is from Legrain and Buttin (1985). The remainder of the sequences are from Meek *et al.* (1987).

sequences. It would follow that an antiidiotypic response to a homogeneous antibody response should be uniform in usage of germline gene segments. Hapten systems are ideal for approaching the question of a germline-encoded network because unlike the responses to many protein antigens, the predominant responses to haptens are often fairly restricted. Structural studies of antiidiotypic antibodies in the antiarsonate system have provided insights to this hypothesis. In addition, immunochemical studies employing these molecules have allowed a fine dissection of the Ars A family of antibodies.

**B. EIGHT MONOCLONAL ANTIIDIOTYPIC ANTIBODIES (Ab2s)
RECOGNIZE PUBLIC IDIOTOPES THAT MAKE UP A PORTION
OF THE CRI-A**

Eight monoclonal antiidiotypic antibodies recognizing distinct public idiotopes which in part comprise the cross-reactive idio type associated with the A/J strain's response to Ars-KLH have been structurally and/or serologically characterized (Moser *et al.*, 1983; Alkan, 1984; Jeske *et al.*, 1986; Meek *et al.*, 1986). Five of the eight Ab2s described are allogeneic. These Ab2s were isolated from BALB/c mice (a strain which normally does not express this idio type) that were first immunized with polyclonal idio type-positive A/J CRI-positive antiarsonate antibodies and then boosted with a monoclonal antiarsonate antibody (Moser *et al.*, 1983). The other three Ab2s studied are syngeneic and were isolated from A/J mice immunized with polyclonal idio type positive A/J antiarsonate antibodies (Alkan, 1984). Table IV shows the reactivities of these Ab2s with a panel of antibodies from the Ars A family. Each varies to some extent, implying that each molecule recognizes a different idio typic determinant on the antiarsonate molecule. The Ars A molecules which express the CRI-A most strongly also express most of the idiotopes defined by these antibodies. Likewise, molecules expressing low levels of CRI-A express only a few or none of the idio typic determinants. This implies that these monoclonals actually recognize structures that collectively comprise the CRI-A.

Through chain recombination experiments, chemical modifications, and sequence information, the amino acid residues in the Ars A molecules which are most important for expression of the idiotypes, defined by the monoclonal allogeneic Ab2s, have been determined (Jeske *et al.*, 1986). The expression of idiotopes H8, E4, and E3 are most affected by amino acid residues in the D_H (Fig. 18). Reactivity with monoclonal 7B7 is most affected by changes in the light chain. Expression of the idio type recognized by 2D3 is strongly affected by the second hypervariable region of the heavy chain. All of the Ars A

TABLE IV
REACTIVITY OF Ab2s WITH ANTIARSONATE ANTIBODIES^a

	CRI	E4	E3	H8	2D3	12S18-1	12S28-16
3665	+	+	+	+	+	+	+
101F11	+	+	-	+	+	+	+
93G7	+	+	+	+	-	+	+
91A3	-	±	-	-	-	-	-
96B8	-	-	-	-	-	-	-
22B5	+	+	-	±	-	-	±
9A5	+	+	+	+	-	±	±

^a Reactivities of polyclonal rabbit anti-CRI antisera (CRI), four allogeneic antiidiotypic antibodies (E4, E3, H8, and 2D3), and two syngeneic molecules (12S18-1 and 12S28-16) with four antibodies of the Ars A family (16.7, 101F11, 93G7, and 91A3), one member of the Ars B family (96B8), and two BALB/c CRI-A-like antiarsonate antibodies as determined by radioimmunoassay using both direct binding and inhibition assays. +, Strong expression; -, no detectable expression; ±, weak, but significant binding. From Jeske *et al.*, 1986.

molecules that have a lysine in position 59 of the heavy chain express this idiotope. Molecules that contain a different amino acid in position 59 do not express the 2D3 idiotope. Two of the syngeneic Ab2s may recognize the same determinant as does E4, because their reactivity patterns are very similar to that of E4 (Table IV).

C. ALLOGENEIC Ab2s IN THE ARSONATE SYSTEM ARE STRUCTURALLY HETEROGENEOUS, WHEREAS SYNGENEIC Ab2s IN THIS SYSTEM ARE HOMOGENEOUS

Four allogeneic Ab2s (Fig. 19) have been analyzed structurally and each derives from different V_H , D_H , J_H , V_κ , and J_κ gene segments (Meek *et al.*, 1986; K. Meek, S. Alkan, J. Urbain, and J. D. Capra, unpublished). The E3 heavy chain derives from the Q52 V_H family. The other three derive from the J558 family, but still are only about 80% similar to one another at the nucleotide level. Since none of these molecules is closely related to any published unrearranged J558 V_H gene segments, we cannot judge whether or not somatic mutation has contributed to their generation. All of these antibodies use different J_H segments within which somatic mutation is apparent. Together, these data argue that the BALB/c strain's response to Ars A molecules is heterogeneous and resembles a response one might expect from any exogenous protein.

In marked contrast, the three syngeneic molecules are nearly identical to one another (Meek *et al.*, 1986; K. Meek, unpublished observations). There is only a single base pair difference between the

A

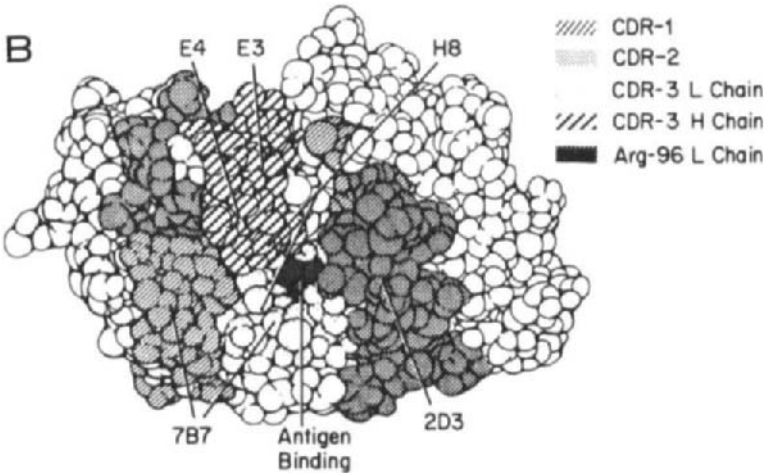
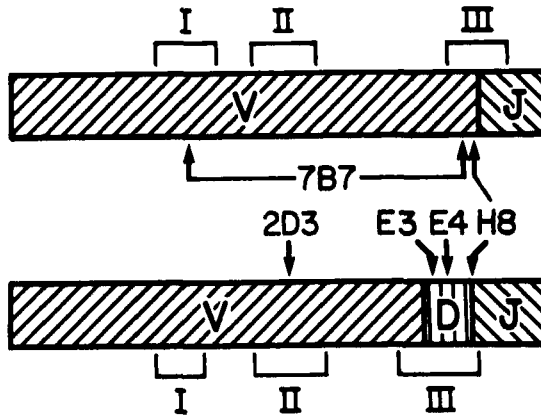


FIG. 18. Proposed localization of idiotopes. (A) The immunoglobulin heavy and light chain variable regions. Arrows point to the proposed location of each idiotope. This representation stresses the relative contribution of each genetically encoded segment in the expression of the CRI. (B) A three-dimensional model of the combining site of 93C7 obtained by superimposing the amino acid residues of 93C7 on the crystallographic structure of McPc 603. This computer generated model was developed by Dr. Charles Mainhardt. The proposed antigen combining site is viewed as a depression at the center of the diagram, with the heavy chain amino acid residues to the right and above and light chain residues to the left and below the site. Complementary determining regions are depicted as labeled. By using the three-dimensional surface as a model, one can visualize how the idiotopes as proposed cluster around the antigen-binding site. This model also stresses the potential interaction of CPR from both heavy and light chains in these determinants. From Jeske *et al.* (1986).

	10	20	30	40	50	60
h8	QVQLQQSGXELVLRP	GTSVKHNSCKASGY	TFNMYMIGWVKORP	GH6LENI	GDTHP	GGGYTNY
2d3	-----A--A--A--L-----S	TS--NX--ES--K-----IY--DY--AR--				
e4	E-----I--EK--A--I-----S	TG--SM--SM--EI-----NID	IYL--G--T--			
e3	-----X--E--SD--LSIT--TV--FSLTSS	GVH--R--P--K-----L--VINA--S--				
	70	80	90	100	110	
h8	MENFRGKATLTADSS	SXTAYNQLSSLTSE	DSXIYYCTRGXIM	VGSYYFAVDYWG	KXFF	
2d3	TQK_KY-----K--S-----A-----AV--A	DDGGSWYFDVNG	AGTTVTVS			
e4	_QK_KXX-----V_K_S-----K--D--AV--A	SGSSYGD_MXQG	TLLTVXS			
e3	_SALNSRL_ISK_N_KSGVFL_MT--RID_TAM--A	DEPLWA_GMDYWGQ	_TSXT			

FIG. 19. Translated amino acid sequences of the heavy chain variable regions of four allogeneic Ab2s. From Meek *et al.* (1986) and K. Meek, S. Alkan, J. Urbain, and J. D. Capra (unpublished).

light chain variable regions of each of these molecules, and the heavy chain variable regions of the three molecules are identical to one another. However, isotypically the heavy chains all differ ($\gamma 1$, $\gamma 2a$, or $\gamma 2b$). These syngeneic molecules derived from 3 of 10 clones selected from a fusion from the pooled spleens of three mice (Alkan, 1984). They were chosen because of their isotypic differences. While clonal relatedness of the three cannot be ruled out, these results argue that the syngeneic Ab2 response in the Ars system is extremely limited in both its level of expression and in its V_H gene segment usage. Again, the degree that these molecules differ from their unrearranged (germline) counterparts cannot be addressed since the latter have not been sequenced.

The observation of restricted use of germline elements in syngeneic Ab2s has been made in two other hapten systems, NP and GAT as well (Dildrop *et al.*, 1984; Legrain *et al.*, 1985). This, in part, supports Jerne's idea of coexistence of complementary sets of variable region genes in the germline. It suggests that within a single strain, a structurally distinct population induces (or is complementary to) a second group of structurally distinct group of antibodies. The extreme heterogeneity of the allogeneic antiidiotypic antibodies would seem to support this idea.

D. THE D_H SEGMENTS OF THE SYNGENEIC AND ALLOGENEIC Ab2s CANNOT BE ACCOUNTED FOR BY ANY KNOWN GERMLINE D_H SEGMENTS

The D_H segments of these molecules cannot be completely explained from existing germline gene segments (Fig. 20). The core of the E4 D_H segment most likely derives from *DFL16.1*. The core of the E3 D_H segment may derive from the noncoding strand of *DSP2.4* (D_H segments are not normally used in both orientations). The 2D3 D_H segment appears to be distinct from any D_H segment sequenced to date. The H8 D_H segment from the H8 hybridoma may be the result of an inverted fusion between the noncoding strand of *DFL16.2* and the coding strand of *DSP2.7*. We have proposed previously that an inverted fusion may explain the conserved "N" segment seen in the majority of Ars A molecules (Milner *et al.*, 1986). Fougereau and colleagues described a similar phenomenon in two GAT antiidiotypic antibodies (Legrain *et al.*, 1985). A similar D_H fusion has been observed in two anticarbohydrate Ab2s (K. Meek, unpublished observations). This mechanism is depicted in Fig. 21.

First, an inverted recombination would occur between the coding strand of *DSP2.7* (using the alternative recombination recognition sequences proposed by Kurosawa and Tonegawa, 1982) and the

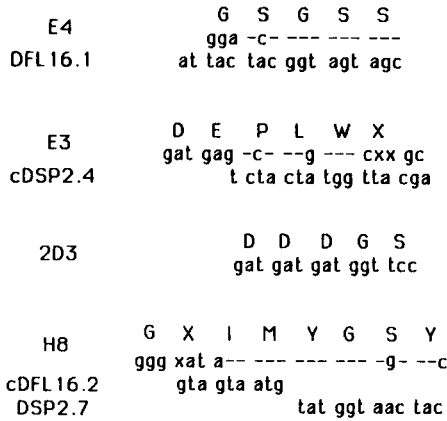


FIG. 20. D_H segments of Ab2 antibodies in the Ars system. Dashes represent identity to the nucleotides directly beneath the Ab2 D_H genes. K. Meek, S. Alkan, J. Urbain, and J. D. Capra (unpublished observation).

noncoding strand of *DFL16.2*. Then, normal D_H to J_H joining between the normal recombination recognition sequences of *DSP2.7* and *JH4* would take place. Finally, V_H to $D_H J_H$ joining would occur between the 5' portion of the noncoding strand of *DFL16.2* and the 3' portion of V_H . The same mechanism must be invoked to explain the D_H segments of all three syngeneic Ab2s.

Since the syngeneic molecules in this system may be clonally related, it is impossible to determine whether or not the event generating the unusual D segment seen occurs repeatedly in Ab2s in the arsonate system as is the case in the GAT system and the anticarbohydrate system. Still, it is remarkable that in three of five Ab2 systems studied, a unique $D-D$ fusion mechanism must be invoked to explain the generation of the D_H sequence.

Although a similar mechanism has been proposed to generate the serine at position 99 in the V_H of Ars A molecules, the proportion of the sequence generated from the inverted fusion in the latter is small compared to the proportion generated from germline elements in the normal orientation (only one amino acid residue). In contrast, nearly *half* of the D_H sequences in these Ab2s appear to be generated from the D_H-J_H fusion process. In order to determine whether or not Ab2s consistently seem to generate their D_H segments through novel mechanisms, a detailed statistical analysis will have to be conducted comparing all known antibody D_H segment structures with their specificities and taking into account the selection pressures put on the system by our sampling method. Still, it is tempting to speculate on

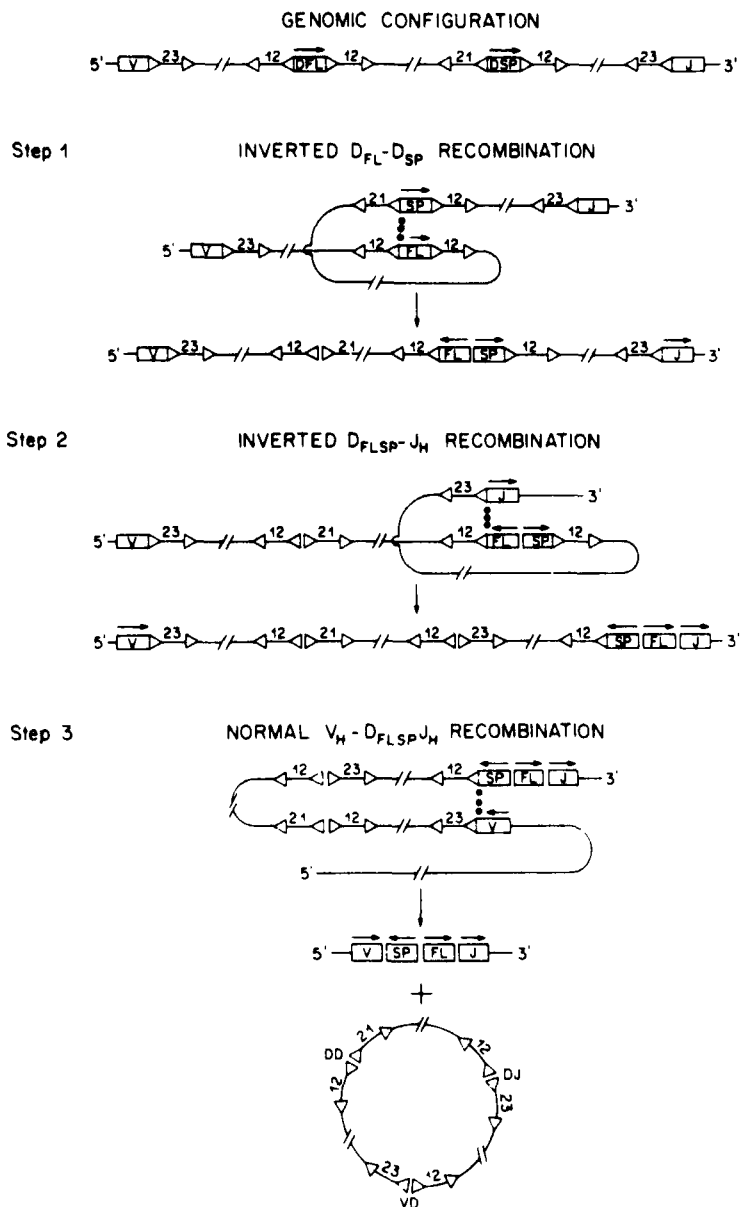


FIG. 21. Diagrammatic model of *D* to *D* joining adapted from Milner *et al.* (1986). Coding regions are indicated by labeled boxes. Arrows above coding regions indicate conventional 5' to 3' coding orientation. Pairs of triangles represent heptamer and nonamer sequences. The lengths of spacers are indicated by numbers between triangles. The positions of the recombinations are identified by vertical dots in steps 1 and 2.

the importance of these novel D_H sequences with respect to anti-idiotypic antibodies.

Several possibilities exist. First, the syngeneic antiidiotypic responses studied to date are all restricted in their level of expression as is the case in the antiarsonate system. If these novel D_H segments are necessary for the Ab2s specificities, and the event that generates these structures represents a relatively rare rearrangement, then molecules with the appropriate specificity would be rare in the B cell repertoire and their expression would be only at low level. But, if the Ars A V_H N segment is derived through this mechanism, it is possible that this unusual rearrangement process is not rare. In fact, before any structural studies of Ab2s had been concluded, several investigators noted that even though isogenic antiidiotypic responses are weak, the potential repertoire of B cells expressing antiidiotypic determinants is relatively large (Schuler *et al.*, 1977; Seppalla and Eichmann, 1979). If this is generally the case, one is left only with idiotypic suppression as the explanation for the low frequency of these molecules.

The allogeneic Ab2s contain a high number of hydrophilic residues in the D_H segments. Of particular interest is the D_H segment of the Ab2 that defines the idiotope that is strongly affected by position 59 in the heavy chains of the Ab1s. The residue which influences expression of this idiotope is the lysine in position 59 of the heavy chains in the Ab1s (Fig. 18). The D_H segment of this Ab2 begins with three aspartic acid residues. Since lysine is positively charged at physiologic pH and three aspartic acids are negatively charged, it is possible that some of the idiotope-antiidiotope interactions in this instance may be ionic in nature.

Thus, in the arsonate system the syngeneic antiidiotypic response is both restricted in its level of expression and restricted in its use of germline elements, whereas, the allogeneic antiidiotypic response is heterogeneous. The heavy chain diversity segments in the majority of both the allogeneic and syngeneic Ab2s appear to have been derived through novel mechanisms.

VIII. Conclusions

In order to understand something of the contribution of germline and somatic events in the generation of a defined immune response, we and others have focused on the antibodies produced by A/J mice in response to immunization with the hapten *p*-azophenylarsonate. The germline elements which give rise to this response have been isolated and structurally characterized. Despite the fact that the

germline genes which give rise to this response derive from relatively large gene families, a large body of evidence argues that a single V_{κ} , J_{κ} , V_H , D_H , and J_H gene segment are utilized almost exclusively. The contribution of junctional diversity, N-segment diversity, and somatic mutation has been analyzed in the expressed antibodies. In addition, the major idiotopes of these molecules have been correlated with the classically defined heterologous idiotype. The regulation of the response has been studied by idiotypic manipulation and the structure of molecules so induced has been characterized. The results of these studies, along with information derived from other model systems, provide important insights into the strategies employed by vertebrates for information storage and retrieval. The mechanisms utilized to generate antiarsonate antibodies are likely similar to those used in other complex systems. Hopefully, information of this type will allow a more complete understanding of pattern recognition in higher organisms.

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The Interleukin 2 Receptor

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I. Introduction

The term lymphokine was coined originally to describe biologic activities discernible using *in vitro* bioassays that were generally complex, comprised of a heterogeneous mixture of target cells and prolonged culture periods. Although it was assumed initially that the various activities found in conditioned media were solely produced by lymphocytes, this idea was broadened subsequently to allow for activities that are produced by nonlymphoid cells, and the term cytokine is now sometimes used. From the very first experiments most investigators implied that the biologic activities they detected were the result of an interaction between a *single* lymphokine molecule and an individual target cell. However, it was difficult to prove this point, owing to the complexities of the bioassays, the difficulties with protein purification, and the growing realization that exceedingly low lymphokine concentrations (i.e., ≤ 1 nM) were usually responsible for the measurable activities.

A pivotal advance that enabled new experimental approaches to these problems came when techniques were developed to culture normal T cells for prolonged periods. Morgan *et al.* (1) reported a method using medium conditioned by phytohemagglutinin (PHA)-stimulated human peripheral mononuclear cells that supported the polyclonal growth of human T cells for intervals exceeding 1 year, while formerly cells died out inevitably after only 1–2 weeks. Using this technique we developed both murine (2) and human (3) cytolytic T lymphocyte lines (CTLL) that differed from the T cell cultures reported by Morgan and co-workers in that they were selected for antigen-specific recognition. Remarkably enough, these cell lines retained their immunologic function of antigen-specific cytotoxicity on repeated passage, which allowed us next to develop methods to derive individual clones from the cell lines (4). For lymphokine experiments the use of cloned cells instead of a heterogeneous population of target cells was the breakthrough needed for unam-

biguous interpretations of experimental results. Thus, the first description of the biologic properties of the lymphokine responsible for T cell growth occurred only after we had developed a T cell growth factor (TCGF) assay using cloned cytolytic T cell lines (5). Moreover, the TCGF bioassay also enabled us to perform biochemical experiments indicating that a single glycosylated 15,500 (M_r) protein is responsible for all of the detectable growth-promoting activity (6). Thus, experimental evidence was finally available supporting the concept that a single molecule is responsible for stimulating DNA replication and division of individual T cells.

Despite these advances, the mechanism whereby this newly discovered lymphokine molecule interacted with the cells to promote mitosis remained to be determined. Clues as to the mechanism were uncovered during the course of the very first experiments on the biologic characteristics of interleukin 2 (IL-2), as the TCGF is now known, since we noted that the activity disappeared from the culture media as the cell density increased (5). This observation suggested that the cells actually consumed the activity in some way, especially as no evidence for extracellular proteolysis or any other form of specific or even nonspecific inhibition could be found. Subsequent adsorption experiments showed that the IL-2 activity was removed from culture media by the cells themselves, and that the adsorption was dependent upon time, temperature, and the cell density (7). Consequently, as a result of these data, we and others proposed that specific cell surface receptors bind and remove the IL-2, most likely via receptor-mediated endocytosis (7-9).

To demonstrate IL-2 receptors directly, we developed a traditional hormone radioreceptor assay based upon the binding of radiolabeled IL-2 to various target cells (10). The problems inherent in the radioiodination of proteins were circumvented by using biosynthetic radiolabeling methods: for radioreceptor assays this approach is superior to external radioiodination procedures in that the molecules are not subjected to oxidative chemical reactions that can result in a mixture of denatured radiolabeled molecules, and native nonradiolabeled molecules. The purity of each radiolabeled IL-2 preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and their specific activities were determined using the bioassay, which continues to be the most sensitive and reproducible method of quantifying IL-2. Given biosynthetically radiolabeled IL-2 capable of promoting T cell growth, a series of experiments established readily and incontrovertably that IL-2 actually binds to antigen/lectin-activated T cells in a manner that is

characteristic for classic endocrine hormones (10). Moreover, using the IL-2 radioreceptor assay, each of the parameters considered essential for a ligand binding site to be termed part of an authentic receptor molecule was satisfied. Thus, radiolabeled IL-2 binds with high affinity, ligand specificity, and target cell specificity to sites that are saturable experimentally. Furthermore, the IL-2 concentrations that bind to IL-2 receptors are identical to those that promote T cell proliferation *in vitro* ($EC_{50} \approx 10^{-11} M$), thereby indicating that the IL-2-receptor interaction itself is the only molecular event responsible for the generation of the signals that ultimately stimulate cell division.

II. IL-2 Receptor Structure

Despite these very promising initial findings the determination of the exact structure of the receptor required a series of experiments performed during the past 5 years, and the studies are even now still ongoing. The authentic, high-affinity IL-2 receptor that functions to signal T cell cycle progression is composed of at least two distinct polypeptide chains, each of which contains an IL-2 binding site (11). In conformity with the convention for nomenclature established for other cell surface receptors, the larger IL-2 binding protein ($M_r = 75,000$) is designated as the α chain, whereas the smaller protein ($M_r = 55,000$) is termed the β chain (12).

The β chain was the first IL-2 binding protein to become recognized and characterized, owing primarily to its reactivity with a monoclonal antibody termed anti-Tac, which was developed by Dr. Takashi Uchiyama and co-workers (13,14). Anti-Tac reacts specifically with antigen/lectin-activated T cells and suppresses IL-2-dependent T cell proliferation at antibody concentrations that coincide with those found to compete with radiolabeled IL-2 for binding (15). However, anti-Tac reacts with an epitope that is expressed only on the β chain as determined by SDS-PAGE. Using anti-Tac to affinity purify the β chain, cDNA clones have been isolated and sequenced (16,17). The nucleotide sequence predicts a protein of only 28,400 Da, most of which appears to be comprised of an extracellular domain: the transmembrane region localized on the basis of its hydrophobicity is situated very near the C-terminus, leaving only 13 amino acid residues that extend into the cell as a cytoplasmic domain. No tyrosine residues are present in this intracellular sequence, and even though serine and threonine residues are present, their phosphorylation does not change the way in which the β chain binds IL-2, nor does it

convey to it signal transduction capabilities, which are entirely lacking as evidenced by cDNA transfection experiments (18).

Little is known about the primary structure of the α chain, since it has been recognized only recently. Actually, IL-2 receptors were thought to be comprised solely of single β chains until experiments with cloned leukemic T cells showed conclusively that a separate and distinguishable cell surface protein also binds IL-2 (11). In those experiments, clones from a T-ALL cell line (YT) were established that express sites that bind IL-2 but these cells lack detectable Tac mRNA and do not express Tac antigen at the cell surface. It is noteworthy that these findings essentially exclude the hypothesis that a "converter" protein, such as a G protein, confers high-affinity IL-2 binding capabilities to the β chain, a hypothesis favored by some investigators prior to the discovery of the α chain (19).

The actual composition of the IL-2 receptor as it exists in the native state has been largely unexplored. Our own studies (11), as well as those of others (20,21), indicate that the minimal structure consists of an α,β heterodimer. This interpretation is based upon quantitative binding assays and ^{125}I -labeled IL-2 cross-linking experiments, where the proteins bound to IL-2 can be visualized by autoradiography after SDS-PAGE. Therefore, when high-affinity IL-2 receptors are occupied, equal densities of α chains and β chains are cross-linked to IL-2. However, until additional approaches are utilized that allow the determination of the size of native IL-2 receptors without the denaturing conditions of SDS-PAGE, the exact composition of the receptor remains conjectural. Moreover, a cautious approach is warranted in this regard in as much as we already have evidence that β chains can undergo disulfide-linked dimerization, forming a complex that migrates at 105,000 (M_r) on SDS-PAGE under nonreducing conditions (22).

III. IL-2 Binding Characteristics Based on the α,β Heterodimer Receptor Structure

Recent experiments exploiting leukemic cell lines that express only one of the IL-2 binding proteins (i.e., either α or β) indicate that each chain binds IL-2 with a distinct affinity, and both binding constants are orders of magnitude lower than the characteristic high-affinity IL-2 receptor (11). Thus, the equilibrium dissociation constant (K_d) for IL-2 binding to α chains is $\sim 10^{-9}$ M, whereas the β chain K_d is $\sim 10^{-8}$ M. By comparison, the K_d for IL-2 binding to high-affinity receptors is $\sim 10^{-11}$ M. Since the equilibrium binding constants indicate the IL-2

concentrations required to ensure half-maximal receptor occupancy, these results demonstrate that high-affinity IL-2 receptors become saturated at IL-2 concentrations that are much lower than those required to saturate individual α chains and β chains. Thus, the distinct advantage of the high-affinity receptor is its remarkable IL-2 binding efficiency compared with either α chains or β chains expressed alone.

The heterodimeric composition of high-affinity receptors and the dynamic nature of the way the α chains and β chains cooperate to bind IL-2 are especially evident and intriguing when the kinetics of IL-2 binding are studied. It is possible to demonstrate the kinetic contribution of each individual chain by capitalizing on leukemic cells that express only one of the chains. Thus, as summarized in Table I, IL-2 binds to α and β chains with markedly differing kinetics: the IL-2- α chain interaction is very slow, whereas IL-2- β chain binding is very rapid. The extent of the differences is marked and readily discernible by comparing the dissociation rate constants, which differ by 3 orders of magnitude. Moreover, the association rate constants follow the same pattern as do the dissociation rate constants, in that the association of IL-2 to α chains is very slow in comparison to its rate of association to β chains. Accordingly, on the basis of these findings, for the first time we have recognized that IL-2 binds to and dissociates from β chains very rapidly, whereas it reacts very sluggishly with α chains (23).

By comparison with the results of kinetic binding experiments performed with the individual chains, even more remarkable and illuminating are the results from kinetic binding experiments with high affinity α,β dimers: the association rate is contributed by the rapidly reacting β chain, whereas the dissociation rate is derived from of the slowly reacting α chain. Since the affinity of IL-2 binding at equilibrium is determined by the ratio of the dissociation rate constant ($k' = 2.3 \times 10^{-4} \text{ sec}^{-1}$) and the association rate constant ($k = 3.1 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$), the kinetic cooperation between α chains and β chains in the formation of α,β dimers results in the very low K_d of 10^{-11} M .

Additional studies indicate that the association between α chains and β chains is noncovalent and itself dynamic, subject to mass action and association-dissociation (23). For example, anti-Tac binding to β chains appears to disrupt the α - β heterodimer, completely preventing IL-2 binding to both high-affinity IL-2 receptors as well as isolated β chains. However, anti-Tac does not interfere with IL-2 binding to α chains. Actually the presence of the antibodies appears to uncover α

TABLE I
KINETIC AND EQUILIBRIUM IL-2 BINDING CONSTANTS^a

Chain ^b	Kinetic binding constants			Equilibrium dissociation constants (<i>M</i>)	
	Dissociation (<i>k'</i>)		Association (<i>k</i>) (<i>M</i> ⁻¹)	Kinetic (<i>k'/k</i>)	Equilibrium
	(sec ⁻¹)	(<i>t</i> _{1/2})			
α	2.5×10^{-4}	(46 minutes)	3.8×10^5	0.7×10^{-9}	1.0×10^{-9}
β	4.0×10^{-1}	(1.7 seconds)	1.4×10^7	2.9×10^{-8}	1.7×10^{-8}
α, β	2.3×10^{-4}	(50 minutes)	3.1×10^7	7.4×10^{-12}	2.6×10^{-11}

^a From Wang and Smith (23).

^b α chain = YT-2C2 membranes; β chain = MT-1 cells; α, β chains = induced YT membranes.

chain binding sites previously associated with β chains in α,β dimers. An example of an experiment (24) demonstrating this phenomenon is shown in Fig. 1. Figure 1A shows ^{125}I -labeled IL-2 binding to a T-ALL cell line (YT) that has been induced to express β chains. There are 8400 high-affinity IL-2 receptor sites/cell, and 9800 lower affinity binding sites that represent an excess of α chains. When ^{125}I -labeled IL-2 is cross-linked to these cells there are two IL-2-membrane protein complexes identifiable of $M_r = 90,000$ and $70,000$. The same population of cells examined by ^{125}I -labeled IL-2 binding and cross-linking performed in the presence of a saturating concentration of anti-Tac (5×10^{-8} M) shows no high-affinity IL-2 binding sites detectable, but an increase in the number of individual α chains

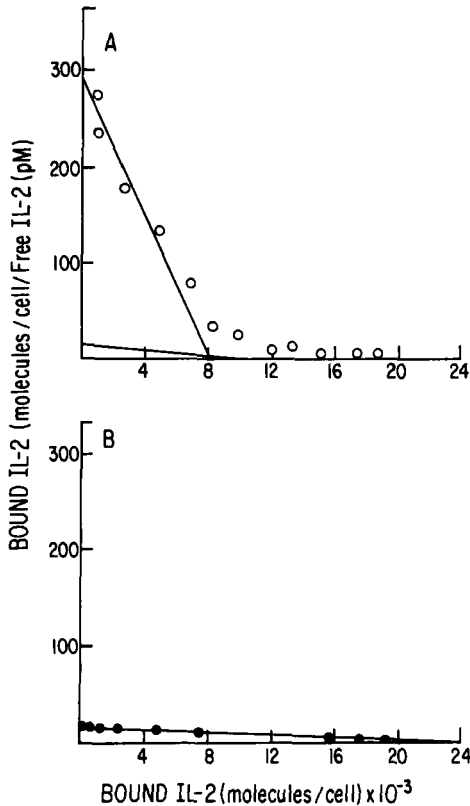


FIG. 1. The effect of anti-Tac on IL-2 receptor expression. YT cells were cultured in 20% ATL-conditioned medium for 24 hours prior to ^{125}I -labeled IL-2 binding assays performed in the absence (A) and in the presence (B) of anti-Tac (50 nM). The data are displayed according to the method of Scatchard. Redrawn from Wang and Smith (23).

(23,000 sites/cell) is readily discernible (Fig. 1B). Moreover, by SDS-PAGE analysis of cross-linked ^{125}I -labeled IL-2, only the α chain is bound (i.e., an $M_r = 90,000$ complex). On the basis of these data, the interpretation that anti-Tac causes dissociation of the α,β heterodimer becomes inescapable.

IV. α Chains Transduce the Growth Signal

Once we realized that the α chain existed, and also that it binds IL-2, it was natural to speculate as to whether the α chain by itself can convey a growth response to the cell, or, alternatively, whether fully formed α,β heterodimers are necessary. Given the disruptive effect of anti-Tac on α,β dimer formation by YT leukemic cells, we explored the characteristics of ^{125}I -labeled IL-2 binding to normal T cells to ascertain whether anti-Tac would effectively uncover α chain binding. Freshly isolated normal human T cells were first activated polyclonally with anti-T3 to stimulate maximum levels of high-affinity IL-2 receptors. Then, ^{125}I -labeled IL-2 binding was performed with and without an excess of anti-Tac present during the equilibrium reaction. The results mirrored those already generated using the leukemic cells: anti-Tac completely masked ^{125}I -labeled IL-2 binding to β chains and α,β dimers, but permitted the easy identification of ^{125}I -labeled IL-2 binding to isolated α chains. Having thus created normal T cells expressing solely α chains capable of binding IL-2, it became feasible to test whether α chains can actually stimulate T cell cycle progression in the absence of any α,β heterodimer-IL-2 binding. The results are interpretable only from the viewpoint that α chains alone are responsible for growth signal transduction: in the presence of anti-Tac the EC_{50} for IL-2-induced T cell proliferation shifted from $\sim 10^{-11}$ to 10^{-9} M, a result entirely predictable from the $K_d \approx 10^{-11}$ M for IL-2 binding to α,β heterodimers, and the $K_d = 10^{-9}$ M for IL-2 binding to isolated α chains (23).

Equally convincing results were obtained when the MLA-144 leukemic cell line was studied. Several years ago we demonstrated that the IL-2 produced constitutively by MLA-144 cells actually binds to IL-2 receptors that are also expressed by these cells, thereby promoting continuous growth by an autocrine mechanism (25). In these earlier experiments, it was only possible to demonstrate that an autocrine mechanism was operative by using glucocorticoids to prevent IL-2 production. Thus, when we learned from a report by Tsudo *et al.* (21) that the kind of IL-2 receptors expressed by MLA-144 cells is restricted to α chains, we repeated our original experiments

using a glucocorticoid-sensitive MLA-144 clone (Fig. 2). It can be seen that IL-2 supports a typical symmetrical sigmoid logarithmic dose-response curve, thereby substantiating our findings with normal T cells: α chains are responsible for signaling cell division.

V. The Regulation of IL-2 Receptor Expression

One of the hallmarks of the immune response is its inducibility. Thus, the vast majority of circulating lymphocytes are metabolically quiescent, and only upon the introduction of antigen do specific clones of cells proliferate and differentiate into effector cells capable of mediating cytolysis, help, or antibody production. It follows that resting T cells cannot express functional IL-2 receptors until they are stimulated via specific antigen. Actually, our very first experiments using the radiolabeled IL-2 binding assay confirmed this prediction: only antigen- and lectin-activated T cells express detectable high-affinity IL-2 receptors (10). Moreover, after polyclonal activation *in vitro*, 72 hours elapse before all of the cells within a cell population express IL-2 receptors (24). Interpreted with the understanding that high-affinity IL-2 receptors are comprised of α,β heterodimers, it

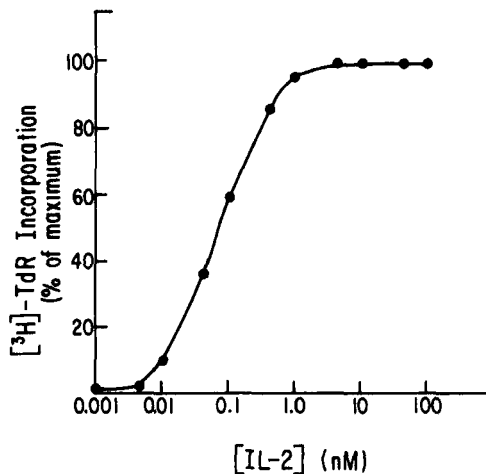


FIG. 2. The α chains expressed by MLA-144 cells transduce a growth response to IL-2. A glucocorticoid-sensitive clone (E7T) of the MLA-144 cell line derived as described previously (25) was cultured (10^4 cells/ml) with dexamethasone (100 nM) in the presence of various concentrations of homogeneous recombinant IL-2. The proliferative response to IL-2 was monitored by [³H]Tdr incorporation during the last 4 hours of a 72-hour culture period.

follows that signals emanating from the T cell receptor complex coordinate the transcriptional activation of the IL-2 gene and the genes encoding both of the IL-2 binding proteins. Although only probes for the IL-2 gene and the β chain are available now, experiments completed thus far are consistent with the concept that unstimulated cells are transcriptionally inactive, and that T cell receptor activation initiates transcription of the IL-2 gene and the IL-2 receptor genes in a coordinate fashion. Even so, it is particularly noteworthy that the 5' flanking regions of the IL-2 gene differ from those of the IL-2 receptor β chain gene, which implies that different promoter/enhancer mechanisms could be responsible for activating each of these genes (26). Such a phenomenon does not preclude a common signal generated via the T cell receptor that subsequently bifurcates into two separate pathways, which then ultimately promote transcription of the IL-2 gene separately from the IL-2 receptor β chain. Also, this would be consistent with some of our recent experiments, which show that cycloheximide prevents the T cell receptor-stimulated expression of IL-2 mRNA transcripts without changing the level of IL-2 receptor β chain mRNA expression (V. Herzberg and K. A. Smith, unpublished).

Since cDNA clones encoding the IL-2 receptor α chain have yet to be isolated, the regulation of this gene can be deduced only from studies of the expression of high-affinity IL-2 binding sites. Thus, it is surmised that T cell receptor triggering must activate transcription of the α chain, particularly as this chain contains the structural information necessary to signal biochemical pathways within the cell, whereas the β chain is impotent in this regard. Moreover, it is predictable that the 5' flanking regulatory regions of the α chain gene may well be found similar to those flanking the IL-2 gene, since our kinetic studies indicate a roughly coordinate appearance of the gene products.

In a series of studies designed to determine the metabolic fate of IL-2, we fortuitously uncovered a unique receptor control system directed by IL-2 itself that suggests a more complex regulation of β chains versus α chains (27). Subsequent to T cell receptor activation, high-affinity α,β dimers are expressed on the cell surface in modest numbers (~ 1000 – 2000 sites/cell). When IL-2 binds to these receptors, several changes take place. First, within 30–60 minutes, the density of high-affinity IL-2 receptors decreases by 40–50%. Subsequently, over the course of the next 24 hours, isolated β chains gradually accumulate on the cell surface, eventually outnumbering the high-affinity IL-2 receptors by as much as 10:1 to 20:1. This IL-2-induced expression of β chains has been traced to a trans-

criptional activation of the β chain gene (28), so that its mRNA accumulates under the influence of IL-2. If IL-2 is withdrawn, high-affinity IL-2 receptors return to their original density within 1–2 hours, whereas the excess β chains persist for many hours before gradually decreasing.

Our most recent experiments have now provided mechanistic explanations for these phenomena. Taking advantage of the leukemic cell lines expressing each isolated chain, we found that α chains convey the information responsible for rapid receptor-mediated endocytosis of radiolabeled IL-2 (23). A representative experiment where the kinetics of ^{125}I -labeled IL-2 internalization were followed using cells expressing solely α chains, β chains, and high-affinity α,β dimers is shown in Fig. 3. It is readily appreciated that IL-2 bound to α chains and to α,β dimers is internalized with a $t_{1/2}$ of 15 minutes. In contrast, the internalization of IL-2 bound to β chains is very slow, making the conclusion inescapable that the α chain must contain structures as part of the cytoplasmic domain that signal rapid IL-2-stimulated internalization. By analogy to other polypeptide hormone receptor systems, these findings suggest that IL-2 binding results in the formation of endocytotic vesicles containing hormone receptor complexes, which upon internalization lead to the phenomenon of peptide hormone-

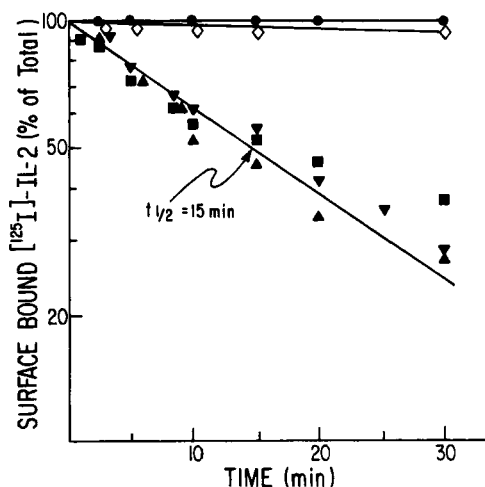


FIG. 3. Chain-specific IL-2 internalization. Cells were incubated with ^{125}I -labeled IL-2 at 4°C for 45 minutes before warming up 37°C to initiate internalization. Cells were chosen to represent high-affinity IL-2 receptors (PHA-activated normal T cells, \blacktriangle and induced YT cells, \blacktriangledown), α chains (YT-2C2 cells, \blacksquare), and β chains (MT-2, \bullet and HUT-102, \diamond). From Wang and Smith (23).

mediated "down-regulation" of homologous receptors. Indeed, the information accumulated so far is consistent with this interpretation: in the presence of IL-2, IL-2 receptors disappear 10 times more rapidly than do unoccupied IL-2 receptors (i.e., $t_{1/2} = 15$ versus 150 minutes respectively) (23,27). Moreover, internalized radiolabeled IL-2 is subsequently degraded by the cell, presumably via lysosomal proteases, as lysosomal blocking agents such as chloroquine prevent ^{125}I -labeled IL-2 degradation (R. Budd and K. A. Smith, unpublished).

Since IL-2 binding to high affinity receptors increases their rate of removal from the cell surface resulting in a readjusted level of surface receptors that is generally only 50% of the original receptor density, IL-2 itself does not appear to markedly influence α chain expression via transcription and translation. Instead, most of the IL-2-mediated regulation of α chain expression occurs at the cell surface, in contrast to its effects on β chain expression, which occur at the genetic level. In any event, the final result of IL-2 binding to high-affinity IL-2 receptors is a gradual decline in their expression. Accordingly, even though IL-2-receptor occupancy results in cell division, it also eventually leads to the loss of the very same receptors that signal the growth response, thereby preventing continuous IL-2 responsiveness.

VI. The Functional Consequences of the Noncovalent Bimolecular IL-2 Receptor Structure

The noncovalent interaction between the α chain and the β chain and the kinetics of IL-2 binding to each chain compared with its binding to the α,β dimer now furnish a picture whereby this receptor system functions. Since the β chain does not contain structures capable of stimulating any known biologic responses, the question arises as to its "raison d'être." It is easy to discern how it functions to change the affinity of IL-2 binding in that the β chain contributes its very rapid association rate to the heterodimer, but it is somewhat more difficult to provide a mechanistic explanation for the phenomenon of the IL-2 induction of a 10- to 20-fold excess of isolated β chains. However, since the α,β interaction is not fixed via covalent bonds, the interaction between the two chains is itself dynamic, and therefore subject to the same laws of mass action that dictate the interaction between any two molecules that bind to one another. Therefore, the induction of an excess of β chains by IL-2 would function to ensure that α chains would always be occupied in the formation of authentic, high-affinity α,β dimers. In effect, the IL-2 induction of an excess of β

chains guards against a premature loss of the most effective IL-2 receptors.

An additional, even more provocative function for the expression of an excess of β chains relates to their rapid binding kinetics. As proposed originally by Adam and Delbruck (29), an excess of rapidly reacting low-affinity receptors can serve to change the search of a hormone for its receptor from an infinitely complex three-dimensional quest to a simple two-dimensional problem in the plane of the membrane. Thus, IL-2 associates and dissociates rapidly from β chains, which do not internalize readily and do not transduce any detectable signal. This phenomenon could serve to localize IL-2 to the membrane, thereby facilitating its binding to high-affinity α,β dimers that are limited in number compared with the density of β chains. A similar phenomenon has been found to be operative when endonucleases interact with DNA. For example, restriction enzymes bind rapidly but with low affinity to nonspecific DNA sequences, then are seen to "slide" along the DNA until the specific sequence is located, where binding occurs with much higher affinity generally as a consequence of a much slower dissociation rate (30,31). In this fashion, the β chain might well function similarly, such that the IL-2- β chain interaction promotes movement of the hormone-receptor complex in the plane of the membrane until an α chain is contacted. In this respect, the β chain could be envisioned to function truly as a "schlepper" binding site, serving to carry IL-2 to authentic, high-affinity, α,β dimers.

VII. Conclusions

The elucidation of the bimolecular structure of the IL-2 receptor provides us with a picture of a very dynamic regulatory system that ultimately controls the magnitude of the T cell immune response. The understanding that IL-2 receptor expression is stimulated initially by environmental antigens emphasizes that the development of IL-2 responsiveness is a pivotal point in the generation of an antigen-specific T cell immune response. Moreover, the dependency of T cell clonal expansion on the rate, extent, and duration of IL-2 receptor expression indicates that effective control of the immune reaction is eventually transferred to the endocrine-like IL-2 hormone-receptor system. Now, with the knowledge that both α and β chain expression is mandatory for formation of the most efficient IL-2 receptors, it is understandable how defects in the ability to adequately express either

chain would lead to severe immunodeficiency: indeed, inability to express IL-2 receptor α chains may be incompatible with life. Moreover, since the IL-2 receptor signal transducing structures are parts of the α chain, future studies elucidating its primary sequence should be quite fruitful and a necessary first step toward understanding the mechanism of signal transduction through the membrane, and the nature of the biochemical pathways activated by IL-2 that lead to DNA replication and cell division. Thus, new IL-2 receptor structure-function studies now opened by our findings promise to provide information crucial to the way the normal immune response functions and central to one of the fundamental life processes, namely, cell division.

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Characterization of Functional Surface Structures on Human Natural Killer Cells

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I. Introduction

In recent years, a small population of normal peripheral blood mononuclear cells (PBMC),³ termed natural killer (NK) cells, has become the subject of extensive laboratory investigation. NK cells have been identified in many vertebrate species and have been operationally defined as cells capable of mediating spontaneous *in vitro* cytotoxicity against a variety of target cell populations without apparent prior sensitization (Takasaki *et al.*, 1973; Peter *et al.*, 1975; West *et al.*, 1977; Ortaldo *et al.*, 1977). NK cells have been considered to be distinct from specific cytotoxic T lymphocytes (CTL) because they have not been shown to have clonally distributed specificity, restriction for products of the major histocompatibility complex (MHC) at the target cell level, or immunologic memory. Despite this apparent lack of specificity, NK cells have been implicated in a large number of diverse immunologic functions. These functions include cytotoxicity against tumor cells and virally transformed cells (Herberman and Ortaldo, 1981), resistance to some microbial (Nencioni *et al.*,

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³ Abbreviations: antibody-dependent cell-mediated cytotoxicity, ADCC; cluster of differentiation, CD; cytotoxic T lymphocytes, CTL; Epstein-Barr virus, EBV; interleukin 2, IL-2; IL-2 receptor, IL-2R; large granular lymphocyte, LGL; lymphocyte-conditioned medium, LCM; lymphokine-activated killer, LAK; major histocompatibility complex, MHC; natural killer, NK; peripheral blood lymphocytes, PBL; peripheral blood mononuclear cells, PBMC; sheep red blood cell, SRBC; T cell receptor, TCR.

1983), fungal, and parasitic agents (Hatcher and Kuhn, 1982; Murphy and McDaniel, 1982), immune regulation through secretion of lymphokines such as interleukin 2 (IL-2) (Kasahara *et al.*, 1983) and interferon (Trinchieri *et al.*, 1978; Timonen *et al.*, 1980), regulation of hematopoiesis (Hanson and Kiessling, 1982; Holmberg *et al.*, 1984; Herrmann *et al.*, 1987), and natural resistance to allogeneic grafts (Kiessling *et al.*, 1977; Warner and Dennert, 1982). Recently *in vitro* activated NK cells, termed lymphokine-activated killer (LAK) cells, have also been utilized in clinical trials of adoptive cellular immunotherapy in patients with metastatic cancer (Rosenberg *et al.*, 1985, 1987). Interestingly, morphologic analysis of NK cells has demonstrated that these cells have the homogeneous appearance of large granular lymphocytes (LGL) and distinctive physical characteristics that allow their enrichment on Percoll density gradients (Timonen and Saksela, 1980; Timonen *et al.*, 1981). Taken together, these morphologic characteristics and unique functional capacities clearly distinguish NK cells from T cells, B cells, monocytes, and other hematopoietic and lymphoid elements.

Despite their homogeneous morphology, it has become apparent that NK cells are extremely heterogeneous and the precise relationships between NK cells, T cells, and myelomonocytic cells have not been clearly defined. In part, the heterogeneity of NK cells has become apparent through characterization of the cell surface antigens expressed by these cells. Using various monoclonal antibodies, studies have shown that the majority of NK cells in human peripheral blood express antigens such as NKH1 (Griffin *et al.*, 1983; Hercend *et al.*, 1985), IgG-Fc receptor (CD16) (Fast *et al.*, 1981; Perussia *et al.*, 1982a,b; Perussia and Trinchieri, 1984), T10 (CD38) (Kung *et al.*, 1979; Ortaldo *et al.*, 1981), Mo1 (C3bi receptor CD11b) (Breard *et al.*, 1980; Kay and Horowitz, 1980; Sanchez-Madrid *et al.*, 1983), and T11 E rosette receptor (CD2) (Ortaldo *et al.*, 1981; Perussia *et al.*, 1984; Hercend *et al.*, 1985). Approximately 60% of NK cells express HNK-1 antigen (Abo and Balch, 1981; Abo *et al.*, 1982) and 20–30% express CD3 (Hercend *et al.*, 1985; Schmidt *et al.*, 1986b; Lanier *et al.*, 1986a) or CD8 antigens (Perussia *et al.*, 1983). Relatively few NK cells express T4 or Ia antigens, and no NK cells are thought to express B cell-restricted markers such as B1 and B2 and monocyte-restricted markers such as MY4 and Mo2. Thus, the majority of NK cells are NKH1⁺ and express markers characteristic of both T cells (CD2) and myeloid cells (CD11b, CD16). Other antigens such as HNK-1, CD3, and CD8 are expressed only on subsets of NK cells.

The heterogeneity of NK cells is also apparent from a wide variety

of immunologic functions that have been attributed to these cells. As noted previously, these functions include resistance to tumor growth and metastasis, resistance to viral infections, immunoregulation, and regulation of hematopoiesis (reviewed in Herberman and Ortaldo, 1981; Trinchieri and Perussia, 1984). In almost all instances, these NK cell activities *in vivo* are inferred from *in vitro* experiments and the precise role of these cells *in vivo* remains to be conclusively established. Moreover, it is not known whether all these functions can be mediated by the same cells or whether distinct functional subsets within the NK cell population have different activities. Since NK cells have been shown to secrete a variety of lymphokines, it is also not known which of these functions is mediated by direct cytotoxicity and which is mediated through lymphokine secretion. At a structural level, it is presumed that the granules in NK cells contain cytotoxins that are capable of mediating target cell lysis (reviewed in Dennert, 1985; Podack, 1985), but the biochemical characterization and purification of these granule constituents have only recently begun (Masson and Tschopp, 1985; Young *et al.*, 1986a-c; Pasternack and Eisen, 1985; Pasternack *et al.*, 1986; Gershenfeld and Weissman, 1986). Moreover, almost all studies of cytolytic granule constituents have utilized either CTL cell lines or rat LGL tumor cells and very few studies have been done with human NK cells (MacDermott *et al.*, 1985).

With regard to the mechanisms whereby NK cells interact with target cells, these are only now beginning to be more fully understood. In almost all instances, the membrane target structures of NK cells have not been identified, and it is not known whether different NK cells react with the same or with different target antigens. At the effector cell level, it is not known which membrane molecules play important roles in effector/target cell binding or if all NK cells utilize the same membrane structures to interact with various target cells. With respect to regulation of NK activity, it has been established that various lymphokines including interferon (Minato *et al.*, 1980; Saksela *et al.*, 1980) and IL-2 (Henney *et al.*, 1981; Trinchieri *et al.*, 1984; van de Griend *et al.*, 1986) are capable of activating NK cells and enhancing their cytolytic function. Moreover, at least some NK cells are capable of secreting interferon and IL-2, as well as other lymphokines (Kasahara *et al.*, 1983; Scala *et al.*, 1984), but the mechanism whereby NK cells can be triggered to secrete these lymphokines is not known.

In this report, we will review a number of recent studies that have begun to characterize the surface antigens of human NK cells. As has

been the case for numerous antigens identified on other lymphoid and myeloid cells, we are now able to begin to associate some surface antigens with specific functional activities of the NK cell. These particular surface structures as well as those that define distinct functional populations of NK cells will be the major focus of this review.

II. Clonal Human NK Cell Lines

Since NK cells represent only a small fraction of PBMC and are themselves heterogeneous, it has been difficult to perform meaningful experiments with NK cells in normal peripheral blood. Physical enrichment techniques such as Percoll density gradients have been very useful for purifying cells with LGL morphology and have therefore been extensively used to obtain NK-enriched populations (Timonen and Saksela, 1980; Timonen *et al.*, 1982). Another strategy that has been useful in the characterization of human NK cells has been the development of methods for *in vitro* clonal expansion of cells with NK activity from normal peripheral blood. Since NK cells as well as T cells proliferate in response to IL-2, this was accomplished primarily through the modification of techniques used to generate clonal T cell populations.

In several laboratories NK clones have been obtained using a limiting dilution technique (Hercend *et al.*, 1982, 1983a; Kornbluth *et al.*, 1982; Allavena and Ortaldo, 1984; van de Griend *et al.*, 1984). In our laboratory, PBMC, LGL, or cells enriched for NK activity by immunofluorescent cell sorting have been cloned at one cell per well on feeder layers usually consisting of irradiated PBMC plus Epstein-Barr virus (EBV)-transformed B cells (Hercend *et al.*, 1983a). Unlike T cell cloning, equivalent results have been obtained using either autologous or allogeneic feeder cells. After seeding, colonies are expanded by the addition of culture medium containing lymphocyte-conditioned medium (LCM) and human AB serum every 3–4 days. NK clones have been identified by screening all colonies for cytotoxicity against an irrelevant allogeneic target cell such as K562. In our studies, all clones showing NK activity against K562 have been NKH1 positive, whether they were derived from PBMC, LGL, or NKH1⁺ sorted cells. Following the identification of clones with NK activity, colonies could not easily be expanded with LCM alone or with recombinant IL-2 (rIL-2) alone. Unlike T cell clones, NK clones appear to require almost continuous contact with feeder cells to maintain consistent proliferation. For this reason, NK clones have

been expanded through frequent subcloning in 96-well plates with irradiated PBMC plus EBV-transformed B cells as feeders. With this procedure, however, it has been possible to maintain the growth of selected clones for several years. More importantly, these NK clones have maintained a stable phenotype and functional activity during this entire period.

Like T cell clones, NK clones that have been generated appear to reflect the extensive phenotypic and functional diversity of NK cells in peripheral blood. Table I presents a summary of the origin and phenotype of a large number of NK clones generated in our laboratory

TABLE I
PHENOTYPE OF HUMAN NK CLONES

Clone	Origin	Phenotype						
		CD2	CD3	CD4	CD8	NKH1	NKH2	NKTA
JT1	LGL	-	-	-	-	+	+	-
JT3	LGL	+	-	-	-	+	+	-
JT9	PBL	+	+	-	+	+	-	+
JT10	PBL	+	+	-	+	+	-	+
JT11	PBL	+	+	-	+	+	-	+
JT15	PBL	+	+	-	+	+	-	+
JT16	PBL	+	-	-	-	+	+	-
JT _A 17	PBL	+	+	-	-	+	+	-
JT _B 18	PBL	+	-	-	-	+	-	-
CNK1	NKH1 ⁺	+	+	-	+	+	-	-
CNK2	NKH1 ⁺	+	-	-	-	+	-	-
CNK3	PBL	+	+	-	+	+	+	-
CNK4	NKH1 ⁺	+	-	-	-	+	-	-
CNK5	NKH1 ⁺	+	-	-	-	+	-	-
CNK6	NKH2 ⁺	+	-	-	-	+	+	-
CNK7	NKH2 ⁺	+	-	-	-	+	+	-
CNK8	NKH2 ⁺	+	+	-	+	+	+	+
CNK9	NKH2 ⁺	+	+	-	+	+	+	+
CNK10	PBL	+	-	-	-	+	+	-
TC12	PBL	+	-	-	-	+	-	-
TC48	PBL	-	-	-	-	+	-	-
TC50	PBL	+	-	-	-	+	-	-
TC59	PBL	+	-	-	-	+	+	-
CNK11	NKTA ⁺	+	+	-	+	+	+	+
CNK12	NKTA ⁺	+	+	-	+	+	+	+
CNK13	NKTA ⁺	+	+	-	+	+	+	+
CNK14	NKTA ⁺	+	+	-	+	+	-	+
CNK15	NKTA ⁺	+	+	-	+	+	-	+
JJ1	NKH1 ⁺	+	-	-	-	+	+	-
JJ3	NKH1 ⁺	+	-	-	-	+	-	-

over a 6-year period. Each of these clones was selected on the basis of exhibiting non-MHC-restricted cytotoxicity and is able to lyse a variety of allogeneic target cell populations. Nevertheless, these clones express a variety of phenotypes and do not have identical cytolytic activity when tested against a large number of different target cells in addition to K562. Given this extensive heterogeneity, NK clones have been very useful in beginning to dissect the functional basis for NK activity. This analysis of NK function at the "single cell" level has been facilitated through the use of homogeneous populations of human NK cells that could be propagated in sufficient numbers to allow phenotypic, functional, and biochemical studies with the same cells. As will be discussed in later sections of this review, NK clones have been utilized to identify and characterize functional surface antigens of human NK clones involved in the effector-target cell interaction and then to analyze individual structures and mechanisms for the regulation of NK cell activity. Because NK clones represent selected individual cells that have been cultured *in vitro* for prolonged periods, it has always been necessary to utilize information obtained from analysis of NK clones to characterize NK cells and NK cell subsets in peripheral blood.

III. NK Cell Phenotype

Studies from several laboratories have demonstrated that NK cells in peripheral blood appear morphologically as a homogeneous population of LGL. However, to define better the entire population of NK cells and also to identify functionally distinct subsets within the LGL population, many investigators have utilized monoclonal antibodies to characterize the phenotype of human NK cells. Table II presents a summary of antigens known to be expressed by NK cells in normal, unstimulated peripheral blood. None of these antigens is totally NK specific and not expressed by other lymphoid or myeloid cells. Since NK cells represent only a small fraction of PBMC, most of the monoclonal antibodies used to characterize NK cells were generated against other lymphoid or myeloid populations and only subsequently tested to determine reactivity with NK cells. Nevertheless, a large number of surface antigens have been identified on NK cells and have been utilized in an attempt to define functionally important structures and functionally distinct subsets of NK cells, as well as for the isolation, purification, and enumeration of NK cells. One issue that has not been resolved by phenotypic analysis is the lineage derivation of NK cells. As summarized in Table II, NK cells express a number of

TABLE II
SURFACE ANTIGENS EXPRESSED BY HUMAN NK CELLS

Antigen	Molecular weight ($\times 10^3$)	Functional epitopes	Reactivity with NK cells (%)	Examples of antibodies	References
NKH1	200		>95	N901, NKH1 _A	Griffin <i>et al.</i> (1983); Hercend <i>et al.</i> (1985)
NKH2	60		20-60	NKH2	Hercend <i>et al.</i> (1985)
CD16	50-60	IgG-FcR	80-90	B73.1, Leu11	Perussia <i>et al.</i> (1982b); Lanier <i>et al.</i> (1983)
CD11a	180, 95	LFA-1 α chain	>95	LFA-1, MHM24	Sanchez-Madrid <i>et al.</i> (1982); Hildreth and August (1985)
CD11b	160, 95	C3biR α chain	80-90	Mo1, MAC1	Todd <i>et al.</i> (1982); Ault and Springer (1981); Kay and Horowitz (1980)
CD11c	150, 95	p150 α chain		LeuM5	Lanier <i>et al.</i> (1985b)
CD18	95	β chain of CD11a,b,c	>95	MHM23	Hildreth and August (1985)
HNK-1	110		50-70	Leu7	Abo and Balch (1981); Abo <i>et al.</i> (1982)
CD2	50	E rosette receptor	80-90	T11 ₁ , T11 ₂	Meuer <i>et al.</i> (1984); Hercend <i>et al.</i> (1985)
CD3	19-29	TCR assoc.	20-30	T3, Leu4, UCHT1	Hercend <i>et al.</i> (1985); Schmidt <i>et al.</i> (1986b); Lanier <i>et al.</i> (1986a)
CD8	32-33	T sup/cyto	30	T8, Leu2	Perussia <i>et al.</i> (1983); Schmidt <i>et al.</i> (1986b)
CD38	45	Activation	75-90	T10, HB7	Kung <i>et al.</i> (1979); Ortaldo <i>et al.</i> (1981)

antigens commonly associated with either myeloid lineage (CD16, CD11b) or T cell lineage (CD2, CD3, CD8). Since all NK active cells do not share a consistent phenotype, the expression of various antigens has thus primarily served to emphasize the extensive heterogeneity of cells capable of mediating non-MHC-restricted cytotoxicity.

A. NKH1 ANTIGEN

Two monoclonal antibodies that have been generated in experiments designed to define antigens selectively expressed on NK cells have been termed anti-NKH1_A and anti-NKH2 (Hercend *et al.*, 1985). These antibodies were generated following immunization of mice with cloned NK cells which were then also used for initial screening of hybridoma supernatants. After identification of hybridomas reactive with the NK clone, antibodies with more selective NK reactivity were readily identified on the basis of lack of reactivity with autologous non-NK cells, such as T cell clones and B cell lines. In addition, immunizing NK clone cells were also used for functional characterization of the reagents, as well as biochemical identification of the target antigens. Using this approach, two monoclonal antibodies termed anti-NKH1_A and anti-NKH2 were chosen for further study.

Anti-NKH1_A (IgM) was found to react with approximately 12% of PBMC and not with any additional hematopoietic cell fraction that was tested. Greater than 90% of circulating NKH1_A⁺ cells are LGL. Treatment of PBL with anti-NKH1_A plus complement resulted in an almost complete disappearance of NK activity in peripheral blood. When tested for reactivity with NK clones, all were found to express NKH1 antigen (Table I). NKH1⁺ clones included cells such as JT3, CNK6, and JT1, which have a common NK cell phenotype (CD2⁺CD3⁻), but in addition, this antigen was found to be present on clones such as JT1 (CD2⁻CD3⁻) and JT9 (CD2⁻CD3⁺), which appear to represent minor populations of NK cells in unstimulated peripheral blood. Biochemical studies indicated that both anti-NKH1_A and a previously described antibody termed anti-N901 (NKH1-IgG₁) (Griffin *et al.*, 1983) were directed at a molecule which migrates at 200,000–220,000 molecular weight on SDS-PAGE analysis. These studies, therefore, confirmed previous results obtained with anti-N901 antibody, which demonstrated that virtually all NK activity present in human peripheral blood was restricted to the N901⁺ subset. More recently, other investigators (Lanier *et al.*, 1986a) have described another antibody, Leu19, with similar specificity. Taken together, these findings suggest that the 200-kDa molecule

(NKH1) can be considered as a pan-NK-associated cell surface marker, useful for defining resting as well as activated NK cells.

Two-color immunofluorescence analysis with other NK associated markers, such as Leu7 and Leu11, has indicated that NKH1 antigen has a distinct pattern of expression that does not overlap entirely with these other NK cell-associated antigens although, as expected, there are significant populations of NKH1⁺ Leu7⁺ and NKH1⁺ Leu11⁺ cells. Nevertheless, NKH1 expression is not restricted only to NK cells since N901 was originally generated following immunization with myeloblasts from a patient with chronic myelocytic leukemia in blast crisis. In addition, some T cell clones as well as long-term cultured T cell lines without NK activity have been found to express NKH1 (Lanier *et al.*, 1987).

Another monoclonal antibody, termed anti-NKH2 (IgM), has also been shown to display selective reactivity for LGL (Hercend *et al.*, 1985). Anti-NKH2 is specific for a 60-kDa surface antigen and reacts with approximately 7% of PBL. Monocytes, granulocytes, red cells, platelets, lectin-activated T cells, thymocytes, and over 20 hematopoietic cell lines tested thus far have been negative. While NKH2⁺ cells could not be distinguished morphologically from NKH1⁺ cells, both complement lysis and two-color immunofluorescence studies demonstrated that distinct subsets of LGL can be either NKH1⁺NKH2⁺, NKH1⁺NKH2⁻ or NKH1⁻NKH2⁺. More importantly, the subsets defined by these antigens were found to be functionally distinct. As opposed to results obtained with anti-NKH1_A, treatment of PBMC with anti-NKH2 plus complement did not significantly alter NK activity in peripheral blood. Moreover, when LGL-enriched fractions were sorted into NKH2⁺ and NKH2⁻ subpopulations, it was shown that high NK activity was still found in the NKH2⁻ fraction and that NKH2⁺ cells from most individuals displayed less NK activity.

These studies suggest that anti-NKH1 and anti-NKH2 are capable of dissecting populations of LGL into functionally distinct subsets with varying levels of NK activity. Using these two antibodies, LGL can be divided into three populations: NKH1⁺NKH2⁺, NKH1⁺NKH2⁻, and NKH1⁻NKH2⁺. The NKH1⁺NKH2⁻ subset appears to contain the most strongly cytotoxic cells in the peripheral blood of most normal individuals. Cultured NK clones expressing both NKH1 and NKH2 antigens can display strong cytotoxicity but the functional activity of the NKH1⁺NKH2⁺ LGLs in unstimulated peripheral blood has not yet been determined. Finally, the function of cells that express NKH2 antigen alone has not yet been evaluated, but there is no evidence to indicate that these cells have significant NK activity in normal

peripheral blood. Thus, the identification of NKH1⁻NKH2⁺ cells suggests that NKH2 may also be expressed on some LGLs that are not NK cells. In addition, anti-NKH2 has been found to be reactive with peripheral blood basophils (J. Griffin, personal communication).

In 1985, Rosenberg *et al.* reported that *in vivo* administration of autologous lymphokine-activated killer (LAK) cells and rIL-2 resulted in regression of metastatic tumors in some patients. Extensive clinical trials using *in vitro* activated LAK cells in patients with metastatic cancer are now in progress and recent studies have attempted to define the populations of cells responsible for this activity. Although LAK cells were initially reported to be a distinct population of effector cells (Mazumder *et al.*, 1983; Grimm *et al.*, 1983; Yang *et al.*, 1986), particular attention has recently focused on the relationship between LAK and NK cells. One characteristic that has been used to distinguish LAK cells from NK cells has been the ability of LAK cells to lyse NK-resistant targets. Nevertheless, LAK cells have not been shown to have a distinct phenotype and their cytolytic activity is not specific for either autologous or allogeneic tumors.

The relationship between LAK and NK has been addressed in a more direct way in recent studies using NKH1 antigen expression to define both cytolytic effector populations. In one study, Phillips and Lanier (1986) demonstrated that following rIL-2 activation almost all LAK activity is mediated by NKH1⁺ cells. Similarly, the precursor cells in peripheral blood responsible for LAK activity were shown to express NKH1 and CD16 (Ortaldo *et al.*, 1986). Schmidt *et al.*, (1987a) have also demonstrated that both before and after rIL-2 activation, the effector population responsible for lysis of NK-sensitive and NK-resistant targets was confined to the NKH1⁺ cells in peripheral blood. These findings are consistent with previous studies demonstrating that IL-2 is able to enhance the cytolytic function of NK cells (Henney *et al.*, 1981) and that following rIL-2 activation, NK cells are able to lyse targets previously resistant to these same effectors (Lanier *et al.*, 1985a). Since the NKH1⁺ population represents only 10–15% of PBMC it is unlikely that LAK represents a distinct effector cell population. In contrast, these studies support the hypothesis that LAK and NK are not distinct effector populations and that LAK primarily reflects the ability of various lymphokines such as IL-2 and interferon to enhance the cytolytic activity of unstimulated NK cells.

B. CD16/FC RECEPTOR FOR IgG

One of the antigens that has been extensively used to define the NK cell population is the Fc receptor for IgG termed CD16 (Table II)

(reviewed in Trinchieri and Perussia, 1984). Prior to the identification of the CD16 antigen, numerous studies had demonstrated that many of the cells capable of NK activity could also display antibody-dependent cell-mediated cytotoxicity (ADCC). In order to mediate this type of cytolytic activity, the effector cell was presumed to express specific Fc receptors for IgG. Antibodies specific for the IgG-FcR (B73.1, 3G8, VEP13, Leu11a, Leu11b) inhibit binding of aggregated IgG to cell membranes and also inhibit ADCC. Several studies have documented the expression of CD16 by the majority of NK cells in peripheral blood (Perussia *et al.*, 1982b; Perussia and Trinchieri, 1984; Rumpold *et al.*, 1982; Lanier *et al.*, 1983). Unlike NKH1 however, the CD16 antigen is also expressed by granulocytes (Perussia *et al.*, 1982b). Although monocytes and B cells also have Fc receptors for IgG, these are apparently distinct antigens not cross-reactive with anti-CD16 antibodies (Perussia *et al.*, 1984).

As noted previously, there is considerable overlap in the expression of NKH1 and CD16 antigen and CD16 has been very useful in the phenotypic identification of NK cells. Nevertheless, it is also evident that not all NKH1⁺ cells express CD16 and not all CD16⁺ cells express NKH1, suggesting that there may be cells capable of mediating NK independently of ADCC as well as cells capable of mediating both functions. This conclusion is supported by studies that have identified a unique population of CD16⁺CD3⁺ effector cells in normal peripheral blood (Lanier *et al.*, 1985c). These cells are able to mediate ADCC but do not have direct cytolytic activity against K562. Similarly, not all NK clones express CD16 and not all NK clones are able to mediate ADCC as well as NK activity. In this regard, recent studies have demonstrated that CD16 antigen can also mediate activation of effector cells and thereby induce cytolytic function of CD16⁺ clones (van de Griend *et al.*, 1987b; Werfel *et al.*, 1987). In the studies by Werfel *et al.*, (1987) F(ab')₂ fragments of anti-CD16 antibodies did not induce cytotoxicity, indicating that both the antigen binding site and the Fc binding site of the anti-CD16 antibody were necessary for activation of effector cells.

C. HNK-1

The HNK-1 antibody (anti-Leu7) is an IgM monoclonal antibody which was produced against a membrane antigen from the cultured T cell line HSB-2 and was originally described as NK cell specific (Abo and Balch, 1981). It precipitates a 110,000 molecular weight surface structure. In peripheral blood, however, HNK-1 reacts with a heterogeneous population of cells comprising some LGL with NK activity

and also a variable proportion of CD3⁺CD8⁺ lymphocytes which have low or no spontaneous cytotoxicity (Abo *et al.*, 1982). Given the expression of HNK-1 by some NK cells, PBMC has been shown to contain subsets that are NKH1⁺HNK-1⁺ and CD16⁺HNK-1⁺ (Hercend *et al.*, 1985; Lanier *et al.*, 1983). HNK-1 is usually not expressed on cultured human NK cell clones and seems to be lost following *in vitro* activation of NK cells. In addition, HNK-1 reacts with cells present in the B cell compartment of lymphoid organs (Banerjee and Thibert, 1983) as well as with several cell types unrelated to the hematopoietic lineage (Bunn *et al.*, 1985; Schuller-Petrovic *et al.*, 1983). These data argue against the NK specificity of this marker and have made it more difficult to interpret data on histologic localization and distribution of HNK1⁺ cells in embryonic tissues to the ontogeny and *in vivo* function of NK cells.

IV. Antigens Involved in NK Effector-Target Cell Interactions

Although NKH1, CD16, and HNK-1 antigens have been useful in defining those cells capable of mediating NK and ADCC, these antigens do not appear to play a significant role in the cellular interactions between NK cells and their targets. More specifically, anti-NKH1 antibodies do not affect proliferation of NK cells, effector cell conjugation with targets, or triggering of NK cell lysis (Schmidt *et al.*, 1986b). Anti-CD16 antibodies inhibit ADCC and have been shown to activate effector cells (van de Griend *et al.*, 1987b; Werfel *et al.*, 1987), but these reagents do not affect NK cell function (Perussia *et al.*, 1982b; Perussia and Trinchieri, 1984). Similarly, anti-HNK-1 has not been found to affect NK cell function or proliferation. Although the function of some NK-associated antigens has not yet been defined, NK cells are known to express a number of antigens in common with other cell types. As summarized in Table II, several of these antigens including CD2, CD3, and CD11 have distinct functional roles in other cells in the immune system and it has therefore been possible to study the function of these structures in NK cells.

A. CD11a/LFA-1

LFA-1 belongs to a family of structurally and functionally related high-molecular-weight cell surface antigens, including Mol (Todd *et al.*, 1982) and p150,95 (Sanchez-Madrid *et al.*, 1983). Each molecule consists of an α subunit and a β subunit noncovalently associated in a dimeric surface structure. All of the three related molecules differ

primarily in their α subunits since all have an identical β subunit of 95 kD. The different α subunits appear to be members of a novel leukocyte adhesion protein family as suggested by protein sequence homology between α subunits of LFA-1 and MAC I/Mo1 (Springer *et al.*, 1985). Following the Third International Workshop and Conference on Human Leukocyte Differentiation Antigens,⁴ antibodies to the distinct α chain structures have been designated CD11a (LFA-1), CD11b (Mo1), and CD11c (p150). The common chain has been designated CD18 (Pallesen and Plesner, 1987). The LFA-1 antigen is widely distributed on T cells, B cells, and myeloid cells (Kurzinger *et al.*, 1981). Analysis of CTL has shown that anti-LFA-1 antibodies inhibit conjugate formation with target cells (Davignon *et al.*, 1981; Krensky *et al.*, 1983; Spits *et al.*, 1986). In addition, it has been shown that anti-LFA-1 antibodies block the allogeneic mixed lymphocyte response (Hildreth and August, 1985) and inhibit the induction of T helper cell response to antigen, presumably at the level of T helper cell macrophage interaction (Fischer *et al.*, 1986). LFA-1 also appears to play a significant role in T helper cell interactions with B lymphocytes (Tedder *et al.*, 1986). Moreover, several patients have recently been described with a Mo1/LFA-1 deficiency who lack a number of phagocyte adhesion functions and, in severe cases, are deficient in NK cytotoxicity (Anderson *et al.*, 1985). This deficiency appears due to a defect in the common β subunit (Springer *et al.*, 1984). Patients' cells lack the expression of both α and β subunits of LFA-1 on T cell blasts and EBV-transformed B cells. However, in mouse/human hybrids formed with patient cells (Marlin *et al.*, 1986) the patient LFA-1 α subunit can form complexes with the mouse β subunit, and the resulting interspecies complex is expressed on the hybrid cell surface. Hybrids expressing the human β subunit associated with the mouse α subunit can be derived from normal human cells but not from patient cells. These results show that the α subunit structure is capable of surface expression if a functional β subunit is present and demonstrates that the β subunit is defective in patients with this genetic disorder. Using these tools, the LFA-1 α and β subunits have been mapped to chromosomes 16 and 21, respectively (Marlin *et al.*, 1986).

Using well-characterized monoclonal antibodies specific for CD11a (MHM24) and CD18 (MHM23) (Hildreth and August, 1985) and NK clones, recent studies have analyzed the role of LFA-1 antigens in NK

⁴ Monoclonal antibodies showing similar reactivity have been given a common CD designation by the Third International Workshop and Conference on Human Leukocyte Differentiation Antigens held in Oxford in September 1986. See reference by Pallesen and Plesner (1987) for the most recent complete listing of CD nomenclature.

cell function (Schmidt *et al.*, 1985b). All NK clones tested expressed LFA-1 antigen and had identical patterns of reactivity with MHM23 and MHM24. With regard to blocking of NK function, both anti-CD11a and anti-CD18 inhibited conjugate formation between NK clone cells and targets resulting in the inhibition of target cell lysis. One additional consistent observation was that the blocking effect of anti-LFA-1 antibodies was target cell dependent. Whereas there was only a relatively minor degree of inhibition of NK clone activity against targets such as K562 and HSB, a more significant blocking effect was identified when NK clones were tested against Molt 4 and CEM target cells. These blocking effects were independent of LFA-1 antigen expression on the target cell membrane since blocking occurred only when antibody was bound to the effector cells. This level of inhibition was demonstrated for CD2⁺CD3⁺NK clones as well as for CD2⁺CD3⁻ NK clones. Similar results were also noted with unstimulated NK cells in peripheral blood and NK effector cells activated with rIL-2 (Schmidt *et al.*, 1987a). These results suggested that despite the phenotypic and functional heterogeneity of the NK cells utilized in these studies, the LFA-1 molecules played a similar role in the cytotoxic mechanism of each of these cells. Moreover, since LFA-1 has been shown to play a similar role in CTL target conjugation, the function of LFA-1 in NK effector target interactions appears to be identical in both MHC-restricted and non-MHC-restricted interactions. Taken together, these studies provide evidence that LFA-1 is not involved in antigen-specific interactions between effector and target cells, but that this structure mediates an important accessory function in the strengthening of nonspecific cell-cell adhesion.

B. CD2/T11 E ROSETTE RECEPTOR

In 1984, Meuer *et al.* demonstrated that the T11 antigen (CD2) functioned as an alternate antigen-independent pathway of T cell activation. In these studies, the 50-kDa CD2 antigen was shown to possess at least three distinct epitopes: T11₁, the sheep red blood cell (SRBC) binding site on all T cells, thymocytes, and the majority of NK cells; T11₂, an epitope unrelated to the SRBC binding site, but with an identical tissue distribution; and T11₃, an epitope not found on resting T11₂⁺ cells but rapidly expressed following activation or in response to anti-T11₂. Addition of anti-T11₂ and anti-T11₃ monoclonal antibodies to resting T cells resulted in the rapid activation and proliferation of these cells, which was mediated through expression of IL-2 receptor and secretion of IL-2 by activated cells. Significantly,

this method of T cell activation was entirely independent of triggering by specific antigens or by antibodies directed against the T cell receptor (TCR) for antigen. This alternate T cell activation pathway has been confirmed by other investigators using anti-T11 antibody pairs (Brottier *et al.*, 1985) or anti-T11 antibody alone and phorbol esters (Holter *et al.*, 1986). Nevertheless, activation through the CD3/TCR pathway was functionally related to CD2 activation since CD3 modulated cells were unable to respond to CD2 triggering (Meuer *et al.*, 1984). Almost all NK cells and NK clones express CD2 and it has therefore been possible to analyze the effects of CD2 triggering on NK cell function and to compare these results to their effects on T cells.

In one series of experiments, different T11⁺ NK clones were used to characterize the results of CD2 activation (Schmidt *et al.*, 1985a). All NK clones expressed T11₁, T11₂, and T11₃ epitopes and following incubation with the combination of anti-T11₂ and anti-T11₃ antibodies, the degree of cell activation was assessed by measuring the surface expression of IL-2 receptor (IL-2R). As previously demonstrated with resting T cells, T11_{2/3} activation resulted in the rapid expression of IL-2R. Moreover, similar results were seen with NKH1⁺CD2⁺CD3⁺ and NKH1⁺CD2⁺CD3⁻ NK clones demonstrating that T11 activation was independent of expression of T cell receptor gene products. In conjunction with the analysis of IL-2R expression, we also examined changes in cell cycle following T11 activation. In NK clone cells cultured with IL-2 alone, the IL-2R was found to be expressed almost exclusively in the G₂ + M phase with no expression of IL-2R by cells during the G₀-G₁ phase. Following activation by anti-T11_{2/3}, a marked increase in IL-2R expression by all cells was observed to the extent that IL-2R expression was no longer dependent on cell cycle. Similar results were obtained by using either CD3⁻ or CD3⁺ NK clones, again indicating that IL-2R expression induced through activation of the T11 antigen complex was not dependent on the expression of a functional T cell receptor. These findings suggest that the CD2 structure functioned in a similar manner in both T cells and NK cells and, in both cell types, the CD2 antigen served as a mechanism for initiation of cell proliferation. The finding that CD2 activation also resulted in the secretion of IL-2 and the induction of T helper activity (Meuer *et al.*, 1984) also suggested that CD2 activation resulted in the induction of T cell function as well as proliferation.

In 1985, Siliciano *et al.* reported that CD2 activation also enhanced the cytolytic function of CTL. In these experiments, MHC-restricted CTL clones were incubated with a combination of anti-T11₂ and

anti-T11₃ antibodies and, following activation, specific CTL effector cells were able to lyse additional targets without MHC restriction. Similar studies performed with NK clones demonstrated that non-MHC-restricted effector cells could acquire the capacity to lyse previously resistant targets following CD2 activation. More recently, Schmidt *et al.*, (1987a.) have extended these observations on clonal NK cells to unstimulated NK cells in peripheral blood. In these experiments, CD2 activation of PBL resulted in a marked enhancement of NK activity that was comparable to that seen with rIL-2. CD2-activated cells were able to display increased killing of sensitive targets as well as lysis of targets that were previously resistant to killing by resting NK cells. Moreover, as previously seen with IL-2-activated killing, CD2 induction of cytotoxicity was exclusively mediated by the small population of NKH1⁺ cells in normal peripheral blood. Taken together, these studies have identified the CD2 antigen as an important regulatory structure for NK cells as well as for T cells and demonstrate that CD2 activation has similar effects in both cell types.

In recent studies (Schmidt *et al.*, 1987b), NK clones have been used to further characterize the mechanisms whereby CD2 activation results in the enhancement of cytolytic effector function. These studies have shown that induction of cytotoxicity was associated with increased formation of effector target cell conjugates and both enhanced conjugate formation and cytotoxicity could be blocked by monoclonal anti-LFA-1 antibody. Ultrastructural analysis of NK cells following CD2 activation demonstrated the increased formation of cell conjugates with resistant targets. Electron micrographs also demonstrated that cell conjugation was associated with the directional reorientation of intracellular granules, centrioles, and Golgi apparatus toward the area of contact between effectors and targets with discharge of granules into pockets within this area of conjugation. When NK clones were activated with anti-CD2 antibodies in the presence of anti-LFA-1, conjugation with targets did not occur and granule contents appeared to be discharged in discrete areas on the surface of the NK clone (Caulfield *et al.*, 1987). These experiments therefore suggest that CD2 enhancement of cytotoxicity is mediated through two distinct mechanisms. The first mechanism is through increased binding of effectors to targets that is at least in part mediated through cell adhesion via LFA-1. The second mechanism is through direct triggering of cytolytic activity resulting in the exocytosis of cytolytic granules. Further support for the direct triggering of the cytolytic mechanism comes from experiments measuring the release of chondroitin

sulfate proteoglycans following CD2 activation. Chondroitin sulfate proteoglycans have been identified in murine mast cell granules (Razin *et al.*, 1982; Seldin *et al.*, 1985) and have also been demonstrated to be one of the constituents of NK cytolytic granules (MacDermott *et al.*, 1985). Exocytosis of proteoglycans has been associated with effector cell lysis of sensitive targets (Schmidt *et al.*, 1985c) and in recent studies (Schmidt *et al.*, 1987b) chondroitin sulfate proteoglycan release has also been demonstrated following CD2 activation of NK clones in the absence of targets.

In conjunction with the functional characterization of CD2 on T cells and NK cells, molecular cloning of the gene coding for CD2 has also been described (Sewell *et al.*, 1986; Sayre *et al.*, 1987). In addition, a number of studies have focused on the identification of LFA-3 as a putative cell surface ligand for CD2 (Shaw *et al.*, 1987; Hünig *et al.*, 1987; Selvaraj *et al.*, 1987; Springer *et al.*, 1987). LFA-3 antigen was initially described (Sanchez-Madrid *et al.*, 1983) as a 55- to 70-kDa protein with wide tissue distribution including endothelial, epithelial, and connective tissue cells. LFA-3 is also expressed on most lymphoid and hematopoietic cells including erythrocytes and, in recent studies (Selvaraj *et al.*, 1987), purified CD2 protein was found to bind to LFA-3 with relatively high affinity ($K_d = 1.9 \times 10^7 M^{-1}$). The CD2 antigen was initially identified as a lymphocyte membrane structure that bound to sheep erythrocytes forming E rosettes and antibodies to CD2 (T11, epitope) inhibit E rosette formation. In this context, Hünig (1985) have identified a molecule on sheep erythrocytes termed T11TS that binds to CD2 and recent experiments (Hünig *et al.*, 1987) indicate that sheep T11Ts and human LFA-3 are homologous structures and that binding of either antigen to CD2 results in T cell activation.

Taken together, these studies provide convincing evidence that interaction of CD2 with a naturally occurring cell surface ligand (LFA-3) provides an activation signal to resting T cells through an antigen-independent pathway. Thus, CD2 appears to be a unique surface structure that has both nonspecific cell adhesion properties as well as providing a pathway for cell activation. It is likely that CD2 plays a similar role on NK cells where the same activation pathway has also been well documented. What remains to be elucidated, however, is the significance of this pathway *in vivo*. Despite the wide cellular distribution of LFA-3 and almost continuous exposure of T cells and NK cells to this structure, almost all T cells and NK cells remain in a resting state *in vivo*. Hünig *et al.* (1987) have postulated that physical contact between CD2 and LFA-3 may be inhibited by

electrostatic forces but further studies will be needed to evaluate this hypothesis.

C. EXPRESSION OF CD3 AND T CELL RECEPTOR (TCR)

The functional definition of NK cells as those cells capable of mediating direct cytolytic activity without MHC restriction implies that NK cells do not mediate their function through a T cell receptor (TCR)-like structure. Nevertheless, the characterization of both murine and human NK clones has suggested that at least some of these cells mediate non-MHC-restricted cytotoxicity through a functional TCR (Hercend *et al.*, 1983b; Yanagi *et al.*, 1985). As shown in Table I, a number of human NK clones express CD3 antigen, a complex structure consisting of at least 3 distinct proteins with molecular weights between 15,000 and 20,000 (Borst *et al.*, 1982, 1983a,b). Expression of CD3 appears to be noncovalently associated with the products of functionally rearranged T cell receptor genes. In most instances, CD3 is associated with a TCR α protein but recent reports have also described CD3 in association with TCR γ homodimers (Moingeon *et al.*, 1987) or TCR γ complexed with another protein (δ) that has yet to be defined (Brenner *et al.*, 1986, 1987a; Borst *et al.*, 1987). There have been no reported instances where CD3 expression is not associated with the expression of a TCR. Interestingly, almost all clones that express TCR γ appear to have non-MHC-restricted cytolytic activity (Nowill *et al.*, 1986; Borst *et al.*, 1987; van de Griend *et al.*, 1987a; Brenner *et al.*, 1987a).

Following the demonstration that some NK clones express CD3, it was determined whether these non-MHC-restricted effector cells expressed and utilized a functional TCR. Using one NKH1⁺CD2⁺CD3⁺ NK clone, JT9, Hercend *et al.* (1983b) generated two monoclonal antibodies, termed anti-NKTa and anti-NKTb, that blocked the cytotoxicity of the clone toward K562 cells. The antigen defined by anti-NKTa and anti-NKTb was identical and was initially identified only on two individual clones, JT9 and JT10. JT10 was derived independently from the same individual as JT9 and was also an NKH1⁺CD2⁺CD3⁺ clone. NKTa was not expressed on other lymphoid and nonlymphoid cells, including a variety of cloned cell lines with either CTL or NK activity. Like the TCR structure on CTL clones, the molecule defined by anti-NKTa was shown to be membrane associated with CD3 in comodulation experiments and immune precipitation experiments demonstrated a heterodimeric structure of 90,000 molecular weight (Hercend *et al.*, 1983b). Subsequent analysis of JT9 cells demonstrated full length transcripts of TCR α and TCR β

mRNA suggesting that this NK clone expressed a TCR α, β protein (Ritz *et al.*, 1985). The blocking capacity of anti-NK1.1 and anti-CD3 antibodies was evaluated in cytotoxicity assays using a panel of target cells, and it was found that both antibodies blocked the cytotoxicity of JT9 and JT10 against all targets. However, in contrast to conventional CTL clones, the expression of cytotoxicity by JT9 and JT10 was not dependent upon recognition of class I or class II MHC gene products on the target cells. Moreover, the cytotoxicity of these CD8⁺ NK clones could not be blocked by anti-T8 antibodies (Hercend *et al.*, 1983b).

Taken together, these results suggested that the specificity of at least some non-MHC-restricted lymphocytes is determined by clonotypic structures. The NK1.1 determinant identified on JT9 and JT10 NK clones appeared to belong to the same family of molecules as TCR structures, previously identified on antigen-specific MHC-restricted T lymphocytes. Although NK clones such as JT9 are thus very closely related to specific CTL, they are nevertheless representative of a unique population of cells that maintain the ability to kill a variety of unrelated target cells in the absence of genetic restriction for class I or II products of MHC.

In order to determine whether NK1.1⁺ cells were derived as a result of *in vitro* stimulation or whether they represent a population with unique specificity *in vivo*, normal peripheral blood of the individual from whom JT9 and JT10 clones were derived was examined for the presence of circulating NK1.1⁺ cells (Schmidt *et al.*, 1986a). Analysis of PBMC from this individual showed that NK1.1⁺ cells occur with a frequency of approximately 0.15%. The existence of NK1.1⁺ cells in peripheral blood was confirmed by the use of immunorosette enrichment techniques, flow cytometric purification, and subsequent clonal expansion of NK1.1⁺ cells. Phenotypic analysis of five additional NK1.1⁺ clones derived in this manner showed that all expressed NKH1 as well as T3, T8, and T11. However, of 10 NK1.1⁺ clones established over a 3-year period from this one individual only five expressed NKH2 antigen. All NK1.1⁺ clones had broad cytolytic activity against a series of different target cells that was similar to that of other NK clones. In addition, cytotoxicity of each clone could be inhibited by preincubation of effector cells with monoclonal anti-NK1.1. Although half of the NK1.1⁺ clones appeared phenotypically different from the other half with regard to the expression of NKH2 antigen, analysis of T cell receptor gene rearrangements indicated that all NK1.1⁺ clones contained identical gene rearrangements of TCR β (Schmidt *et al.*, 1986a). In summary, these results indicated

that NKTa⁺ cells were present in this individual's peripheral blood prior to *in vitro* stimulation and suggest that the expression of the NKTa clonotype defines a distinct population of non-MHC-restricted cytolytic effector cells. The functional role of these cells *in vivo* remains to be determined.

Following the identification of NKH1⁺CD3⁺ non-MHC-restricted cells at the clonal level, studies have also been undertaken to determine whether cells with similar phenotype and cytolytic function are present in unstimulated peripheral blood. Using direct two-color immunofluorescence, normal PBMC from all individuals tested appears to contain a subset of NKH1⁺CD3⁺ cells that represent approximately 2% of PBMC (Schmidt *et al.*, 1986b). This phenotypic subset has LGL morphology and represents approximately 20–25% of the NKH1⁺ population. Following flow cytometric purification of these cells, cytotoxicity assays confirmed their non-MHC-restricted cytolytic activity. In addition, this cytolytic activity could be inhibited by prior incubation with anti-T3 monoclonal antibody. These studies therefore confirmed the presence of NKH1⁺CD3⁺ cells in normal PBMC and also suggested that as with NKH1⁺CD3⁺ clones, the target specificity of these cells was at least in part determined by CD3 associated TCR. In most individuals, the NKH1⁺CD3⁺ subset represents only a small fraction of the NK population but, in one instance, patients with chronic EBV infection syndrome, NKH1⁺CD3⁻ cells are markedly reduced and NKH1⁺CD3⁺ cells represent the major NK active population in peripheral blood (Caligiuri *et al.*, 1987).

In recent studies, *in vitro* culture of normal thymus has been utilized to examine the derivation of NKH1⁺CD3⁺ cells. Following stimulation of thymocytes with concanavalin A and subsequent culture in lymphocyte conditioned medium, Blue *et al.* (1987) have demonstrated that NKH1⁺CD3⁺ cells can be generated *in vitro* and that these cells exhibit broad cytolytic activity in a non-MHC-restricted fashion. In subsequent studies NKH1⁺ cells have been generated from thymic cultures following stimulation with rIL-2 alone (Michon *et al.*, 1987). Prior to stimulation, NKH1⁺ cells could not be detected, but proliferating NKH1⁺ cells rapidly appeared and after 14 days represented greater than 60% of the cultured population. As in previous studies, almost all non-MHC-restricted cytolytic effector function was contained within the NKH1⁺ population. As expected given the thymic origin of these cells, the majority of NKH1⁺ cells in culture expressed CD3. However, all cultures also contained NKH1⁺CD3⁻ effector cells which represented 20–40% of the NKH1⁺ population. As in peripheral blood, both NKH1⁺CD3⁻ and

NKH1⁺CD3⁺ populations exhibited non-MHC-restricted cytotoxicity, but only CD3⁺ effectors could be inhibited by anti-T3 monoclonal antibody. The demonstration that NKH1⁺CD3⁺ effectors could be derived from thymic cultures is consistent with the supposition that the thymus plays a central role in T cell maturation and the acquisition of T cell specificity. The finding that NKH1⁺CD3⁺ cells do not exhibit MHC restriction despite their thymic origin does not necessarily argue against the role of the thymus in the development of MHC-restricted specificity. In contrast, it is evident that under normal circumstances, NKH1⁺CD3⁺ cells are relatively rare and only when thymocytes are cultured under *in vitro* conditions do these cells appear as a major population of cytolytic effectors.

V. Target Antigen for NKH1⁺CD3⁺NKTa⁺ Clones—TNK_{TAR}

In 1984, Hercend et al. described a monoclonal antibody termed anti-TNK_{TAR} that was able to block cytotoxicity of the NKH1⁺CD3⁺ NK clone JT9. Analysis of the functional effects of anti-TNK_{TAR} indicated that, unlike anti-NKTa and anti-T3, blocking of cytotoxicity resulted from the binding of the antibody to the membrane of target cells. Immunoprecipitation experiments indicated that TNK_{TAR} antigen is a heterodimeric structure, which resolves as a single band at 140,000 MW under nonreducing conditions and as two bands at approximately 97,000 and 40,000 MW under reducing conditions in SDS-PAGE. Further experiments demonstrated that the TNK_{TAR} antibody recognized the same antigen as antibody 4F2 (Haynes *et al.*, 1981; Moingeon *et al.*, 1985). This heterodimer is present on lymphocytes and monocytes in human peripheral blood and perhaps, more importantly, the membrane density of TNK_{TAR} antigen rapidly increases following lymphocyte activation. Although the density of this antigen varies greatly from one cell line to another, TNK_{TAR} is expressed on all cultured cell lines that have been tested. In functional assays, anti-TNK_{TAR} and 4F2 block, in an identical fashion, cytotoxicity of all NKTa⁺ clones (Schmidt *et al.*, 1986a). Anti-TNK_{TAR} and 4F2 have no blocking effects on cytotoxicity mediated by other NK or CTL clones that do not express the NKTa clonotype. These data strongly support the view that a surface antigen of 140,000 MW, linked to cell activation, is the specific target structure for these NK-active T lymphocytes. The widespread distribution of this antigen on activated and rapidly proliferating cells is consistent with the broad specificity of NKTa⁺ NK clones and may provide a structural

framework for understanding how CD3⁺ NK cells can mediate their various functions *in vitro*.

VI. Rearrangement and Expression of T Cell Receptor Genes in NK Cells

Following the identification of the T cell receptor proteins in (TCR α and TCR β) (Meuer *et al.*, 1983; Acuto *et al.*, 1983) and the molecular cloning of the genes encoding TCR (Hedrick *et al.*, 1984; Yanagi *et al.*, 1984) it was possible to analyze NK cells and NK cell subsets for rearrangement of TCR genes, production of TCR mRNA, and expression of TCR gene products on the cell membrane in association with CD3 proteins. This analysis has been complicated by the presence of a third (TCR γ) (Saito *et al.*, 1984) and fourth (TCR δ) T cell receptor structure (Brenner *et al.*, 1986, 1987a). Nevertheless, TCR proteins have now been clearly identified and monoclonal antibodies specific for clonotypic TCR epitopes (Meuer *et al.*, 1983; Samuelson *et al.*, 1983; Hercend *et al.*, 1983b; Moingeon *et al.*, 1986) as well as V region determinants (Acuto *et al.*, 1985) and constant region determinants (Tax *et al.*, 1984; Brenner *et al.*, 1987b) have been described. The analysis of TCR expression has been facilitated by the availability of α WT31 monoclonal antibody which recognizes a constant region determinant on the TCR α,β structure that is not expressed by cells expressing TCR γ (Tax *et al.*, 1984; Brenner *et al.*, 1986; Lanier and Weiss, 1986; Nowill *et al.*, 1986).

Analysis of TCR on NK cells has been reported by several laboratories that have studied both murine and human NK clones (Yanagi *et al.*, 1985; Ritz *et al.*, 1985), NK cell lines (Tutt *et al.*, 1986), LGL tumor cells (Rambaldi *et al.*, 1985; Loiseau *et al.*, 1987), and purified NK populations in peripheral blood (Lanier *et al.*, 1986b,c). As expected, TCR expression has been exclusively associated with expression of CD3. CD3-negative cells which represent the majority of NK-active cells in peripheral blood do not express TCR α or β proteins. Consistent with this finding, TCR α , β , or γ gene rearrangements have not been demonstrated, and functional TCR mRNA species have not been identified. Some NKH1⁺CD3⁻ clones have been found to have a truncated TCR β 1.0-kb mRNA, but these cells do not have expression of a functional TCR β protein (Ritz *et al.*, 1985).

In all cases reported, NKH1⁺CD3⁺ cells have been shown to have functional rearrangements of TCR genes and expression of a TCR protein in association with CD3. NKH1⁺CD3⁺ WT31⁺ cells have been found to have rearrangement of TCR β , expression of full length TCR

α and TCR β mRNA, and surface expression of a TCR α,β heterodimer similar to that observed in MHC-restricted T cells (Ritz *et al.*, 1985). A minority of NKH1⁺CD3⁺ cells do not express WT31 antigen and have been found to have full length TCR γ mRNA as well as expression of TCR γ protein (Moingeon *et al.*, 1987). In some instances, the TCR γ protein appears to be associated with another protein (TCR δ) (Brenner *et al.*, 1986, 1987a; Borst *et al.*, 1987), but in other cases TCR γ appears to be expressed as a γ - γ homodimer (Moingeon *et al.*, 1987). In this latter instance, anticlonotypic antibodies specific for TCR γ have been shown to inhibit the cytolytic effector function of these cells suggesting that TCR γ is, in fact, a functional receptor on these unique cells (Moingeon *et al.*, 1986). Of interest, almost all cells that express TCR γ have been found to display non-MHC-restricted cytotoxicity and this, therefore, appears to represent a consistent functional property of this receptor. Nevertheless, a putative target antigen for TCR γ,γ or TCR γ,δ has not yet been identified.

It should also be emphasized that both NKH1⁺CD3⁺ TCR α,β and NKH1⁺CD3⁺ TCR γ cells as well as NKH1⁺CD3⁻ cells have similar cytolytic activity that is not MHC restricted. Except for those NKH1⁺CD3⁺ NKTa⁺ clones that appear to be specifically reactive with TNK_{TAR} (4F2) antigen, putative target cell antigens have not been identified for any non-MHC-restricted clone. Despite the numerous similarities between NKH1⁺CD3⁻ and NKH1⁺CD3⁺ clones, some authors have advocated the redefinition of "natural killer" to include only CD3⁻TCR⁻ effectors and have defined CD3⁺ effectors as non-MHC-restricted CTL (Lanier and Phillips, 1986). This change in nomenclature has some advantages since it more clearly distinguishes TCR⁺ from TCR⁻ effectors and classifies all TCR⁺ effectors as mature T cells. Nevertheless, it is also based on the assumption that all CD3⁺TCR⁺ cells utilize this receptor structure to exhibit non-MHC-restricted cytotoxicity. Despite the demonstration that anti-CD3 and anti-TCR antibodies inhibit cytolytic activity of these cells against all targets, this hypothesis has not been conclusively proven. In fact, there is some evidence to suggest that at least some CD3⁺TCR⁺ effectors may exhibit cytolytic activity through an independent mechanism. One specific example of this possibility has been the identification of CD3⁺CD16⁺ effector cells capable of mediating ADCC (Lanier *et al.*, 1985c). Presumably, these cytotoxic effector cells exhibit ADCC through the CD16 antigen which has recently been shown to be capable of mediating effector cell activation (van de Griend *et al.*, 1987b; Werfel *et al.*, 1987). Thus, ADCC, which is mediated through a distinct cell surface receptor for IgG (CD16),

appears to function independently of the conventional T cell receptor and this functional activity can therefore be exhibited by both CD3⁺ and CD3⁻ effector cells. Although this has not yet been directly examined, there is no evidence to suggest that ADCC mediated by CD16⁺CD3⁺ cells is functionally distinct from ADCC mediated by CD16⁺CD3⁻ cells or that expression of CD3/TCR influences ADCC.

Although it is evident that CD3⁻ NK cells do not utilize conventional TCR, the mechanism whereby CD3⁻ NK cells interact with targets in a non-MHC-restricted fashion (i.e., an NK receptor) has not yet been identified. In this context, there remains the possibility that NKH1⁺CD3⁻ and NKH1⁺CD3⁺ cells utilize a similar receptor for non-MHC-restricted interactions and that inhibition of cytolytic effector function by anti-CD3/TCR does not result from a direct inhibition of an antigen-specific interaction but rather through an indirect inhibition of cell activation. One example of such an indirect inhibition is the ability of anti-CD3 antibodies to block the subsequent activation of T cells via CD2 (Meuer *et al.*, 1984), an alternate pathway of antigen-independent cell activation.

VII. Summary

Recent studies on human NK cells have identified a number of surface antigens that can be utilized to define this population of cells and to identify functionally distinct subsets within this heterogeneous population. In addition, it has been possible to associate specific functional activities with several antigens expressed on NK cells as well as other hematopoietic cells. This information, which is summarized in Table III can be utilized to develop a framework for the classification of cytolytic effector cells. Of primary importance, this classification identifies subsets of cytolytic cells with distinct functional repertoires and distinct cytolytic mechanisms. The majority of NK cells in unstimulated peripheral blood and the majority of NK clones express NKH1 and CD2 antigens but do not express CD3 antigen. These cells morphologically appear as large granular lymphocytes and have broad cytolytic activity against a variety of allogeneic targets without primary sensitization. Consistent with the finding that these cells are CD3 negative, they have not been found to have rearrangement of genes encoding for TCR, or functional mRNA transcripts of either TCR α , TCR β , or TCR γ genes. In addition, these cells do not express heterodimeric surface proteins similar to those that have now been demonstrated to be MHC-restricted T cell

TABLE III
CYTOLYTIC EFFECTOR CELLS

	NK MHC nonrestricted		CTL MHC restricted
Phenotype	NKH1 ⁺ CD2 ⁺ CD3 ⁻	NKH1 ⁺ CD2 ⁺ CD3 ⁺	NKH1 ⁻ CD2 ⁺ CD3 ⁺ CD4 ⁺ or CD8 ⁺
Morphology	LGL	LGL	LGL
Activation pathways	CD2	CD2, CD3	CD2, CD3
TCR gene expression	None	$\alpha\beta$, $\gamma\gamma$, or $\gamma\delta$	$\alpha\beta$
Target antigen	Unknown	?Activation antigen	Antigen + MHC

receptors for antigen. Taken together, these findings provide strong evidence that NKH1⁺CD3⁻ NK clones do not interact with target cells through a T cell receptor-like structure. Nevertheless, these NK cells do share several properties with conventional CTL. These functional T cell characteristics include (1) expression of CD2-T11/E rosette receptor antigen, and (2) utilization of LFA-1 surface antigen to enhance effector cell adhesion to target cells. As previously demonstrated for T cells, NK cells can be activated through the CD2 molecule and this has recently been shown to result in the enhancement of cytolytic function by these effectors. Since CD2 can also function as a cell surface ligand for LFA-3, an antigen expressed on NK targets, the CD2 molecule may be considered as a potential NK receptor structure. The fact that a very small subset of NK cells ($\approx 10\%$) as well as some NK clones (JT11) does not express CD2 argues against a potential role for CD2 as the NK cell receptor. Certainly, further studies will be necessary to clarify the role of CD2 on NK cells and to identify the mechanisms whereby NKH1⁺CD3⁻ NK cells interact with targets in a non-MHC-restricted fashion.

In addition to the majority of NK cells which are NKH1⁺CD3⁻, a subset of NK clones has been shown to express CD3 antigen. These clones appear to be derived from a small subset of NK cells in peripheral blood with similar phenotype and can be distinguished from conventional CTL because they express NKH1. NK cells of this type have (1) functional rearrangement and expression of either TCR α , TCR β , or TCR γ genes, (2) surface membrane expression of TCR proteins, and (3) clonotypic membrane antigens associated with CD3 antigen. CD2 and LFA-1 surface antigens also appear to play a functional role on these cells. The target specificity of these cells therefore appears to be mediated through conventional T cell-like

mechanisms, but these cells can nevertheless be distinguished from CTL because they have broad cytolytic activity that is independent of MHC restriction. The recent identification that a subset of T3⁺NKH1⁺ clones which express the NKTa clonotype is specific for a target antigen termed TNK_{TAR} that is widely expressed on hematopoietic cells further substantiates the MHC-independent specificity of these cells. Nevertheless, the role of NKH1⁺CD3⁺ cells *in vivo* remains to be determined as does the possibility that these cells may function independently of other types of non-MHC-restricted effectors.

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The Common Mediator of Shock, Cachexia, and Tumor Necrosis

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I. Introduction

A large number of pathologic states may arise when immune mechanisms that evolved for the protection of the host are expressed in an unrestrained manner. Complement-mediated injury, anaphylactic shock, and cell-mediated hypersensitivity reactions are often cited as disease processes in which the immune system has worked to the detriment of the host. During the past few years, several of the primary mediators of inflammatory response have been isolated, and it has become clear that certain host proteins can, of themselves, cause injury through direct and indirect effects on host tissues, and through their effects on host metabolism. Prominent among these proteins is a macrophage-derived hormone known as cachectin.

Cachectin, acting alone and in concert with other mediators, is capable of evoking a "shock" state, in which hypotension, derangements of lipid and glucose metabolism, metabolic acidosis, and end-organ damage may injure or kill the host. When administered at low doses over a prolonged period of time, cachectin induces a state of anorexia and wasting similar to cachexia as it occurs in chronic infection or cancer. In part through its effects on vascular endothelial cells, cachectin can induce a coagulopathic state localized to certain vascular beds, leading to hemorrhagic necrosis of various tissues. This effect is particularly pronounced in certain tumor vessels, such that the hormone was originally recognized by its ability to provoke the hemorrhagic infarction of transplantable neoplasms, and was termed "tumor necrosis factor." It is now evident that cachectin/tumor necrosis factor serves as a general mediator of inflammatory processes, and that cachectin, along with several other cytokines, may be an

essential element in the pathogenesis of many human and animal diseases.

II. The Dual Nature of Cachectin (TNF)

Reflecting its important role as a modulator of numerous physiologic processes, cachectin was isolated as the result of two separate paths of scientific inquiry. On the one hand, as "cachectin," the molecule was purified as a mediator of shock and wasting in chronic disease. On the other hand, as "tumor necrosis factor," the molecule was isolated as a mediator of one of the "beneficial" effects of bacterial endotoxin (induction of tumor necrosis). A brief review of these efforts is presented here.

A. THE SEARCH FOR "CACHECTIN"

In the mid 1970s, Cerami and co-workers studied cachexia (wasting) in chronic disease, utilizing trypanosomiasis as a model system. Trypanosome-infected cattle, rabbits, and certain other species succumb to a marked wasting diathesis which occurs despite the presence of a low parasite burden *in vivo*. Only a few grams of parasite may be sufficient to provoke wasting in a large cow. Thus, in this model of cachexia, a competitive mechanism cannot explain the consumption of lipid and protein mass that is observed.

During the terminal phase of infection, trypanosome-infected rabbits were found to become anorectic, and to lose over half of their initial body mass. Paradoxically, such animals also developed an impressive hypertriglyceridemia (1,2). Plasma triglycerides, principally in the form of very-low-density lipoprotein of (VLDL), may be elevated by a factor of 10, with respect to levels observed in noninfected controls. Rouzer and Cerami determined that the rise in triglycerides resulted from a clearing defect, caused by systemic suppression of the enzyme lipoprotein lipase (LPL) (1).

Subsequently, Kawakami and Cerami demonstrated that LPL suppression also occurred in endotoxin-treated mice, and that LPL suppression was conferred by a transferable serum factor (3). This factor, termed "cachectin," was shown to be produced principally by the macrophage.

Cachectin was also found to be capable of suppressing LPL expression by adipocytes *in vitro* (4). LPL suppression was dependent upon inhibition of biosynthesis, rather than upon a direct interaction between cachectin and the LPL molecule. Moreover, cachectin appeared to suppress the biosynthesis of enzymes required for *de*

novo triglyceride synthesis (e.g., acetyl-CoA carboxylase and fatty acid synthetase) (5).

While a variety of invasive stimuli were shown to induce the biosynthesis and release of cachectin by macrophages, endotoxin was the most potent stimulus identified (6). Quiescent macrophages produced no detectable cachectin (7).

Cachectin was purified to homogeneity by Beutler *et al.* who showed that the LPL-suppressing activity emanated from a polypeptide hormone with a molecular weight of approximately 17,000 (8). Cachectin was shown to be produced in considerable abundance by LPS-activated RAW 264.7 (mouse macrophage) cells. Approximately 1% of the secretory protein derived from cultured LPS-stimulated RAW 264.7 cells, or natural mouse peritoneal macrophages, is cachectin. Correspondingly, large quantities of cachectin are produced *in vivo* (9), particularly in animals primed with agents capable of causing reticuloendothelial hyperplasia.

Purified cachectin was shown to bind to a high-affinity plasma membranae receptor, present on a wide variety of cultured cells and tissues obtained from living animals (8). Approximately 10^4 receptor sites were identified per 3T3-L1 cell *in vitro*, and an affinity constant of $3 \times 10^9 M^{-1}$ was determined by Scatchard analysis. Cachectin was shown to prevent the morphologic differentiation of adipocytes *in vitro*, and to suppress the expression of mRNA molecules produced specifically by mature adipose tissue cells (10). Cachectin was also shown to cause anorexia and weight loss in mice, and to produce acute toxic effects in a variety species, as detailed below.

B. THE SEARCH FOR "TUMOR NECROSIS FACTOR" (TNF)

Late in the nineteenth century, Coley reported the induction of hemorrhagic necrosis of human tumors by bacterial broths derived from the culture of *Streptococcus* and *Serratia* organisms (11–15). Infusion of such broths could lead to a reduction of tumor mass; however, the toxic effects associated with this form of therapy led to its eventual abandonment.

Shear and Andervont isolated the bacterial product responsible for induction of hemorrhagic necrosis of transplantable tumors in mice, and termed this material the "bacterial polysaccharide" (16). Now known as "lipopolysaccharide," the agent responsible for induction of tumor necrosis is unfortunately also the most toxic fraction of Coley's broth.

In 1962, O'Malley *et al.* (17) observed that serum derived from LPS-treated mice contained an endogenous factor capable of inducing

hemorrhagic necrosis of a tumor grown in a second animal. In 1975, Carswell *et al.* reported a similar observation (18), and noted that postendotoxin serum derived from mice treated with *Bacillus Calmette-Guerin* (BCG) was cytotoxic for a variety of tumor cells *in vitro* (19). The activity they described was dubbed "tumor necrosis factor." TNF, as such, was isolated by Aggarwal *et al.* (20), who also succeeded in isolating the related cytolytic polypeptide hormone lymphotoxin (21). The cDNA sequence was reported by several groups (22–24). Details of the structure of TNF (cachectin) and the related hormone lymphotoxin are discussed below.

C. LYMPHOTOXIN

In the 1960s, Ruddle and Granger independently reported the existence of a lymphocyte-derived cytolytic activity (25,26) which was produced in response to specific antigenic or nonspecific mitogenic stimuli (27–29) in an H-2-restricted manner (30). This activity was observed in the course of an investigation of cell-mediated hypersensitivity reactions. Not only T lymphocytes, but also B-lymphoblastoid cells were found to be capable of producing lymphotoxin. Macrophages do not produce lymphotoxin in response to stimuli capable of eliciting its production by lymphocytes, nor do lymphocytes produce lymphotoxin when exposed to endotoxin. The structure of lymphotoxin was found to be homologous to that of cachectin/TNF, as detailed below.

D. IDENTITY OF CACHECTIN AND TNF

The amino-terminal sequence of mouse cachectin, when determined, displayed a high degree of homology to the sequence of human tumor necrosis factor (31). When mouse TNF cDNA was cloned, its sequence exactly predicted the amino-terminal sequence of mouse cachectin (32–34). Moreover, immunologic studies, and reciprocal measurements of bioactivity indicated that cachectin and TNF were identical molecules. A variety of exciting possibilities were suggested by this observation.

III. The Structure of Cachectin/TNF

PRIMARY STRUCTURE AND CHROMOSOMAL LOCATION

Cachectin and the related cytotoxic polypeptide hormone lymphotoxin are encoded by closely linked genes lying within the major

histocompatibility complex [chromosome 6 in man (35,36) and chromosome 17 in the mouse (37)]. In the mouse, these genes have been mapped to a position 70 kb proximal to the *D* locus (38).

Human cachectin and human lymphotoxin are approximately 28% identical at the amino acid level, display a highly concordant set of bioactivities, and bind to a common receptor. Structural dissimilarities include the possession, by cachectin, of an internal disulfide linkage (absent in lymphotoxin), and extensive glycosylation (absent in cachectin). The close physical proximity of the genes encoding these two proteins, the developmental proximity of the cells that produce the hormones, and the relative disparity of stimuli required to evoke their production are all subjects of considerable interest.

Mouse cachectin is initially synthesized as a polypeptide hormone containing 235 amino acids, but is secreted as a 156 amino acid protein, after processing to remove 79 amino-terminal residues. Human cachectin (157 residues in length) contains a propeptide segment consisting of 76 amino acids. While the mature polypeptide hormones are highly conserved among species (79% conservation between human and murine forms), the propeptide segment is even more conserved: 86% of the amino acids present in this portion of the molecule are shared by proteins derived from human and murine sources. It has been proposed that the propeptide may fulfill a distinct, yet-to-be-identified hormonal function. However, it is known that the propeptide is extensively cleaved in processing to yield the mature 17-kDa protein (39,40).

Cachectin aggregates *in vitro* to yield a protein of variable size. Dimers, trimers, pentamers, and perhaps higher order multimers have been reported. Baglioni and Smith have recently suggested, based on cross-linking studies, that the active form of the protein is trimeric (41). Extensive β -sheet content is thought to be present in the native molecule (42,43).

Inferences concerning the location of the active site have been made, based on analyses of interspecific homology, and the structural resemblance between cachectin and lymphotoxin, both of which bind to a common receptor (44). Amino acids 115 through 130 (with respect to the sequence of the mature human protein) have been highly conserved among human, rabbit, and mouse proteins. Genetic manipulation of the primary structure has revealed that the carboxy-terminus of the cachectin molecule is particularly crucial for bioactivity; amino-terminal deletions several amino acids in length may be sustained without loss of bioactivity.

IV. Toxic Effects of Cachectin

A. MEDIATION OF THE EFFECTS OF ENDOTOXIN

Many of the clinical features of gram-negative septicemic shock may be reproduced in animals by administration of bacterial LPS. Yet, for over a decade, it has been clear that LPS works its toxic effects indirectly. Michalek and colleagues demonstrated that the lethal effect of LPS is conferred by cells of hematopoietic origin (45). LPS-resistant mice were shown to be rendered sensitive to LPS by transplantation with marrow obtained from sensitive, histocompatible donors. For a variety of reasons, the macrophage was thought to be important in the mediation of endotoxicity. LPS-stimulated macrophages were shown to produce a factor capable of killing LPS-resistant mice (46). Yet the factor responsible for mediating the effects of LPS remained obscure until recent times.

Determination of the identity of cachectin and TNF suggested that cachectin might be an important mediator of the lethal effect of LPS, since this single molecule was responsible for two very different effects of LPS (hypertriglyceridemia and tumor necrosis) in mice, was produced in large quantities in response to LPS, and was highly catabolic. Accordingly, Beutler *et al.* sought to determine whether inhibition of cachectin action *in vivo* might attenuate the effects of LPS.

Passive immunization against cachectin was shown to protect against the lethal effect of LPS in mice (47). Subsequently, similar observations have been made in primates (K. J. Tracey *et al.*, unpublished data) and in rabbits (Matheson and Ulevitch, personal communication). Such experiments have confirmed that cachectin is an essential element in the response to LPS.

When large quantities of recombinant human cachectin became available, it also became clear that cachectin could, by itself, induce a state of shock that closely resembled shock induced by endotoxin. Indeed, the toxicity of cachectin, in some species, is approximately 100,000-fold greater than cyanide when compared on a molar basis. When pretreated with a variety of agents that sensitize to LPS [including galactosamine (48) and lead (B. Beutler, unpublished data)], animals are rendered still more sensitive to cachectin.

The most comprehensive studies of the acute toxic effects of cachectin have been carried out in rats (49). Here cachectin was found to cause metabolic acidosis, transient hyperglycemia followed by hypoglycemia, hypotension, hemorrhagic infarction of the gastrointestinal tract, adrenal hemorrhage, pancreatic hemorrhage, acute renal

tubular necrosis, and a severe interstitial pneumonitis. The latter lesion was a frequent cause of mortality, since it led to ventilatory arrest.

In dogs (50), a variety of similar changes were observed, together with an acute stress response characterized by elevations in plasma catecholamines, glucagon, and glucocorticoid hormones. Thus, cachectin was shown to be both necessary and sufficient to induce many of the features of endotoxin poisoning.

Many of the effects noted in the above-mentioned studies have been confirmed by other workers (51,52), who have also noted that cyclooxygenase inhibitors diminish the lethal effect of cachectin *in vivo* (52). It is likely that arachidonate metabolites do, indeed, act as final effectors following administration of cachectin, and may lead to many of the histopathologic changes that are observed. For example, it has been shown that cachectin is a potent inducer of cysteinyl leukotriene and PAF release *in vivo*, and it is widely assumed that these metabolites play an important role as terminal mediators of endotoxic shock (D. Keppler, personal communication).

B. MEDIATION OF CACHEXIA

Chronic exposure to low levels of cachectin leads to a state of anorexia and wasting, which occurs over a period of months. This state closely resembles cachexia as it is observed in association with invasive neoplastic diseases and a variety of infectious disorders. Perhaps the clearest demonstration of cachectin's "cachexia-inducing" potential was provided by Oliff and colleagues, who produced a genetically altered Chinese hamster ovary (CHO) cell line that secreted recombinant human cachectin *in vitro* and *in vivo*. (53). Nude mice inoculated with cachectin-secreting tumor cells, like control animals inoculated with cells bearing a vector that did not encode cachectin, developed a nonmetastatic tumor, confined to the hind limb. However, cachectin-secreting tumors caused a state of progressive wasting, despite the presence of relatively low levels of cachectin in their serum. Tumors that did not secrete cachectin were compatible with continued weight gain.

It has now become possible to detect cachectin in the serum of human patients suffering from a variety of diseases, including gram-negative septic shock (54) and certain malignancies (F. Balkwill, personal communication). Further work will be required to establish a precise correlation between cachectin levels and pathophysiologic effect in man; however, it seems likely that such measurements will provide a valuable clinical tool in the future.

C. OTHER TOXIC AND INFLAMMATORY ACTIVITIES

Cachectin has also been implicated in the pathogenesis of malaria (particularly the cerebral complications of malaria) (55,56), and more recently, in the early changes that characterize graft-versus-host disease (57). Thus, a wide variety of pathologic states may result from the action of this protein, depending upon the kinetics of its production, and the context in which it is produced.

V. Cachectin Biosynthesis: Cellular Source, Inducing Stimuli, and Kinetics of Synthesis and Release

A. CELL OF ORIGIN

Cachectin is produced by all types of macrophages thus far tested, including macrophages of pulmonary, hepatic, peritoneal, and bone marrow origin (58). The latter, however, like peripheral blood monocytes, respond weakly to LPS, at least in the absence of a "priming" signal such as interferon- γ . Thus, it would seem that cell maturation, as well as differentiation along the mononuclear phagocytic line, is important in cachectin biosynthesis.

Several other cell types have also been reported to produce cachectin. These include T lymphocytes, which produce abundant quantities of cachectin mRNA and protein when induced with the calcium ionophore A23187 in conjunction with phorbol myristate acetate (59). However, these cells fail to respond to inducing stimuli such as LPS, or to various mitogens, by producing cachectin. Natural killer cell colony inhibiting activity (NK-CIA) is thought to be cachectin (60). At least a fraction of the cytotoxic activity produced by mast cells is thought to be cachectin (61,62), and it has been reported that smooth muscle cells are capable of producing cachectin mRNA (63). To date, however, smooth muscle cells have not been shown capable of elaborating cachectin protein. The quantity of cachectin released by each of these cell types in response to natural inducing stimuli is very small compared to that produced by macrophages, and in the absence of evidence to the contrary, it must be considered that the macrophage is the principal source of cachectin *in vivo*.

A variety of invasive stimuli are capable of causing macrophage cachectin production (6). Among these, LPS is by far the most potent. Certain viruses, including Sendai virus (64) and influenza virus (65), are also capable of inducing cachectin secretion. Trypanosomal lysates, plasmodial lysates, and certain gram-positive organisms including staphylococci induce cachectin production as well, but only in

rather small amounts. Interferon- γ , while incapable of causing cachectin biosynthesis on its own (66), appears to increase the rate of cachectin gene transcription (67), and when administered in conjunction with LPS, augments cachectin biosynthesis (66). Interleukin 2 (IL-2) may have similar effects (68). Recently, it has been reported that cachectin mRNA production is also stimulated in cultured macrophage cell lines by granulocyte macrophage colony-stimulating factor (GM-CSF) (69). It is not clear whether this cytokine leads to the synthesis of cachectin protein.

B. CONTROL OF CACHECTIN BIOSYNTHESIS

Cachectin biosynthesis is controlled chiefly at a posttranscriptional level (70). Cachectin gene transcription increases some 3-fold in response to stimulation of the macrophage by LPS; however, cachectin mRNA levels may increase 100-fold, and cachectin protein production may increase 1000-fold or more. In part, this posttranscriptional control may depend upon variable instability of the cachectin mRNA, which, in turn, may be attributable to the presence of an AU-rich 3'-untranslated sequence, first identified in a variety of cytokine messages by Caput *et al.*, (32). Shaw and Kamen (71) demonstrated that such sequences are highly destabilizing *in vivo*, and it would appear that they comprise a recognition site for the action of a selective ribonuclease (B. Beutler, unpublished data).

Among the physiologic stimuli that influence cachectin biosynthesis, glucocorticoid hormones may occupy a dominant position. Glucocorticoids strongly suppress cachectin biosynthesis *in vitro* if administered to macrophages prior to contact with LPS and may have a similar effect *in vivo*; this fact may largely explain their highly protective effect when administered to animals prior to an otherwise lethal dose of LPS (70). Adrenalectomized animals, which are highly sensitive to the lethal effect of LPS, might be so as a result of their inability to suppress cachectin biosynthesis, in the course of the stress response that normally occurs under conditions leading to cachectin release. Cachectin itself causes elevation of glucocorticoid levels in dogs (50); it is probable that this represents a physiologic feedback loop.

C. KINETICS OF CACHECTIN SYNTHESIS AND RELEASE

In vitro, cachectin mRNA concentrations rise detectably within macrophages minutes after exposure to LPS; detectable quantities of cachectin protein are secreted within 20–30 minutes following induction. mRNA levels peak 2–3 hours after LPS induction, and,

thereafter, decline slowly to approximate levels observed within noninduced cells after 15–20 hours, despite the continued presence of an inducing stimulus (B. Beutler, unpublished data). Cachectin production itself ceases within 4–6 hours following contact with LPS. Continuous contact with LPS is essential for cachectin biosynthesis over the period of time during which synthesis occurs (72). A fixed quantity of cachectin may be produced by LPS-activated macrophages, and this production ceases if LPS is removed at any time following induction.

In rabbits, cachectin levels reach detectable concentrations within the plasma space within 20 minutes of an intravenous injection of LPS (9). Levels peak between 90 minutes and 2 hours, and then fall to approach baseline values within 5 to 6 hours. In animals primed with agents causing reticuloendothelial hyperplasia, cachectin concentrations may approach micromolar levels (73). In view of the fact that the hormone is cleared from the blood with a short half-life (9) (6–7 minutes in mice), such concentrations reflect the net production of milligram quantities of cachectin per kilogram of body mass.

Primates (D. G. Hesse *et al.*, unpublished data), as well as mice (74), exhibit similar kinetics of cachectin production, and are capable of producing quantities of cachectin that, with respect to body size, are similar to those produced by rabbits.

VI. The Effects of Cachectin on Various Cells and Tissues

A. RECEPTOR DISTRIBUTION

Virtually all somatic tissues, with the exception of erythrocytes, possess a receptor for cachectin. Receptor number is highly variable, ranging from a few hundred copies on some cells (75) to over 20,000 on others. Receptor number, in most cases, bears no relationship to susceptibility to cytotoxic effect.

In some instances, as in T lymphocytes, the receptor may be absent (or present in an inactive form) in resting cells, but can be induced by primary activation (75). Reflecting its importance as a general mediator of inflammatory response, cachectin exerts an effect on the metabolic activities of most tissues exposed to it, and alters immune function in most, and perhaps all, classes of leukocyte.

B. METABOLIC EFFECTS OF CACHECTIN

Perhaps the best studied target of cachectin action is adipose tissue. Cachectin was originally isolated by virtue of its ability to suppress

expression of LPL in cultured fat cells. It is now clear that cachectin is also capable of enhancing glycerol release (presumably through activation of the hormone-sensitive lipase) (76,77), suppressing adipocyte differentiation (10), and suppressing the expression of a variety of adipose tissue-specific proteins at a transcriptional level. These include glycerolphosphate dehydrogenase (10), the fatty acid binding protein (10), and adipsin (78), a serine protease produced largely by adipocytes. A number of mRNA molecules specifying yet-to-be-identified proteins found specifically in mature adipocytes are also suppressed (10). Administration of cachectin to cultured adipocytes prevents or reverses morphologic differentiation *in vitro* (10), and antagonizes the lipogenic effect of insulin (79). Moreover, administration of cachectin to animals results in elevation of plasma triglyceride, suppression LPL, and resorption of fat *in vivo* (80).

A variety of other tissues also express LPL, and in these tissues, as in plasma, LPL levels tend to increase (80). The reason for this different pattern of response has not been clearly delineated.

Muscle cells exhibit a fall in transmembrane potential (81) following contact with cachectin, and an increase in glucose transport (82). The significance of these changes remains to be determined; however, they have been noted in endotoxemic states, and likely reflect increased metabolic activity of a type associated with shock and wasting.

C. OTHER TISSUE-SPECIFIC EFFECTS OF CACHECTIN, AND THEIR IMPLICATIONS

Among the most important cell types affected by cachectin is the neutrophil. Neutrophils are activated by cachectin according to a number of criteria (83–89). Cachectin augments neutrophil phagocytic activity, increases cytotoxicity to certain microorganisms, enhances production of superoxide anion and H_2O_2 , stimulates degranulation, inhibits neutrophil migration under agarose, and causes neutrophil adhesion to endothelial cells. Cachectin also has been reported to exert a chemotactic effect.

The propensity of cachectin-activated neutrophils to adhere to endothelial surfaces may be particularly important, since induction of pulmonary leukostasis appears to be one of the major toxic effects of the hormone (49). In *in vitro* systems, cachectin prompts the disruption of endothelial monolayers by activating neutrophils (89). Cachectin-induced neutrophil adhesion is very rapid in onset (full effect observed in less than 5 minutes) and does not require protein synthesis on the part of the neutrophil (84).

Cachectin has a separate, slower effect on endothelial cells themselves, which also results in neutrophil adhesion. This effect requires protein synthesis, and may involve the expression of a number of antigens related to intercellular adhesion (84).

Cachectin is directly cytotoxic to endothelial cells (90), and it is also reported that cachectin causes an endothelial cell rearrangement (91) in tissue explants *in vitro*. When exposed to cachectin, endothelial cells produce a procoagulant factor (92,93), and show decreased expression of thrombomodulin (92). These two effects permit the accretion of thrombus on endothelial surfaces, a phenomenon which may be related to the pathogenesis of disseminated intravascular coagulation, hemorrhagic necrosis of tumors, and the migratory thrombosis that accompanies certain neoplastic diseases. In addition to these direct effects, cachectin prompts endothelial cell production of interleukin 1 (IL-1), which can, in turn, trigger leukocyte activation and initiate coagulation.

Eosinophils (96) kill schistosomula more effectively when exposed to the cachectin. In addition, monocyte-mediated tumor cell killing (97) and macrophage IL-1 production (98) are stimulated by cachectin. Cachectin augments lymphocyte expression of IL-2 receptor and HLA-DR antigen (75), suggesting that it may play a role in the response of these cells to antigenic stimulation.

In addition to its effects on mature leukocytes, cachectin profoundly influences the development of hematopoietic progenitor cells (60,99–101). The hormone also suppresses erythropoiesis *in vivo* (102,103), and it has been suggested that its release may be involved in the anemia of chronic disease.

Cachectin is a potent endogenous pyrogen, capable of causing fever through a direct effect on hypothalamic neurons, and, indirectly, by triggering peripheral production of IL-1. Hence, a biphasic fever curve is generated following intravenous administration of cachectin to rabbits (104). Cachectin stimulates bone resorption (105), a property that it shares with IL-1 and lymphotoxin. By some estimates, IL-1 is approximately 1000-fold more potent than cachectin in its ability to trigger the release of calcium from bone *in vitro* (106). However, cachectin also appears to synergize with IL-1, increasing the osteolytic effect of IL-1 as much as 10-fold. Lymphotoxin (but not cachectin) release by myeloma cells has been reported, and may be involved in the hypercalcemia and osteolytic lesions that prevail in multiple myeloma (G. Mundy, personal communication).

Cachectin also causes the degradation of proteoglycan in cartilage, and inhibits its synthesis *in vitro* (107). It has a catabolic effect on

synovial cells and dermal fibroblasts, triggering the production of PGE-2 and collagenase by both (108). These effects on connective tissues may indicate that cachectin plays an important role in local inflammatory processes, particularly those involving the bones and joints. Indeed, it has recently been reported that cachectin may be detected in synovial fluid in a variety of inflammatory joint diseases (109), and that it is produced *in vitro* in response to stimuli that trigger joint inflammation (e.g., monosodium urate and hydroxyapatite crystals) (110,111).

Cachectin, like IL-1, prompts the production and release of various acute-phase reactants from hepatocytes (112–114). These include complement proteins factor B and C3, α_1 -antichymotrypsin, and serum amyloid A. Cachectin suppresses albumin and transferrin biosynthesis *in vitro*. Such changes in hepatic protein synthesis, commonly noted in the course of acute infectious diseases, may assist the host in its effort to deal with invasive organisms.

VII. Characterization of the Cachectin Receptor

A. RECEPTOR NUMBER AND AFFINITY

The cachectin receptor was first identified on C2 myotubules and 3T3-L1 cells, where it was represented at a density of approximately 10,000 binding sites per cell, and exhibited an affinity constant of approximately $3 \times 10^9 M^{-1}$ (8). Similar receptors, varying in number between a few hundred and as many as 50,000 per cell, have been found on a wide variety of tissues.

B. RECEPTOR STRUCTURE AND SIGNAL TRANSDUCTION

Several investigators, using cross-linking techniques, have reported that the binding subunit of the cachectin receptor is approximately 75 kDa in size (41,115–119). A second subunit may also be required for cytotoxic activity. It appears to be larger (approximately 138 kDa) (117). The molecular size of the intact receptor, as assessed by gel filtration, has been reported to be approximately 300 kDa (120).

It would appear that the binding of the cachectin to a target cell is not, by itself, sufficient to produce a response. Thus, cachectin derived from different species may bind to target tumor cells with identical affinity, yet produce responses of different magnitude (120). It would also seem that the cytotoxic effect of cachectin may proceed through a different postreceptor pathway than the cytostatic effect of the hormone.

The nature of the signal transduced following hormone–receptor association has not yet been clarified, and, indeed, the response to this signal may differ considerably in different tissues.

VIII. The Beneficial Effect of Cachectin

Since the inflammatory response benefits the organism, cachectin most likely serves a beneficial function when produced in appropriate quantities. Indeed, it would seem that cachectin must fulfil a beneficial function in order to have been conserved throughout mammalian evolution.

In murine malaria, cachectin has been associated with the pathogenesis of cerebral complications (55), but also appears to fulfill a protective function. Cachectin allows neutrophils to kill *Candida albicans* organisms *in vitro* (87), and also inhibits the growth of certain viruses in culture, apparently by directly killing virus-infected cells (121–123).

In the absence of a survival advantage to the individual, cachectin may benefit the kindred of an infected individual by assuring his rapid removal from a population, thereby eliminating a source of contagion, and a source of competition for shared resources. While this aspect of the protein's function cannot account for its conservation throughout mammalian evolution, the "detrimental" effects of cachectin may not always be detrimental to the species.

Cachectin is now generally regarded as an essential mediator of the inflammatory response, and its beneficial effects must surely reflect those of inflammation itself. Once a thorough understanding of cachectin action has been attained, we come to understand the function of the inflammatory response, its role in immunity, and its manipulation for the benefit of the host.

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Myasthenia Gravis

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I. Introduction

A. OVERVIEW OF MYASTHENIA GRAVIS AND EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

The muscular weakness and excessive fatigability that characterize myasthenia gravis (MG)¹ are caused by an antibody-mediated autoimmune response to nicotinic acetylcholine receptors (AChRs) in skeletal muscle. MG is the best characterized autoimmune disease of the nervous system; and, arguably, the best characterized autoimmune disease.

Discovery of the autoimmune nature of MG was made possible by advances in molecular studies of the AChR. When AChRs were first purified from electric eels, it was observed that rabbits immunized with purified AChRs developed muscular weakness (Patrick and Lindstrom, 1973). This was termed experimental autoimmune MG (EAMG). Subsequently, autoantibodies to AChRs were detected in MG patient sera (Almon *et al.*, 1974; Lindstrom *et al.*, 1976d), and the pathological mechanisms by which autoantibodies impaired neuromuscular transmission in MG and EAMG were shown to be quite similar (Lindstrom, 1979; Engel, 1984).

An appreciation of the development of ideas about MG may be obtained through the volumes that record proceedings of meetings on MG held at 5-year intervals since 1954 by the New York Academy of Sciences. By 1971 (Vol. 183), it was clear that MG involved a defect in neuromuscular transmission, but although there was some recent evidence of a postsynaptic defect (Engel and Santa, 1971), a presynaptic abnormality was still suspected. In the 1976 volume (#274),

¹ Abbreviations: α Bgt, α -bungarotoxin; AChR, acetylcholine receptor; APC, antigen-presenting cell; Bis Q, trans-3,3'-bis[α -(trimethylammonium)methyl] azobenzene bromide; DM, daunomycin; EAMG, experimental autoimmune myasthenia gravis; GABA, γ -aminobutyric acid; LEMS, Lambert Eaton myasthenic syndrome; mAb, monoclonal antibody; MG, myasthenia gravis; MIR, main immunogenic region; MW, molecular weight; SDS, sodium dodecyl sulfate.

discovery of EAMG (Lindstrom *et al.*, 1976c; Seybold *et al.*, 1976; Lennon *et al.*, 1976; Lambert *et al.*, 1976; Engel *et al.*, 1976c) and autoantibodies to AChRs in MG patients (Almon and Appel, 1976; Drachman *et al.*, 1976; Lindstrom *et al.*, 1976d) made it clear that the defect in neuromuscular transmission was caused by an autoimmune response to AChRs. The next volume (#377), in 1981, recorded advances in immune diagnosis and antigenic structure of the AChR, studies of pathological mechanisms, and great interest in immunosuppressive drugs and plasmapheresis. With the discovery of the autoimmune nature of MG, it became possible to clearly distinguish MG from rare myasthenic syndromes caused by genetic defects in the AChR or other components of neuromuscular transmission, or from autoimmune responses to other components of the neuromuscular junction (Vincent and Newsom-Davis, 1979; Engel *et al.*, 1981; Mossman *et al.*, 1986). In the 1976 volume, passive transfer of MG from patients to mice by IgG was reported (Drachman *et al.*, 1976). By the 1987 volume (#505), passive transfer by IgG was used to demonstrate that Lambert Eaton myasthenic syndrome (LEMS) is caused by an autoimmune response to voltage-sensitive calcium channels on the presynaptic membrane, which impairs release of ACh (Roberts *et al.*, 1985; Fukuoka *et al.*, 1987a,b). Thus, not only has knowledge of the autoimmune nature of MG increased in depth, but concepts and methods used in its study have increased our knowledge of other diseases of neuromuscular transmission.

Several important aspects of MG are clearly known. It is clear that neuromuscular transmission is impaired by an antibody-mediated autoimmune response to muscle AChRs, that assay of serum antibodies to AChR can improve diagnosis, and that immunosuppressive drugs and plasmapheresis are useful in the treatment of MG. Advances in the study of AChRs and in the techniques for determining antibody specificity have permitted much greater definition of the antigenic structure of AChRs and the effects of antibodies bound to AChRs. Advances in cellular immunology and in the techniques for determining T cell specificities are permitting much better definition of the cellular interactions involved in production of antibodies to AChRs.

However, two important aspects of MG remain mysterious: (1) what initiates and sustains the autoimmune response to AChRs, and (2) how to specifically suppress this autoimmune response. It is in these areas that the next big breakthroughs in MG research remain to be made.

The best clue to the cause of MG may be that the autoimmune

response in another antibody-mediated disease of neuromuscular transmission is a paraneoplastic response. Evidence that the autoantibodies which impair neuromuscular transmission in LEMS are provoked by calcium channels on a slowly growing small cell carcinoma and that they cross-react with similar calcium channels in the presynaptic membrane (Roberts *et al.*, 1985; Fukuoka *et al.*, 1987a,b) is especially intriguing when considering the high incidences of thymoma and thymic hyperplasia in MG (Castleman and Norris, 1966; Schluep *et al.*, 1987).

Another interesting hint of a cause for MG is that D-penicillamine treatment of some rheumatoid arthritis patients induces MG, which remits after withdrawal of the treatment (Russell and Lindstrom, 1978; Vincent and Newsom-Davis, 1978). Penicillamine has been shown to react with AChR (Bever *et al.*, 1982) and has been reported to enhance EAMG (Bever *et al.*, 1984), suggesting that one possible cause of MG is alteration of AChR by an exogenous or endogenous agent which renders it immunogenic.

Development of a specific immunosuppressive therapy for MG should be aided by several experimental advantages. First, a well-characterized antigen is available in large quantities. Second, EAMG is a well-characterized animal model of the pathological effects of anti-AChR antibody on neuromuscular transmission (Lindstrom, 1979). Third, naturally occurring canine MG closely resembles human MG, not only in its effects on neuromuscular transmission, but also, presumably, in the mechanisms which initiate and sustain the autoimmune response (Lennon *et al.*, 1978a; Garlepp *et al.*, 1979, 1984; Pflugfelder *et al.*, 1981; Dau *et al.*, 1979; Palmer and Barker, 1974). It provides a system for testing experimental therapies that appear effective on EAMG on "real" MG without risk to humans and with the possibility of invasive analysis. Finally, advances in the knowledge of cellular immunological mechanisms, immunotoxin therapeutic approaches, and molecular genetic techniques for manipulating AChRs all provide more sophisticated approaches to the development of a practical, specific immunosuppressive therapy for MG than were available when the autoimmune nature of MG was first discovered. Although current therapies have reduced mortality to less than 10%, which precludes the use of very risky therapies, the patient's quality of life can be compromised for many years by MG, and current nonspecific immunosuppressive drugs often produce severe side effects. The methods by which an effective specific immunosuppressive therapy for MG might be achieved may also prove applicable to

other antigen-specific autoimmune diseases with fewer experimental advantages.

B. OVERVIEW OF THE ACETYLCHOLINE RECEPTOR

In order to understand MG, it is necessary to understand the basic features of neuromuscular transmission, and the function, basic structure, antigenic structure, and regulation of nicotinic AChRs in skeletal muscle. These areas are briefly reviewed in the following paragraphs.

The function of neuromuscular transmission is to amplify the very small currents involved in conducting an action potential down the small motor nerve axon in order to ensure that an action potential is initiated in the much larger muscle fiber to conduct the signal to the contractile apparatus. Numerous reviews on neuromuscular electrophysiology are available (e.g., in Engel and Banker, 1986).

Depolarization of the nerve ending causes exocytosis of ACh from vesicles at active zones located adjacent to sites of AChR concentrations at the crests of folds in the postsynaptic membrane. Typically, 10–200 vesicles, each containing about 10^4 ACh molecules, might be released at a neuromuscular junction.

Two molecules of ACh must bind simultaneously to the two ACh binding sites on an AChR to activate it (this is probably a noise-suppression mechanism to prevent spontaneous activation of the AChR). In response to binding ACh, the AChR cation channel opens for a few milliseconds, permitting the passive flow of 5×10^4 cations per millisecond along their electrochemical gradient. This cation channel is an integral component of the AChR molecule. ACh is usually rapidly eliminated through diffusion and through hydrolysis by ACh esterase located in the basal lamina between the nerve and muscle. In the continued presence of ACh, AChRs in their resting state (with low affinity for ACh and a closed channel) are activated to flicker open and shut several times before assuming a desensitized conformation (with higher affinity for ACh and a closed channel). The desensitized conformation persists for many seconds before relaxing to the resting state.

In MG, insufficient AChRs are available for activation, an action potential is not triggered, and muscle contraction does not occur. Transmission may fail the first time the nerve fires, but failure becomes more likely on successive firings as the number of readily releasable ACh quanta decreases and desensitized AChRs accumulate.

Under normal conditions, a safety factor for neuromuscular transmission of about 10-fold exists in most muscles in the form of excess

ACh released and excess AChRs. Because of this safety factor, a substantial loss of AChRs can occur without obviously impairing transmission. Subclinical loss of AChRs can be detected if curare is added to further reduce the effective amount of AChRs. In the face of AChR loss, transmission is further threatened by rapid restimulation, which depletes the amount of ACh released at each stimulus. An increased probability of transmission failure on successive stimulae is probably the mechanism by which the excess fatigability characteristic of MG occurs.

Inhibitors of ACh esterase are used for symptomatic therapy of MG because slowing the removal of ACh from the synapse partially compensates for loss of AChRs. Excessive inhibition of ACh esterase, however, overexposes the AChRs to ACh, causing desensitization of AChRs and impairment of neuromuscular transmission. It has also been noted that commonly used esterase inhibitors have weak agonist properties (Pascuzzo *et al.*, 1983).

Because of their critical role in neuromuscular transmission, AChRs are the target of many naturally occurring toxins. Curare, cobra toxin, and α -bungarotoxin (α Bgt) are competitive antagonists of AChRs that have proven especially useful in studying AChRs and MG. α Bgt is an 8000 molecular weight (MW) basic polypeptide snake venom toxin which binds with great specificity and affinity to the ACh binding sites of AChRs. Treatment of animals with α Bgt produces weakness similar to MG (Satyamurti *et al.*, 1975), but does not cause the pathological effects of autoantibodies to AChRs. Unlike α Bgt, most antibodies to AChRs do not bind at the ACh binding site, and do not directly affect AChR function. Instead, antibodies to AChRs act primarily by causing loss of AChRs and disruption of the postsynaptic membrane (reviewed in Drachman, 1981; Lindstrom, 1985; Drachman *et al.*, 1987).

Numerous reviews on the structure of AChRs are available (Popot and Changeux, 1984; Stroud and Finer-Moore, 1985; Maelicke, 1987; Lindstrom *et al.*, 1988).

The AChR molecule is about 110 Å long, with 55 Å extending above the membrane in a large rigid extracellular domain and about 15 Å extending below the membrane in a smaller, looser structure (Kistler *et al.*, 1982). Viewed from above, the AChR looks like an 85-Å-diameter doughnut with a central cation channel (Kistler *et al.*, 1982). AChRs in skeletal muscles and fish electric organs are composed of four kinds of subunits in the mole ratio $\alpha_2\beta\gamma\delta$ (Reynolds and Karlin, 1978; Lindstrom *et al.*, 1979; Raftery *et al.*, 1980). The subunits are organized like barrell staves around the central cation channel in the

order $\alpha\beta\gamma\delta$ (Kubalek *et al.*, 1987). The ACh binding sites are on top of the α subunits (Kubalek *et al.*, 1987). Binding of ACh causes a subtle conformation change that permits channel opening.

All of the AChR subunits exhibit sequence homologies throughout their sequences, indicating that they evolved from a primordial subunit by gene duplication and indicating that the fundamental orientation of the polypeptide chain in each subunit is the same (Raftery *et al.*, 1980; Noda *et al.*, 1983b). Muscle AChR structure had evolved by the time of elasmobranchs and has changed little since then. There is 80% sequence homology between α subunits of AChR from *Torpedo* electric organ and human muscle (Noda *et al.*, 1983a), and about 55% homology between the other subunits (Takai *et al.*, 1984; Tanabe *et al.*, 1984; Kubo *et al.*, 1985). AChRs at mature neuromuscular junctions are thought to contain ϵ subunits, whereas extrajunctional AChRs present before innervation or after denervation have γ subunits (Mishina *et al.*, 1986). The apparent and calculated protein molecular weights of these subunits from *Torpedo* AChR are α = 38,000 and 50,116; β = 49,000 and 53,681; γ = 57,000 and 56,279; and δ = 64,000 and 57,565 (Noda *et al.*, 1983b).

Skeletal muscle AChRs are part of a superfamily of ligand-gated ion channels that includes neuronal nicotinic AChRs, neuronal α Bgt binding components that are not ACh-gated cation channels, glycine receptors that have anion channels, GABA receptors that have anion channels, and probably other ligand-gated ion channels (Grenningloh *et al.*, 1987; Schofield *et al.*, 1987; reviewed in Lindstrom *et al.*, 1987b). All the subunits of these receptors have in common four hydrophobic domains, termed M1, M2, M3, and M4, and a disulfide-linked loop between cysteines at about 128 and 142, with an N-glycosylation site included in this loop (reviewed in Lindstrom *et al.*, 1988). Several of the AChR subunits are glycosylated at more than one site (Nomoto *et al.*, 1986). Some of the conserved domains in the subunits of all receptors in the family may be involved in the specific interactions between subunits that lead to their proper association around a central ion channel, which is the characteristic feature of this family (Lindstrom *et al.*, 1988). The transmembrane orientation of the AChR subunit polypeptide chains is uncertain, but it is evident that much of the N-terminal part is extracellular (including amino acids 128–142 and 192–193) (Anderson *et al.*, 1983; Kao *et al.*, 1984; Mishina *et al.*, 1985; Criado *et al.*, 1986) and that much or all of each subunit C-terminal of M3 (about amino acid 300 and beyond) is on the cytoplasmic surface (La Rochelle *et al.*, 1985; Ratnam *et al.*, 1986b,c). The ACh binding site is on α subunits near disulfide-linked cysteines

at positions 192 and 193, which are unique to α subunits (Kao *et al.*, 1984, Kao and Karlin, 1986).

In neurons, there are nicotinic AChRs which belong to the same gene family as nicotinic AChRs from muscle (reviewed in Lindstrom *et al.*, 1988). Ganglionic nicotinic AChRs have μM affinity for nicotine, as do muscle AChRs, whereas brain nicotinic AChRs have nM affinity for nicotine. Unlike muscle AChRs, neuronal AChRs do not bind αBgt . Neuronal AChRs have only two kinds of subunits: one is structural like the β , γ , or δ subunits from muscle AChRs; the other is ACh binding like α subunits of AChRs from muscle (Whiting and Lindstrom, 1987).

In neurons there are also αBgt -binding proteins of unknown function and uncertain subunit composition, which are members of the AChR superfamily, but which are not ACh-gated cation channels (reviewed in Lindstrom *et al.*, 1987b). These proteins and AChRs may occur on the same neuron (Jacob *et al.*, 1984).

Muscarinic AChRs typical of smooth muscles and neurons are not structurally related to nicotinic AChRs from skeletal muscle or neurons. Muscarinic AChRs belong to the superfamily of receptors that interact with G proteins, which includes adrenergic receptors and rhodopsin (Kubo *et al.*, 1986), rather than to the superfamily of ligand-gated ion channels.

The human neuronal cell line TE671 produces muscle-like AChRs as shown by (1) αBgt binding (Syapin *et al.*, 1982), (2) the reaction of these AChRs with monoclonal antibodies to muscle AChRs and MG patient autoantibodies (Lindstrom *et al.*, 1987a), (3) single channel electrophysiological properties (Y. Blatt, M. Montal, and J. Lindstrom, unpublished), and (4) the sequence of their α subunits (Schoepfer *et al.*, in press). The anomalous expression of these muscle-type AChRs in a neuron-like cell is quite pragmatically useful because, for example, it provides a much larger and more uniform source of AChR than does the normal source—muscle from amputated human legs.

Muscle AChR subunits are synthesized in the endoplasmic reticulum as precursors with a signal peptide (Anderson and Blobel, 1981; Merlie *et al.*, 1983). They are cotranslationally glycosylated, but changes in glycosylation and acylation may occur later (Olson *et al.*, 1984). Association of the subunits begins some 30 minutes after synthesis. During the interval before assembly, conformational maturation occurs (Merlie and Lindstrom, 1983). In α subunits, partial conformational maturation is marked by the acquisition in nearly mature form of two important features of the extracellular surface: (1)

the ACh binding site, and (2) the main immunogenic region. AChR synthesis is largely regulated at the level of transcription (Merlie *et al.*, 1984). AChRs are phosphorylated, and this may be important in regulating their function and turnover (Huganir *et al.*, 1986; Smith *et al.*, 1987).

AChRs at mature neuromuscular junctions turn over with a half-time in excess of 5 days, whereas extrajunctional AChRs formed before innervation or after denervation turn over with a half-time less than 20 hours (Fambrough, 1979). AChR turnover occurs by a process of internalization and lysosomal destruction (Merlie *et al.*, 1979a,b). The immune assault on AChRs in MG can cause local denervation, and cross-linking of AChRs by antibody increases their internalization rate, resulting in a net loss of AChR, as will be detailed later (reviewed in Drachman, 1981; Lindstrom, 1985).

The neuromuscular junction is capable of a wide range of responses when the system is disrupted, as in MG. The all-or-none nature of neuromuscular transmission, its large safety factor, the lack of direct antagonistic effects of most antibodies on AChR function, and the wide range of regulation of AChR synthesis and destruction under the influence of many factors all contribute to the complexity of the effects of the immune attack on AChR in MG.

C. ANTIGENIC STRUCTURE OF THE AChR

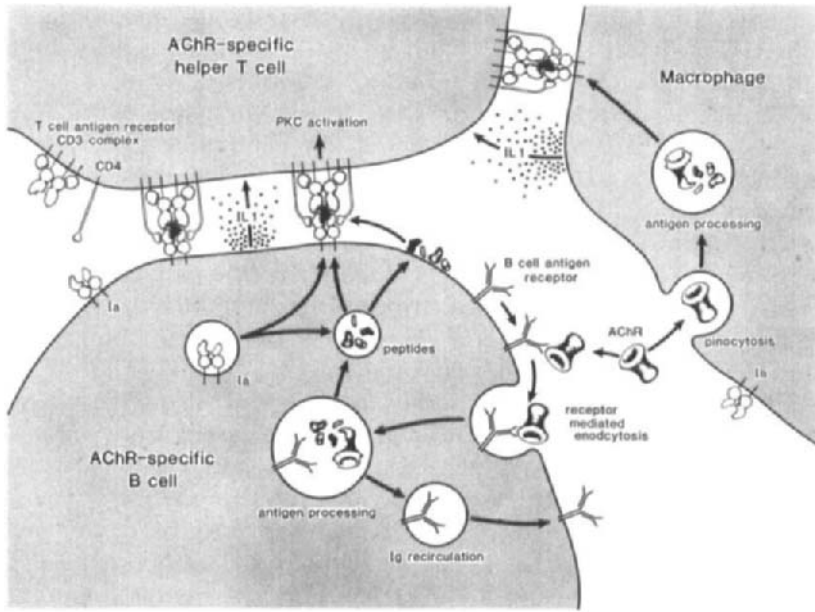
Antibodies made in animals immunized with native AChRs are directed primarily at the extracellular surface of the AChR (Froehner, 1981). Although antibodies are made to many parts of the AChR, >50% are directed at the main immunogenic region (MIR) on the extracellular surface of α subunits (Tzartos and Lindstrom, 1980; Tzartos *et al.*, 1981, 1983). The MIR is defined by competition with monoclonal antibodies (mAbs) and is a complex, conformation-dependent set of epitopes. The MIR has been mapped to the general region α 46–127 (Ratnam *et al.*, 1986c; Barkas *et al.*, 1987), but it has thus far eluded precise mapping using denatured peptides, due to its conformation dependence (Ralston *et al.*, 1987). It is a rather well-conserved antigenic feature of AChRs, but the normal functional role of this region is unknown. mAbs to the MIR have no direct effect on AChR function (Blatt *et al.*, 1986), but they can passively transfer EAMG (Tzartos *et al.*, 1987) and cause antigenic modulation of AChRs (Conti-Tronconi *et al.*, 1981b; Tzartos *et al.*, 1985). The MIR is located on the outside of the extracellular surface of α subunits, and AChRs in membranes can be arrayed with the sides of α subunits of adjacent AChRs in close proximity (Kubalek *et al.*, 1987). These observations

explain why antibodies to the MIR are very effective at cross-linking adjacent AChRs [and, thereby, inducing antigenic modulation of AChRs (Tzartos *et al.*, 1985)] but cannot cross-link the α subunits within an AChR (Conti-Tronconi *et al.*, 1981b).

When AChRs are denatured by SDS, the conformation of the highly immunogenic MIR is destroyed and less antigenic determinants remain. The remaining determinants are primarily on the cytoplasmic surface of the AChR (Froehner, 1981; Ratnam *et al.*, 1986b,c). There are also prominent immunogenic sequences on denatured subunits that provoke antibodies which do not react with the native AChR (e.g., α 46–53 and α 156–163; J. Lindstrom, unpublished). On all of the denatured subunits, there is a prominent series of epitopes in the C-terminal third of the sequence (Ratnam *et al.*, 1986b,c; Souroujon *et al.*, 1986a) which provoke antibodies that can react strongly with both denatured subunits and peptides and with the cytoplasmic surface of native AChR. This suggests that much of the cytoplasmic surface of the AChR is in a rather flexible conformation (Kordossi and Tzartos, 1987). By contrast, the extracellular surface and much of the rest of the AChR exists in conformations not recognized by antibodies to denatured peptides or inaccessible to such antibodies. Synthetic peptides from many other parts of the AChR are effective at provoking subunit-specific mAbs, but most of these do not bind to native AChR (Ralston *et al.*, 1987), although some extracellular sequences, such as α 127–143, can be weakly recognized in the native AChR by a fraction of antipeptide antibodies (Neumann *et al.*, 1985; Criado *et al.*, 1986; McCormick *et al.*, 1987). In addition to epitopes in the C-terminal third, there are several epitopes in the remainder of the subunits which are detectable by reaction with synthetic peptides, but antibodies made against these epitopes cannot bind to native AChRs (Criado *et al.*, 1985; Ralston *et al.*, 1987; J. Lindstrom, unpublished). Antibodies to a similar pattern of epitopes on denatured α subunits are detectable in antisera to native AChRs or to denatured AChRs, indicating that low titers of antibodies to these epitopes are made in antisera to native AChR as a result of traces of denatured AChR in the immunogen and/or degradation of the AChR in the animal prior to antigenic recognition (Ratnam *et al.*, 1986b; Ralston *et al.*, 1987).

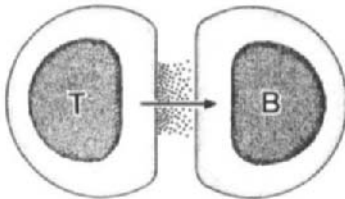
AChR epitopes recognized by T cells are just beginning to be investigated, as will be detailed in the section on the cellular immunology of antibody production, but several generalizations can be made: (1) denatured subunits are nearly as effective as T cell immunogens, as is native AChR (DeBaets *et al.*, 1982; Fujii *et al.*, 1988); (2) there are multiple T cell epitopes, but α subunits predom-

I. Antigen processing and recognition



II. Activation and growth of B cells

BCGF and BCDF secretion by T cells



III. Differentiation of B cells to plasma cells

Ig secretion

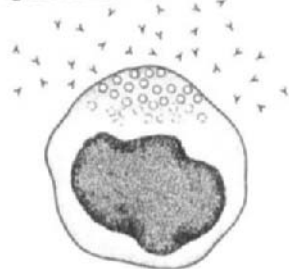


FIG. 1. AChR-specific T cell and B cell interaction in MG. I. Processing of AChR by B cells or macrophages and binding of T cell receptor (TCR) to AChR-Ia complex leading to T cell activation. AChR-specific T cells can be activated in two ways. One way is by conventional macrophages or by dendritic cells. These cells internalize, digest, and present the fragments of AChR to AChR-specific T cells in association with an Ia molecule. T cell receptors bind the AChR-Ia complex (Buus *et al.*, 1987) and this binding is stabilized by the binding of the CD4 molecule to Ia. Macrophages do not discriminate AChR from other antigens, and need a high concentration of antigen to stimulate T cells (Chesnut and Grey, 1986). A second pathway to activation of T cells involves AChR-specific B cells (Zhang *et al.*, 1987). B cells can use their surface receptors (anti-AChR immunoglobulin, Ig) to bind, internalize, and concentrate the

inate (Tami *et al.*, 1987; Hohlfeld *et al.*, 1987); (3) there is much cross-reaction between subunits (Tami *et al.*, 1987; Fujii *et al.*, 1988); (4) epitopes for T cells and B cells are different, for the most part (Ralston *et al.*, 1987; Hohlfeld *et al.*, 1987; Fujii *et al.*, 1988); and (5) T cell epitopes appear to be limited to sequences which include binding sites for class II MHC proteins on antigen-presenting cells (Fujii *et al.*, 1988; Hohlfeld *et al.*, 1988). These generalizations are consistent with the conventional wisdom concerning T cell responses in the general case (Berzofsky, 1988). T cell receptors in general see proteolytic fragments of antigens displayed on the surface of antigen-presenting cells by Ia proteins. The purpose of antigen processing may be to reveal buried sequences that can interact with presenting proteins rather than to create small fragments (Allen, 1987).

Inevitably, the requirement that T cells interact with denatured antigens means that AChR T cell epitopes will differ from the dominant B cell epitope, which is the conformation-dependent MIR (Tzartos and Lindstrom, 1980; Tzartos *et al.*, 1982). In EAMG most of those synthetic subunit peptides which are recognized by antibodies to native AChR or denatured α subunits (Ratnam *et al.*, 1986b; Ralston *et al.*, 1987) are not recognized by T cells (Fujii *et al.*, 1988). T cells, by contrast, recognize some peptides (e.g., α 101–116 in Lewis rats) which are not recognized by antibodies. This is consistent with the idea illustrated in Fig. 1, that there is no direct linkage between AChR epitopes recognized by T cells and the B cells they help, but that the linkage between these cells is simply their close proximity to the

antigen from very low environmental concentrations more effectively than the conventional macrophages (Chesnut and Grey, 1986). In the acidic environment of the endosome AChR dissociates from Ig and Ig is recirculated to the B cell surface. AChR is digested and presented to AChR-specific T cells in the same way as in the macrophages. Initial association of AChR fragments with Ia may occur within intracellular vesicles (Cresswell, 1985) or on the cell surface of macrophages or B cells (see Germain, 1986). Activation of helper T cells usually needs an additional signal (soluble factors such as IL-1 secreted by antigen-presenting cells) and occurs through activation of protein kinase C (PKC) (Droge, 1986). The AChR epitopes recognized by T cells and B cells are most often different (Yeh and Krölick, 1987; Fujii *et al.*, 1988). The specificity of their interaction depends primarily on their close topological association around the AChR and its fragments. II. Activation of B cells by lymphokines secreted by T cells. Once T cells are activated, B cell growth and differentiation factors (BCGF, BCDF) are secreted. These factors act nonspecifically, but B cells that are actually in contact with activated T cells via their receptors receive these factors more effectively (Kupfer *et al.*, 1986). III. Differentiation of B cells into Ig-secreting plasma cells. B cells differentiate into plasma cells which secrete immunoglobulins. In MG, this occurs not only in lymph nodes, bone marrow, and blood, but also in the thymus (Scadding *et al.*, 1981; Fujii *et al.*, 1986).

AChR antigen. The B cells may even process and present the antigen to T cells, using their high-affinity membrane-bound antibodies to concentrate the antigen. The close association of the two cell types around the antigen puts the B cell within range of T cell lymphokines during the time that the B cell antigen receptors are being stimulated.

The synthetic α subunit peptides recognized by T cells vary remarkably with the strain of rat (Fujii *et al.*, 1988) or the HLA type of the MG patient (Hohlfeld *et al.*, 1988), suggesting that the binding specificity of the antigen-presenting protein determines which epitopes can be detected by the T cells. This is consistent with the observation that susceptibility to EAMG maps to the I-A region in mice (Christadoss *et al.*, 1981b).

AChR-specific T cell lines frequently react with more than one subunit, but almost all react with α subunits (Tami *et al.*, 1987; Hohlfeld *et al.*, 1987; Fujii *et al.*, 1988). However, although there is sequence homology between the AChR subunits (Noda *et al.*, 1983b), cross-reactions are difficult to predict; for example, AChR-specific T cell lines from Lewis rats which recognize the synthetic peptide [Tyr 100] α 100–116 cross-react with other subunits, even though there is quite limited sequence homology between subunits at this sequence (Fujii *et al.*, 1988).

Several groups seeking to identify AChR T cell epitopes in MG patients by stimulating lymphocyte cultures with synthetic α subunit peptides have obtained varying results (Harcourt *et al.*, 1988; Hohlfeld *et al.*, 1987; Berrik-Aknin *et al.*, 1988). In one study, two peptides (α 169–181, α 351–368) of the eight tested provoked significant proliferation in less than 25% of the 34 patients tested (Berrik-Aknin *et al.*, 1988). Thus, no one of these peptides encodes an immunodominant sequence. α 351–368 is interesting because this immunogenic sequence (Souroujon *et al.*, 1986) is on the cytoplasmic surface (Ratnam *et al.*, 1986b). In MG, pathologically significant T cell epitopes, unlike B cell epitopes, need not be on the extracellular surface of the AChR, because antibodies produced by B cells are the effector of the autoimmune response and T cells need recognize only processed antigen. Another study with 12 peptides on 27 patients found two sequences that provoked proliferation (Harcourt *et al.*, 1988). One of these, α 251–271, was stimulatory in 5 of 27 patients. This is interesting because this sequence is the same in human and *Torpedo* AChRs. Given the much lower specificity of T cell reaction than B cell reaction [illustrated for example by the high cross-reactivity of T cells among AChR subunits (Tami *et al.*, 1987) and the low cross-reactivity of antibodies among subunits (Gullick and Lindstrom,

1983; Tzartos *et al.*, 1986a)] it would not be surprising if future experiments revealed that AChRs are also much more cross-reactive among species at the T cell level than at the B cell level. Harcourt *et al.*, (1988) also reported that peptides including the sequence α 125–143 were stimulatory in 8 of 27 MG patients, but were also stimulatory in controls. This is interesting because others have reported that large doses of this peptide can provoke mild EAMG (McCormick *et al.*, 1988). The results of Harcourt *et al.*, (1988), showing that α 125–143 can be nonspecifically mitogenic, and Criado *et al.*, (1986), showing that most mAbs to this peptide do not react well with native AChR, suggest caution in estimating the pathological significance of α 125–143, or any other sequence.

In summary, the results of studies of AChR T cell epitopes suggest that there are many and that there is probably no single dominant epitope. A large fraction of AChR T cell epitopes may be found on α subunits. There is much cross-reaction between subunits. The results suggest that no single sequence should be expected to be especially potent at stimulating both T cells and B cells. Pathologically significant T cell epitopes could correspond to sequences revealed only on denaturation of the AChR, whereas pathologically significant B cell epitopes must be on the extracellular surface of the AChR. The most frequent pathologically significant B cell epitopes, the MIR, has been found to be conformationally dependent.

Most serum antibodies and mAbs to AChR do not block the function of AChRs (Wan and Lindstrom, 1985). mAbs to the MIR alter neither the conductance nor duration of AChR channel opening (Blatt *et al.*, 1986).

Some mAbs have been produced which do block function, either by competing for the ACh binding site or by allosteric mechanisms (Mochly-Rosen and Fuchs, 1981; Mihovilovic and Richman, 1984; Fels *et al.*, 1986; Souroujon *et al.*, 1986b; Whiting *et al.*, 1985; Donnelly *et al.*, 1984).

Most mAbs that bind at or near the ACh binding site bind to only one of the two sites (Mihovilovic and Richman, 1984; Fels *et al.*, 1986; Whiting *et al.*, 1985; Claudio and Raftery, 1980). This is also true of some MG patient autoantibodies (Gu *et al.*, 1985). Similarly, curare has much greater affinity for one of the two sites (Neubig and Cohen, 1979; Sine and Taylor, 1980), and affinity labeling reagents are much more effective on one of the two sites (Damle and Karlin, 1978; Wolosin *et al.*, 1980). These results indicate that, although the sequence of the two α subunits is the same, the two α subunits must have a prominent conformation difference as a consequence of

the different subunits which surround each α in the native AChR (Kubalek *et al.*, 1987) and/or as a result of different posttranslational modifications (Ratnam *et al.*, 1986a.).

Most antibodies to AChR are quite species specific. Thus, although there is 80% sequence homology between α subunits of AChRs from *Torpedo* electric organ and human muscle (Noda *et al.*, 1983a), and although some mAbs to the MIR made against AChRs from either species cross-react well between species (Tzartos *et al.*, 1981, 1983), nonetheless there is only about 2% cross-reaction of serum antibodies between these species (Lindstrom *et al.*, 1978a). However, because the native AChR is highly immunogenic, it is easy to achieve serum antibody titers of many μM , and even though there is <5% cross-reaction between AChRs from *Torpedo* and rats, it is easy to achieve autoantibody concentrations in rats with EAMG of 10–100 nM, which are typical of patients with MG and in large excess over the amount of AChRs in rat muscles (Lindstrom *et al.*, 1976b).

Autoantibodies to AChR in MG patients closely resemble those in rats immunized with native AChR (Tzartos *et al.*, 1982). MG patient autoantibodies to AChR are primarily IgG, polyclonal, heterogeneous, and of high affinity (Lindstrom *et al.*, 1976d; Tzartos *et al.*, 1982; Bray and Drachman, 1982; Compston *et al.*, 1980). Using rat mAbs to various epitopes as competitive inhibitors of patient antibody binding to human AChR, it was found that the average distribution of antibody specificities in all clinical groups was similar, and an average of >50% was directed at the MIR (Tzartos *et al.*, 1982). In a smaller, similar study using mouse mAbs, some variation in autoantibody specificity was found among clinical groups (Whiting *et al.*, 1986a). In a study of 35 cases of naturally occurring canine MG, rat mAbs to the MIR inhibited an average of 68% of serum antibodies from binding to canine AChR (Shelton *et al.*, 1988a). A monovalent Fab fragment of an mAb to the MIR could inhibit an average of 68% of the antigenic modulation of AChRs on human muscle cells caused by MG patient sera (Tzartos *et al.*, 1985). Thus, antibodies to the MIR are the most frequent specificity of autoantibody to AChR (Tzartos *et al.*, 1982) and, although they do not directly inhibit AChR function (Blatt *et al.*, 1986), they can passively transfer EAMG (Tzartos *et al.*, 1987) and cause antigenic modulation (Conti-Tronconi *et al.*, 1981b; Tzartos *et al.*, 1985, 1986b), thereby accounting for the prominent effects of serum antibodies to AChR.

Some MG patient's sera react better with AChRs from denervated muscle (Weinberg and Hall, 1979; Gu *et al.*, 1985). mAbs have also been reported that can distinguish between normal and denervated

AChRs (Whiting *et al.*, 1986b). Patients with symptoms of MG confined to ocular muscles have been suggested to react somewhat better with AChRs from ocular muscle (Vincent and Newsom-Davis, 1982a). This experiment must be interpreted cautiously because it is based on comparison of AChR preparations from only a few individuals and small differences in autolysis between various samples could easily confuse the results.

In MG patients, it is possible to detect antibodies to AChR that bind at or near the ACh binding site and directly impair AChR function (Whiting *et al.*, 1983; Gu *et al.*, 1985). In most patients, these are a small fraction of the total, and for this reason it is difficult to quantitate these accurately (Whiting *et al.*, 1983). For example, in immune precipitation assays, large excesses of antibodies to other sites can form microprecipitates of AChRs that may not bind α Bgt well, even though the antibodies do not bind at the ACh binding site.

The observation that the specificities of the autoantibodies to AChR in human and canine MG patients closely resemble those in animals immunized with native AChR (rather than sera from animals immunized with denatured AChR subunits or with another protein that has antigenic similarity at only a single unusual epitope) suggests that MG patients are responding to some endogenous source of native AChR (Tzartos *et al.*, 1982). In some cases, the endogenous source of AChR may be extrajunctional AChR (Weinberg and Hall, 1979), as might be found on denervated muscle, thymic myoid cells (Kao and Drachman, 1977; Schlupe *et al.*, 1987), or a transformed cell that expressed AChR. Autoantibodies to AChR are clearly not the result of a monoclonal gammopathy from a transformed B cell clone. Molecular mimicry is an interesting hypothesis for explaining autoimmune disease (Oldstone, 1987), and it may well be that in some diseases an immune response directed at a bacterial or viral epitope crossreacts with a similar or identical sequence in a host protein. However, in MG it is highly unlikely that the immunogen is a single, similar epitope on a bacterial or viral coat protein, because the autoantibody specificities are identical to those obtained by immunization with intact AChR. Rats with active or passive EAMG are not triggered to a sustained autoimmune response to AChR of the type seen in MG patients, even though endplate AChRs may be exposed to macrophages during acute or passive EAMG (Lindstrom *et al.*, 1976a,b). Thus, it is difficult to argue that limited cross-reaction of antibacterial or anti-viral antibodies to a single epitope on AChR could ignite a full-blown autoimmune response involving many specificities of antibodies to AChR, or ignite an autoimmune response that persisted long after the bacterial or viral

infection. Antibodies to contractile components of myoid cells (Peers *et al.*, 1977) and other components (Fischbach *et al.*, 1981) are also frequently found in MG patients, which probably not only suggests the patient's genetic proclivity to an autoimmune response (Compston *et al.*, 1980), but also suggests that AChRs are just the critical immunogen on a muscle-like cell that is the target of the initiating immune response in MG. Although the specificities of the autoantibodies in MG suggest that the immunogen is native AChR, often extrajunctional AChR, the source of the AChR that initiates and sustains the immune response is unknown. Thymic myoid cells in MG patients have been shown to produce extrajunctional AChRs, but a detailed histological study concluded that the myoid cells do not appear to be the focus of immunological stimulation (Schluep *et al.*, 1987).

There is very limited cross-reaction between nicotinic AChRs from muscle and neurons (Lindstrom *et al.*, 1987b). mAbs to human neuronal nicotinic AChRs did not react with human muscle AChRs (Whiting *et al.*, 1987). MG patient serum antibodies did not react with either nicotinic neuronal AChRs or α Bgt-binding proteins from human brain (Whiting *et al.*, 1987). Thus, although antibodies to AChR are found in the CSF of MG patients at about 1% of the serum titer (Keeseey *et al.*, 1978), these are unlikely to have effects in the central nervous system.

II. Etiology

A. EAMG

All species of animals tested have been found to be susceptible to EAMG, including rabbits (Patrick and Lindstrom, 1973), rats (Lindstrom *et al.*, 1976a,b,c), mice (Christadoss *et al.*, 1979a,b, 1981a, 1983a,b, 1984; Berman and Patrick, 1980; Berman *et al.*, 1981; Berman and Heinemann, 1984), and monkeys (Tarrab-Hazdai *et al.*, 1975). However, some strains of mice and rats are relatively resistant to clinically apparent EAMG, although they may have significant titers to electric organ AChRs (Christadoss *et al.*, 1979a; Berman *et al.*, 1981; Berman and Heinemann, 1984).

EAMG can be induced by immunization with either purified syngeneic AChR or with AChR purified from the electric organs of *Electrophorus electricus* or *Torpedo californica* (Patrick and Lindstrom, 1973; Lindstrom *et al.*, 1976a,b). Purified AChRs in complete Freund's adjuvant are usually used for immunization, but it

is possible, although less effective, to immunize with syngeneic AChR in membranes without adjuvant (Scadding *et al.*, 1986). If the animal survives EAMG, the autoimmune response to AChR diminishes in parallel with the response to the immunogen.

EAMG can also be passively transferred with AChR antibody into animals (Lindstrom *et al.*, 1976a; Engel *et al.*, 1978). If the animal survives, the weakness remits and no active immune response to AChR persists.

Mild EAMG has been reported after immunization of rats with synthetic peptides corresponding to the sequence 125–147 of α subunits of AChRs from *Torpedo* or humans (Lennon *et al.*, 1985; McCormick *et al.*, 1987). Only nM titers of antibody to native AChR were detected after immunization with more than 400 times as many moles of peptide as are required of native AChR to produce fatal EAMG. Two other groups did not notice EAMG in rats or rabbits immunized with overlapping sequences (Neumann *et al.*, 1985; Criado *et al.*, 1986). Most of the mAbs to these peptides did not react well with native AChR. Antibodies capable of binding to most parts of the extracellular surface of AChRs in muscle membranes should be able to cause EAMG. However, it is difficult to find peptides which induce antibodies that react well with the extracellular surface (Ralston *et al.*, 1987). Also, in EAMG induced with native AChR (and probably MG) B cell and T cell epitopes are not on the same small peptides (Ralston *et al.*, 1987; Fujii *et al.*, 1988).

The T cell response to *Torpedo* AChR is controlled by an H-2-linked *Ir* gene that maps to the I-A subregion of the mouse MHC (Christadoss *et al.*, 1979b). Ia molecules are essential for accessory cells to present polypeptide antigens to helper T cells. A mouse strain with a I-A β chain mutation was found to be less responsive to immunization with *Torpedo* AChR than the original strain (Christadoss *et al.*, 1985a). Monoclonal antibodies to murine I-A have been shown to block the antibody responses to AChR *in vivo* and prevent the onset of clinical EAMG (Waldor *et al.*, 1983). The human analog of I-A is DQ, and HLA-DQ polymorphism has been linked to human MG (Bell *et al.*, 1986).

Induction of EAMG by means of anti-idiotypic antibodies has been reported. Antibodies were prepared in rabbits against trans-3,3'-bis[α -(trimethylammonio)methyl] axobenzene bromide (Bis Q), a structurally constrained AChR agonist (Wassermann *et al.*, 1982; Cleveland *et al.*, 1983). Another group of rabbits was then immunized with the anti-Bis Q, generating a population of anti-idiotypes specific for the binding sites of anti-Bis Q. Several of these rabbits were reported to

exhibit transient muscular weakness. Mice were immunized with Bis Q, hybridomas made, and wells scored for those producing antibodies to Bis Q (14%), to rabbit anti-Bis Q (7.4%), and to AChR (2.4%) (Cleveland *et al.*, 1983). An IgM mAb anti-id was produced which exhibited low affinity for the ACh-binding site of the AChR. Transient mild EAMG was also reported after immunization with conjugates of choline, and both anti-ACh and antiidiotype antibodies were detected (Souan and Geffard, 1985). In MG patients who have been treated with high concentrations of esterase inhibitors for prolonged periods, it would not be surprising to detect low concentrations of antibodies to these cholinergic molecules, and such antibodies might be mistaken for part of an antiidiotype network involving antibodies to ACh.

While antiidiotype antibodies might be a mechanism for initiating some disorders, such as insulin-resistant diabetes mellitus (Schechter *et al.*, 1982), the mechanism is not likely in MG because the antibodies are directed primarily to sites other than the ACh binding site.

An early report (Goldstein and Whittingham, 1966) described features of MG in rodents following immunization with extracts of thymus glands. The results could not be duplicated in a critical study using currently available methods for evaluating physiological, biochemical, and immunological changes of EAMG (Murphy *et al.*, 1980). Now there is some evidence that the impairment of transmission caused by thymopoietin resulted from some affinity of this peptide for the ACh binding site of the AChR (Morel *et al.*, 1987). The ACh-binding site of the AChR is rather promiscuous, and interacts significantly with cations as simple as tetramethylamine.

In summary, EAMG provides little information about what initiates the immune response in MG, although it provides a very good model of the effects of the autoimmune response. EAMG does show that the specificities of the immune response in MG are those that would be obtained by immunization with native AChR, and EAMG shows that an autoimmune response to AChR is not self-sustaining once initiated, implying that MG must have both an initiating and a sustaining immune response to persist for years.

B. MG

Several clinical groups of MG patients have been identified, suggesting that multiple factors are involved in the pathogenesis of the various forms of MG (Compston *et al.*, 1980).

Thymic abnormalities, including thymic hyperplasia and thymoma, are present in a large fraction of MG patients (Castleman and Norris,

1966; Bofill *et al.*, 1985). Although the precise role of the thymus in the autoimmune process is not yet defined, it is well documented that the thymus contains small amounts of AChRs (Engle *et al.*, 1977a; Kao and Drachman, 1977; Lindstrom *et al.*, 1976c) and is one of the sites of AChR antibody production (Scadding *et al.*, 1981; Fujii *et al.*, 1986), and that some patients improve clinically following thymectomy (Buckingham *et al.*, 1976; Vincent *et al.*, 1983; Rodriguez *et al.*, 1983; Kagotani *et al.*, 1985). The thymus is also believed to be the site of tolerance induction to self-antigens (Kappler *et al.*, 1987). AChRs on thymic myoid cells could be the initiating or sustaining immunogen, but there is no evidence for this (Schluep *et al.*, 1987). Viral infections have been postulated to trigger loss of tolerance to AChR (Aoki *et al.*, 1985; Bottazzo *et al.*, 1983), but direct evidence is lacking. In one study, attempts at virus isolation from myasthenic thymuses by various techniques failed to detect or isolate virus (Klavinskis *et al.*, 1986).

The increased incidence of some HLA markers such as B8 and DR3 in some patient groups suggests that patients with certain immune response genes are more susceptible to MG, however the relative risks associated with these markers (~ 4) are not large, and different HLA markers are associated with patient groups varying in age, sex, race, and the presence of thymoma (Compston *et al.*, 1980). Thus, patients are not genetically doomed to MG, and an unknown environmental factor or factors must be important in triggering the autoimmune response to AChR. Allotypes of Gm, a polymorphic serologic marker on the Fc portion of immunoglobulins, have been associated with MG and LEMS (Willcox *et al.*, 1984; Nakao *et al.*, 1980). Restriction fragment length polymorphisms detected no particular association of a heavy chain marker with MG, although some effect was seen with LEMS (Demaine *et al.*, 1988). Idiotypes also provide serologic markers of antibodies and, in some autoantibody systems, recurrent idiotypes have been found (Shoenfeld *et al.*, 1983). In EAMG and MG, however, there is no compelling evidence for dominant idiotypes or of sharing of anti-AChR idiotypes between different MG patients (Killen *et al.*, 1985; Lang *et al.*, 1985).

Penicillamine, a drug used in the treatment of rheumatoid arthritis and Wilson's disease, induces MG in some patients (Bucknall *et al.*, 1979). The disorder is reversible and indistinguishable from naturally occurring MG in its clinical features and electrophysiology (Buchnall *et al.*, 1975). Autoantibodies to AChR can be demonstrated (Russell and Lindstrom, 1978; Vincent and Newsom-Davis, 1978). Serum autoantibodies to AChR and AChR loss from neuromuscular junctions

were reversed within 8 months of stopping penicillamine (Kuncl *et al.*, 1986). It has been postulated that D-penicillamine chemically alters host proteins, and that loss of tolerance is triggered by the presence of the neoantigens (Noor and Galili, 1972; Dawkins *et al.*, 1981; Bever *et al.*, 1982). In one study, D-penicillamine was shown to bind to *Torpedo* AChR and alter agonist binding, providing biochemical evidence for antigenic alteration (Bever *et al.*, 1982), and it was reported that penicillamine treatment facilitated induction of EAMG (Bever *et al.*, 1984). If it could be established that the mechanism by which penicillamine induces MG was alteration of the AChR molecule, it would be important not only for explaining this rare form of MG, but also to suggest that other forms of MG might be initiated by a response to modified AChRs.

Several cases of MG have been reported after bone marrow grafting in humans (Smith *et al.*, 1983; Bolger *et al.*, 1986), and one canine case has been reported (Cain *et al.*, 1986). The incidence of AChR antibodies has also been reported to be high following bone marrow transplantation (Lefert *et al.*, 1988); the majority of patients with AChR antibodies, however, had undergone transplantation because of aplastic anemia or acute nonlymphocytic leukemia. What activates the AChR-specific cell clones is not known.

Transient neonatal MG develops in about 12% of infants born to myasthenic mothers (Namba *et al.*, 1970). The disorder is a result of the passive transfer of antibodies from the mother to the child, and the disease usually remits as the maternal antibodies are spontaneously removed by the child (Keeseey *et al.*, 1977).

Some patients with clinical signs of MG lack detectable antibodies to AChR, but respond to immunosuppressive therapy and plasmapheresis, and their antibodies passively transfer weakness to mice (Mossman *et al.*, 1986; Newsom-Davis, 1988; Drachman, 1988). These patients clearly have antibodies to a component of the neuromuscular junction, but have neither anti-AChR MG nor LEMS.

One recent finding has shed some light on the origin of autoantibodies—the description of autoantibodies to tumor-associated calcium channel determinants that cross-react with similar determinants at the motor nerve terminal in LEMS (Roberts *et al.*, 1985; Fukuoka *et al.*, 1987a,b). Studies such as this are a beginning in the understanding of mechanisms by which autoantibodies are initiated, and of the etiology of “remote” neurological paraneoplastic syndromes.

Several congenital myasthenic syndromes of nonimmune etiology

have been described in humans (Vincent *et al.*, 1981; Engel *et al.*, 1981). Human congenital myasthenic syndromes have been described with defects in ACh resynthesis or mobilization, ACh esterase deficiency, AChR deficiency, AChR ligand binding affinity, and AChR channel function. In one canine study of a congenital myasthenic syndrome (Oda *et al.*, 1984), the low junctional membrane density of AChR observed was attributed to a low insertion rate of AChR in the postsynaptic membrane.

Studies of naturally occurring canine autoimmune MG may be valuable. Factors that initiate and sustain the autoimmune response to AChRs are probably similar in humans and dogs. The clinical signs of MG, methods of diagnosis, and forms of therapy are also similar. Dogs would also provide an excellent model for testing new forms of therapy.

In summary, what initiates and sustains the immune response to AChR in MG is unknown. There may be multiple mechanisms in various patient groups with a final common pathway of an antibody-mediated autoimmune response. There are multiple genetic factors, probably involving both the immune and neuromuscular systems, which effect both susceptibility and the course of the disease. In the case of penicillamine-induced MG, the triggering environmental insult may be the covalent reaction of penicillamine with the AChR which renders it immunogenic. In other cases, it could be that virally induced modification of the AChR renders it immunogenic, but there has been no direct evidence for viral involvement. What induces the immune response to AChR in MG must continue to sustain the response for years. Penicillamine-induced MG remits when penicillamine is withdrawn. Myoid cells in the thymus provide a potential source of immunogen, and thymic hyperplasia, thymoma, and a beneficial therapeutic response to thymectomy all suggest an important role for the thymus in MG, yet not all patients respond to thymectomy and the immune response to AChR can persist after thymectomy, so it is neither the sole site sustaining the immune response nor the sole site of synthesis of antibody to AChR. Autoantibodies to muscle contractile proteins are found in some MG patients, especially those with thymoma, which suggests that in at least some cases, multiple antigens on a muscle cell are involved in the immune response. This could be the case if there were chronic infection of a muscle cell, but there is no direct evidence for this. Thus, the cause of MG remains a mystery, but there are many tantalizing clues scattered about.

III. Diagnosis

A. EAMG

Diagnosis of EAMG is based on evaluation of clinical findings, measurement of the antibody response to AChR, electrophysiological testing, and demonstration of characteristic pathologic changes at the neuromuscular junction. Muscular weakness can be evaluated subjectively (Lennon *et al.*, 1975) or by pragmatic tests of endurance such as how long the animals can swim (Berman and Patrick, 1980). Since weakness inhibits feeding and drinking, weight loss is a convenient quantitative measure (Lindstrom *et al.*, 1976b). Electromyography can be used, often with curare challenge to make the decrement more obvious (Seybold *et al.*, 1976). Animals can be sacrificed for electrophysiological studies (Lambert *et al.*, 1976; Olsberg *et al.*, 1987) or electron microscopy (Engel *et al.*, 1976a–c). Measurement of AChR content in total muscle and the fraction of AChRs bound by antibodies is an especially good and convenient quantitative measure (Lindstrom *et al.*, 1976a,b). Clearly, diagnostic approaches in animals can be a good deal less subtle, more intrusive, and more informative than is possible with MG patients.

B. MG

MG has frequently been diagnosed by subjective evaluation of muscle weakness and fatigability, by subjective evaluation of relief of muscle weakness by a short-acting inhibitor of ACh esterase, or by a decrementing electromyogram response that can be repaired by an ACh esterase inhibitor.

The best diagnostic technique for MG is detection of serum antibodies to human muscle AChRs (Lindstrom *et al.*, 1976c; Vincent and Newsom-Davis, 1985b). This is objective and quantitative, and proves an autoimmune response to AChR, as opposed to other causes of muscle weakness such as an autoimmune response to another component of the neuromuscular junction (Mossman *et al.*, 1986; Drachman *et al.*, 1987) or congenital myasthenia (Vincent and Newsom-Davis, 1979; Engel *et al.*, 1981; Engel, 1984; Mora *et al.*, 1987).

Using ^{125}I -labeled αBgt human AChR in indirect immune precipitation assays, serum autoantibodies can be detected in about 90% of patients thought to have generalized MG (Lindstrom, 1977; Vincent and Newsom-Davis, 1985b; Limburg *et al.*, 1983; Oosterhuis *et al.*, 1983). Background is about 0.6 nmol of αBgt binding sites precipitated

per liter of serum. The average titer is about 50 nM and titers in excess of 1000 nM have been observed. Solid phase assays for MG patient autoantibodies have also been described (Dwyer *et al.*, 1983b; Kobayashi *et al.*, 1984; Smith *et al.*, 1984; Hinman *et al.*, 1986; Jaikhani *et al.*, 1986). Most of these do not offer the direct quantitation and specificity of immune precipitation, but offer convenience, and may be better for measuring antibodies to the ACh binding site.

Using human AChR as antigen in diagnostic assays is important because cross-reaction of MG patient antibodies with AChRs from other species is limited and variable among patients (Lindstrom *et al.*, 1978a; Oda *et al.*, 1980; Savage-Marengo *et al.*, 1979). The usual source of human AChRs is amputated limb muscle. This is inconvenient, limited in quantity, and subject to the vagaries of autolysis. Muscle-type AChRs from TE671 cells will offer a much larger and more uniform source of AChR for diagnostic assays (Lindstrom *et al.*, 1987a).

Sensitive detection of autoantibodies to AChR is important for diagnosis, but precise quantitation is of limited value. Antibody titers in MG patients vary widely, though patients with only ocular symptoms have lower average titers (Lindstrom *et al.*, 1976c; Limburg *et al.*, 1983; Vincent and Newsom-Davis, 1985b), and changes in an individual's titer correlate with changes in clinical state (Newsom-Davis *et al.*, 1978; Dau *et al.*, 1977; Seybold and Lindstrom, 1981; Oosterhuis *et al.*, 1983; Hohlfeld *et al.*, 1985b). In the future, routine quantitation of both the total anti-AChR antibody concentration and the fraction of the antibody that is directed at the MIR, that is directed at the ACh binding site, or that is capable of causing antigenic modulation may somewhat increase the value of quantitation; but these additional approaches have not yet proven remarkably informative or practical for routine assays.

Attempts have been made to measure functional properties of subfractions of MG patient serum autoantibodies to AChR in hopes of obtaining better correlation between titer and severity of muscle weakness. For example, the ability of MG patient sera to cause antigenic modulation or inhibit binding of α bungarotoxin to AChRs of rat muscle cells was measured (Conti-Tronconi *et al.*, 1981a; Drachman *et al.*, 1982). These studies were compromised by the use of rat AChR as antigen. Using AChRs of human myotubes, the rate of antigenic modulation was found to be simply proportional to the titer of the sera against AChR from human muscle (Tzartos *et al.*, 1986b).

MG occurs at a frequency of about 1 in 20,000 in the population, and is more frequent in younger females and older males (Compston *et al.*,

1980). About 10–15% of patients have thymoma, and these patients as a group have higher titers of antibody to AChR (Lindstrom *et al.*, 1976c; Compston *et al.*, 1980; Limberg *et al.*, 1983). The different clinical groups of patients, distinguished by sex, age, HLA types, presence of thymoma, and antibody titers, may reflect different mechanisms involved in triggering the immune response to AChR (Compston *et al.*, 1980).

IV. Pathology

A. CELLULAR IMMUNOLOGY OF AUTOANTIBODY PRODUCTION

Several lines of evidence suggest that antibody production against AChR is T cell dependent. Neonatally thymectomized rats or lethally irradiated rats that are reconstituted with B cells do not respond to AChR (Lennon *et al.*, 1975; Fuchs *et al.*, 1976). Lymph node cells from AChR-immunized rats do not produce antibodies against AChR *in vitro* after treatment with anti-rat brain serum plus complement (DeBaets *et al.*, 1982), but antibody production could be reconstituted by adding back T cells (Krolick and Urso, 1987).

T cells are activated only when their surface receptors bind the antigen in association with an MHC encoded molecule of their own type on the surface of an antigen-presenting cell (APC)—this is termed MHC-restricted antigen recognition (Schwartz, 1985) (see Fig. 1). This complex phenomenon is partially explained by recent biochemical studies showing that a T cell antigen (e.g., hen egg lysozyme peptide) can bind to detergent solubilized Ia from a responder strain of mice, but cannot bind to Ia from a nonresponder strain of mice (Babbitt *et al.*, 1985). In EAMG, T cells from AChR-immunized rats proliferate only in the presence of syngeneic antigen-presenting cells (Hohlfeld *et al.*, 1981).

Using mice congenic in their MHC region, susceptibility to EAMG has been mapped to the I-A subregion (Christadoss *et al.*, 1979a), and a monoclonal antibody to an allele of I-A was reported to suppress anti-AChR antibody production (Waldor *et al.*, 1983). However, in human MG, DR3-, 5-, 7-positive cells are all capable of stimulating the AChR-reactive T cell lines (Hohlfeld *et al.*, 1985a), and it is surprising that in some mice, the other Ia molecule, I-E (which is homologous to human DR), does not seem to play any role in the immune response to AChR. In another study, all 14 strains of mice studied (including the low responder in Christadoss *et al.*, 1979a) showed comparable antibody response to AChR (Fuchs *et al.*, 1976). Recently, the

bm12 mouse strain bearing a mutant I-A β chain was shown to respond to AChR to a somewhat lesser degree than the original strain (Christadoss *et al.*, 1985b), although the T cells did respond well to AChR (stimulation index = >10). Four rat strains showed equally high responses to AChR by measures of both T cell proliferation and antibody production, although the T cell epitopes recognized by the various strains differed (Fujii *et al.*, 1988). Complete lack of response to a protein as large as the AChR seems unlikely because of the variety of peptide sequences available for interaction with various Ia types and T cell receptors and the variety of sequential and conformation-dependent epitopes available for interaction with B cells.

AChR-immune T cells respond well to SDS-denatured subunits (DeBaets *et al.*, 1982; Krolick and Urso, 1986). Despite their ability to stimulate T cell proliferation, SDS-denatured subunits do not effectively induce AChR antibody production *in vitro* (Fujii *et al.*, 1988). Studies of cloned T cell lines from rats immunized with AChR indicate that there is a significant degree of crossreaction between subunits at the T cell level, and that more cloned lines react with α than any other subunit (Tami *et al.*, 1987; Fujii *et al.*, 1988). In *Torpedo* AChR-specific human T cell lines, the α subunit also seems to be preferred (Hohlfeld *et al.*, 1987).

T cell antigens can usually be substituted for by proteolytic fragments of the protein or small peptides (Shimonkevitz *et al.*, 1984; Allen and Unanue, 1984), and synthetic peptides have been used to identify the epitopes in AChR that can stimulate T cells, as was previously discussed in Section I,C (Lennon *et al.*, 1985; Atassi *et al.*, 1987; Fujii *et al.*, 1988; Harcourt *et al.*, 1988; Berrik-Aknin *et al.*, 1988; Zhang *et al.*, 1988). It is evident that there are many T cell epitopes on the AChR which differ from the B cell epitopes, and it seems likely that T cell recognition is restricted by Ia proteins.

If the antigen must interact with Ia to stimulate T cells (Babbitt *et al.*, 1985; Buus *et al.*, 1987), then different peptides may be recognized in different species or strains where they interact with different Ia molecules. This seems to be true in mice (Atassi *et al.*, 1987) and rats (Fujii *et al.*, 1988), where different sets of peptides were recognized by AChR-immune T cells in different strains. The situation is more complex in humans because each individual has several Ia molecules, and finding one or two immunodominant T cell epitopes common to many patients is unlikely.

Activated T cells help B cells to produce antibodies by secreting lymphokines such as B cell growth and differentiation factors (see Fig. 1). B cells also seem to need to bind the antigen to be efficiently

stimulated by T cells (linked recognition). This is suggested by the observation that SDS-denatured subunits of AChR or the synthetic peptide [Tyr 100] α 100–116 that stimulate T cells of Lewis rats in culture fail to induce antibody production by AChR-immune lymph node cells (Fujii *et al.*, 1988). This can be explained by the fact that most antibodies against native AChR, hence the receptors on B cells from AChR-immune rats, have been shown to bind poorly to denatured subunits (Tzartos *et al.*, 1982; Tzartos and Lindstrom, 1980) or the synthetic peptide [Tyr 100] α 100–116 (Ralston *et al.*, 1987).

AChR-specific T cell clones have been demonstrated to help antibody production when added to T cell-depleted lymph node cells with antigen (Krolick and Urso, 1987). In passive transfer experiments using AChR-specific T cell clones plus unfractionated B cells from AChR-immunized animals, no T cell clone specificities were found to preferentially cause muscle weakness (Olsberg and Krolick, 1988). This could be interpreted to mean that B cell AChR epitope specificities determine the pathological significance of the effector autoantibodies, while T cells with any AChR epitope specificity could provide the necessary T cell help required for antibody production.

Suppressor T cells are believed to down-regulate the immune response by a complex mechanism. Several reports have appeared of AChR-specific suppressor cells and suppressor factors in mice and rats (Bogen *et al.*, 1984; Pachner and Kantor, 1984; Sinigaglia *et al.*, 1984; McIntosh and Drachman, 1986). However, in some studies, nonspecific suppression is apparent (Bogen *et al.*, 1984; Pachner and Kantor, 1984). It is possible that once AChR-specific suppressor cells are activated specifically, they secrete factors that nonspecifically suppress the immune response (Asherson *et al.*, 1986). Cyclosporin A is thought to inhibit immune responses while permitting antigen-induced activation of suppressor T cells. It has been used to treat EAMG (Drachman *et al.*, 1985). Treatment of lymphocytes from AChR-immunized rats with cyclosporin produced large macrophage-like cells, not T cells, which strongly suppressed the response to AChR, but not KLH, of other cultures (McIntosh and Drachman, 1986). These are interesting results which might conceivably offer the distant possibility of treating MG with a patient's own cyclosporin A-stimulated suppressor cells. In one report, which has not been followed up, a *Torpedo* AChR-specific suppressor factor which did not suppress the reaction to fetal calf AChR was secreted by a retrovirus-transformed murine T cell line (Sinigaglia *et al.*, 1984). To react to millions of naturally occurring antigens with this fine specificity, such suppressor factors would have to be encoded by a set of

genes like T cell receptors. In contrast to the search for T cell receptor genes, the search for suppressor factor genes has not been successful (DeSantis *et al.*, 1987; Mengle-Gaw and McDevitt, 1985).

In MG, genetic restriction of the response to AChR has been suggested by the association of HLA B8 and DR3 with young female patients (Naeim *et al.*, 1978; Compston *et al.*, 1980). Recently, T cell lines were generated from MG patients by *in vitro* stimulation of peripheral blood cells using *Torpedo* AChR and autologous blood cells as antigen-presenting cells (Hohlfeld *et al.*, 1984, 1985a, 1987). These T cells have been shown to be stimulated by *Torpedo* AChR and to a lesser extent by human AChR. This activation was observed only when T cells were cultured with blood cells from individuals who share one of the DR phenotypes with the patient from whom the T cells were derived, demonstrating that the T cell response to AChR is also MHC restricted in humans. It must be pointed out that DR5- and DR7-bearing antigen-presenting cells could also present AChR to T cells, and apparently DR3 is not the only MHC class II molecule involved in the response to AChR.

Peripheral blood lymphocytes from MG patients have been reported to proliferate weakly in response to purified AChR from fish electric organ (Abramsky and Fuchs, 1975; Conti-Tronconi *et al.*, 1979; Richman *et al.*, 1979). The magnitude of response has never been high (stimulation index = <4-5), and the fraction of patients which respond at all is less than half. This could be due in part to low cross-reactivity of T cell response to AChR from different species. It is almost certainly due in part to the low frequency of AChR-sensitive lymphocytes in peripheral blood.

Thymoma and thymic hyperplasia are common in MG patients (Castleman and Norris, 1966), and thymectomy is beneficial to many patients (Buckingham *et al.*, 1976; Rodriguez *et al.*, 1983), suggesting the involvement of thymus and T cells in this disease. Thymic lymphocytes from MG patients have been shown to spontaneously produce AChR antibodies (Scadding *et al.*, 1981) and stimulate antibody production by peripheral blood cells (Newsom-Davis *et al.*, 1981), suggesting that production of antibodies to AChR in MG is also dependent on T cells. But this stimulatory effect was later shown to be unaffected by killing T cells in the thymic lymphocytes with anti-T cell monoclonal antibody and complement (Willcox *et al.*, 1984). AChR antibody production by lymphocytes from thymus, bone marrow, or lymph nodes from MG patients occurs spontaneously in culture without adding any antigen, and (except for blood lymphocytes) is not further stimulated by pokeweed mitogen (Fujii *et al.*,

1986). Although the initiation of the disease is probably T cell dependent, it seems that most of the AChR antibodies in MG patient thymuses are produced by fully differentiated plasma cells (Willcox *et al.*, 1984).

AChR-specific T cell lines from MG patients stimulated with *Torpedo* AChR have been shown to enhance production of antibody to human AChR by autologous blood lymphocytes (Hohlfeld *et al.*, 1986).

An AChR-specific B cell line has also been established from an MG patient (Kamo *et al.*, 1982). Antibody from this line was shown to passively transfer MG to rats.

B cell lines which secrete monoclonal antibodies to myosin, α -actinin, or actin have been prepared from thymic lymphocytes of MG patients (Williams and Lennon, 1986). The observation in MG patients of autoantibodies to components of muscle striations is consistent with the hypothesis that the immune response in MG may often be directed at several components of lysed muscle cells involved in the initiating events, but only autoantibodies to AChRs are pathologically significant in impairing neuromuscular transmissions.

More than 10% of MG patients have other autoimmune diseases such as Graves' disease (Simpson, 1960; Osserman *et al.*, 1967), suggesting that MG may be caused by a generalized immunological hyperreactivity. However, results from studies of suppressor T cell populations (Skolnik *et al.*, 1982; Berrih *et al.*, 1981; Miller *et al.*, 1982) or suppressor function (Shore *et al.*, 1979; Koethe *et al.*, 1981; Mischak and Dau, 1981) are conflicting. As mentioned before, it is difficult to think about the role of suppressor cells in MG in particular when the precise mechanism of suppressor cell function in the general case is unclear.

B. AUTOANTIBODY EFFECTS ON MUSCLE AChRs

The time course of EAMG in rats has been studied by electrophysiological (Lambert *et al.*, 1976) and immunochemical methods (Lindstrom *et al.*, 1976a,b), and by electron microscopy (Engel *et al.*, 1976a,c, 1977b, 1978).

An acute phase of EAMG is observed 8–12 days after immunization of rats with AChR in complete Freund's adjuvant, if pertussis is given at other sites as additional adjuvant (Lennon *et al.*, 1975). At this time, serum antibody titers to AChR are very low (Lindstrom *et al.*, 1976b). An acute phase is not observed after active immunization if the pertussis is omitted. An acute phase response is also observed 1–2 days after passive transfer of anti-AChR antibodies (Lindstrom *et al.*,

1976a; Lambert *et al.*, 1976). The acute phase is characterized by an intense phagocytic invasion of the endplates, which may cause local denervation (Engel *et al.*, 1976a,c; Lambert *et al.*, 1976). This phagocytic invasion is dependent on antibody binding (Lennon *et al.*, 1978b) to a small fraction of the AChRs (Lindstrom *et al.*, 1976a,c), causing complement fixation, which results in focal lysis of the postsynaptic membrane and release of chemotactic fragments that attract phagocytes (Engel *et al.*, 1976a,c, 1978; Sahashi *et al.*, 1978). In the absence of complement, a large fraction of the AChRs can be bound by antibodies without triggering a phagocytic invasion (Lennon *et al.*, 1978b). Phagocytic invasion is probably caused by rapid fixation of a bolus of complement due to a rapid change in antibody concentration. Chronic EAMG occurs in the absence of an obvious acute phase if pertussis is omitted. The acute phase, if not fatal, is self-limiting over 2–3 days.

In MG patients, there is no obvious equivalent of the acute phase of EAMG. Phagocytic invasion of endplates is not observed (Engel *et al.*, 1976c). This is probably because, in most patients, antibodies to AChRs build up insidiously over a prolonged period. Also, if a transient acute phase does occasionally occur, it would probably pass by the time of diagnosis, and in any case would not be easily seen by microscopy.

The “chronic phase” of EAMG begins about 30 days after immunization with AChR (Lindstrom *et al.*, 1976b). At this time, serum antibody titers after a single immunization are maximal, and cross-reacting antibodies are present in large excess over AChR. If the chronic phase is not fatal over the following few weeks, it remits as the response to the immunogen diminishes, unless booster immunizations are given. Chronic EAMG is characterized by loss of 50–70% of muscle AChRs, antibodies are bound to most of the AChRs that remain, the folded structure of the postsynaptic membrane is simplified, and there is evidence of continuing focal lysis (Engel *et al.*, 1976a,c, 1977b, 1978; Sahashi *et al.*, 1978).

Endplates in MG patients closely resemble those in rats with chronic EAMG. Serum antibody levels to AChR may be in large excess over the amount of AChR (Lindstrom *et al.*, 1976d). The amount of muscle AChR is reduced by half or more, and much of what remains has antibodies bound (Fambrough *et al.*, 1973; Lindstrom and Lambert, 1978; Pestronk *et al.*, 1985). The postsynaptic membrane is simplified, and there is evidence of complement fixation (Engel *et al.*, 1977a,b, 1979; Sahashi *et al.*, 1980). In MG patients, the autoimmune response persists for years. This can result in a process of

focal denervation and sprouting of the nerve endings, which can account for the enlarged endplates observed in MG.

There may be a natural history of MG in which, for some patients, the immune response intensifies over a period of a year or two, plateaus, and then slowly decreases (Grob *et al.*, 1987). However, this is difficult to document in individual cases, and the remissions and exacerbations characteristic of the disease often complicate the interpretation of therapeutic measures applied at varying times along its course.

The antibody dependence of EAMG and MG is demonstrated by the effectiveness of passive transfer with Ig (Toyka *et al.*, 1977, 1978) and by the effectiveness, although transient, of plasmapheresis (Newsom-Davis *et al.*, 1978, 1979; Dau *et al.*, 1977). Antisera to AChR have little direct effect on AChR function, but are quite potent at decreasing the amount of AChR (Conti-Tronconi *et al.*, 1981a; Drachman *et al.*, 1982; Tzartos *et al.*, 1986a). This process of antigenic modulation depends on the ability of antibodies to cross-link AChRs, and thereby facilitate their normal pathway of lysosomal destruction (Heinemann *et al.*, 1977; Lindstrom and Einarson, 1979; Drachman *et al.*, 1978, 1980). The amount of AChR loss that can be caused by antigenic modulation is sufficient to account for the degree of AChR loss observed, but focal lysis of the postsynaptic membrane also contributes to loss of AChR and further hinders transmission by disrupting the relationship between presynaptic sites of ACh release and postsynaptic sites of AChR concentration. The amount of AChR loss observed in most patients is sufficient to account for the weakness observed, though even low levels of antibodies to the ACh binding site in some patients would potentially further inhibit transmission.

Antibodies to the MIR are the most frequent specificity of antibody to AChR in both MG and EAMG (Tzartos and Lindstrom, 1980; Tzartos *et al.*, 1982). The antibodies have the characteristic properties of antisera to AChRs: (1) they do not directly affect AChR function (Blatt *et al.*, 1986), (2) they can passively transfer EAMG (Tzartos *et al.*, 1987), and (3) they efficiently cause antigenic modulation (Tzartos *et al.*, 1985).

The characteristic vulnerability of extraocular muscles in MG may be caused by a decreased safety factor for neuromuscular transmission in these muscles. These endplates have an unusually simplified structure and mediate one of the highest average firing rates of any muscle (Oda, 1986).

Variation in patients' symptoms might be caused by many factors. Both MG patients in remission and rats immunized with AChR can tolerate substantial titers of antibody to AChR, probably due to the

high safety factor for neuromuscular transmission. Neonates of mothers with MG usually have a substantial fraction of the maternal anti-AChR concentration, yet often are not grossly impaired (Keesey *et al.*, 1977). This may be because developing neuromuscular junctions can readily respond to the transient immune assault by increased AChR synthesis and reinnervation. Spontaneous and therapeutically induced variations occur in concentration of antibodies to AChR (Seybold and Lindstrom, 1981; Oosterhuis *et al.*, 1983). Patients may differ in the fraction of antibodies that directly inhibits AChR function. Such limited evidence as exists suggests that the pattern of antibody specificities produced is constant over prolonged periods, despite large changes in concentration of antibodies to AChR (Tzartos *et al.*, 1982). Neuromuscular junctions may respond to the autoimmune assault on AChRs by budding of the nerve ending or increasing the rate of AChR synthesis (Wilson *et al.*, 1983a,b). The rate of AChR synthesis, or factors effecting transmission, conductance, or contractility may be susceptible to change under hormonal influences, as might occur during menstruation or pregnancy. Antibody production may also be affected. Because antibodies to AChR do not act primarily as simple competitive AChR antagonists, but act primarily through the slower, more indirect mechanisms of AChR loss and morphologic disruption, it is difficult to quantitatively compare chronic autoantibody concentration with severity of muscle weakness.

V. Therapy

A. EAMG

Since the humoral and cellular mechanisms of the pathogenesis of EAMG are well described, EAMG is a good experimental model for the initial development and testing of new therapies for MG. It has the advantage that many animals can be studied. A disadvantage of using EAMG as a model is that it is an exogenously initiated cross-reacting response and not a self-sustaining autoimmune response.

Several drugs have been tested in the EAMG animal model both to suppress the antibody response to AChR during primary immunization and to suppress an ongoing antibody response. Although some of these drugs are effective, they rely on general suppression of the immune system, thus leaving the animal unable to mount an immune response to other antigens. Single high-dose cyclophosphamide therapy was tested in rats with ongoing EAMG to eliminate B lympho-

cytes. (Pestronk *et al.*, 1982). Although it produced a rapid fall in antibody titer against the immunogen, the treatment necessitated bone marrow transplantation due to the myelosuppressive effects of the drug, and did not eliminate the anamnestic response to antigen. The anamnestic response was eliminated in a later study that added total body irradiation to the preceding protocol (Pestronk *et al.*, 1983). The risks of this type of treatment seem to outweigh any benefits for MG patients. Dimethyl sulfoxide (DMSO) has been shown to either suppress or enhance humoral immune responses to AChR, depending on the time of treatment in the course of the disorder and on the strength of the antigenic stimulation (Pestronk *et al.*, 1985). The mechanism of action of DMSO has not been elucidated. Finally, the effects of cyclosporin A were studied during the various phases of EAMG (Drachman *et al.*, 1985). Cyclosporin A is thought to act by selectively inhibiting T helper cell production of growth factors essential for B cell and cytotoxic T cell differentiation and proliferation, while allowing expansion of suppressor T cell populations (Cohen *et al.*, 1984). Again, while relatively effective in suppressing anti-AChR antibody production during primary immunization or ongoing EAMG, the treatment was not specific and the drug has significant side effects, notably nephrotoxicity.

One approach to partial antigen-specific therapy has used monoclonal antibodies directed to products of the immune response (*Ir*) genes. In the murine system, susceptibility to EAMG has been mapped to the I-A subregion of the *Ir* gene complex within H-2 (Christadoss *et al.*, 1979a). It was reported that treatment of mice with a monoclonal antibody to I-A suppressed both humoral and cellular responses to AChR, and also suppressed the clinical manifestations of EAMG (Waldor *et al.*, 1983). In outbred humans with a variety of antigen-presenting molecules, this approach seems unlikely to be effective.

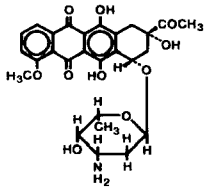
Another approach to therapy of EAMG with monoclonal antibodies used an mAb to the L3T4 antigen on murine helper lymphocytes to treat murine EAMG (Christadoss and Dauphine, 1986). Such an approach is not antigen specific.

The goal of therapy for MG is to specifically eliminate the anti-AChR autoimmune response without affecting the patient's general immune status. In recent years, much work has focused on the development of immunotoxins in an effort to specifically target therapy to elimination of defined populations of cells (Thorpe *et al.*, 1982; Vallera *et al.*, 1983; Quinones *et al.*, 1984; Gilliland *et al.*, 1980; Krolick *et al.*, 1980). Immunotoxins are cell-binding antibodies or

antigens covalently bound to plant or microbial toxins (Vitetta *et al.*, 1983; Vitetta and Uhr, 1985). The specific antibody or antigen directs the toxin to target cells and kills them, while leaving nontarget cells unaffected (Fig. 2). Conjugates of native ricin molecules (Thorpe *et al.*, 1982; Vallera *et al.*, 1983; Quinones *et al.*, 1984) or ricin A chain (Gilliland *et al.*, 1980; Krolick *et al.*, 1980, 1982; Oeltmann and Heath, 1979; Hashimoto *et al.*, 1984; Rennie *et al.*, 1983) and antibodies or antigens have been used with some success in *in vitro* studies to selectively kill cells bearing specific antigenic determinants or hormones. Following treatment of human autoimmune lymphocyte cultures from patients with Hashimoto's thyroiditis with ricin A chain thyroglobulin conjugates, the production of autoantibodies against thyroglobulin by lymphocytes was suppressed (Rennie *et al.*, 1983). Earlier studies from this laboratory had demonstrated the effectiveness of holoricin-AChR conjugates in suppressing B cell antibody synthesis in lymph node cultures from rats with EAMG following addition of AChR to the culture (Killen and Lindstrom, 1984). Similar studies that used ricin A chain AChR conjugates to treat isolated lymphocytes resulted in suppression of both T cell proliferation and B lymphocyte antibody production (Olsberg *et al.*, 1985). The ribosomal-inactivating proteins gelonin (Thorpe *et al.*, 1981) and saporin (Glennie *et al.*, 1987), after covalent linkage to monoclonal antibodies, have been shown to acquire potent and specific cytotoxic activity in other systems. These single chain proteins have the advantage of enzymatic activity similar to ricin A chain without the nonspecific binding of ricin B chain. Conjugates of gelonin and AChR were effective at inhibiting the antibody response to AChR in cultures of sensitized lymph node cells from AChR-immunized rats (G. D. Shelton, Y. Fujii, and J. M. Lindstrom, unpublished results). If an autoantigen such as AChR coupled to a toxin could induce specific immunological unresponsiveness in whole animals, this could provide a highly selective means of deleting autoantibody-secreting B lymphocyte clones in patients with MG, and could also have a more general significance in the treatment of any autoimmune disease where the autoantigen is known.

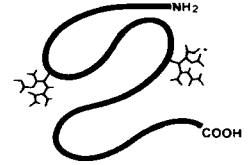
Chemotherapeutic agents such as daunomycin (DM) have also been coupled to antigens or antibodies with some success (Hurvitz *et al.*, 1978; Levy *et al.*, 1975; Belles-Isles and Page, 1980). Diener *et al.* (1986) showed that DM coupled to hapten conjugates of ovalbumin by an acid sensitive spacer caused hapten-specific immunosuppression of murine B lymphocytes *in vitro* and *in vivo*. The effectiveness of conjugates of DM and AChR in specifically suppressing the antibody

DAUNOMYCIN (DM)

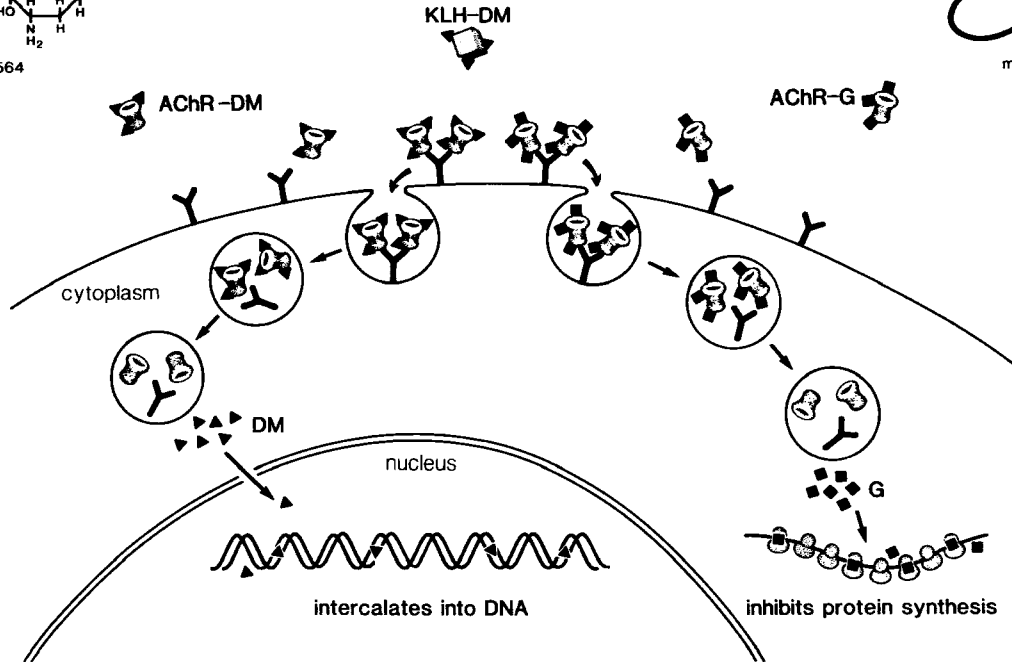


mw = 564

GELONIN (G)



mw = 30,000



response in AChR-sensitized lymph node cells and in preventing the onset of EAMG following immunization of AChR has recently been tested both in cultures and in rats (Shelton *et al.*, 1988b). In cultures of sensitized lymphocytes from rats immunized with AChR, DM-AChR conjugates with a ratio of 40–60 : 1 resulted in 50–90% suppression of AChR antibody production, without suppressing the immune response to keyhole limpet hemocyanin (KLH), and DM-KLH conjugates did not effect the response to AChR. The T cell response was not suppressed by DM-AChR conjugates. This is not surprising, since T helper cells recognize proteolytic fragments of a protein in association with Ia molecules, and it is unlikely that the toxin would stay bound to the immunogenic peptide while AChR is being processed. This treatment resulted in very little if any nonspecific toxicity as determined by thymidine uptake of T cells following Con- A stimulation. These conjugates were also shown to be effective in preventing the onset of EAMG in rats pretreated with conjugate prior to immunization with AChR. The effect was antigen specific and did not reduce the immune response to KLH.

Although antigen-specific prevention of EAMG by pretreatment with DM-AChR is encouraging, attempting to treat chronic EAMG or MG with toxin conjugates is likely to be much more difficult. Potential problems include binding of circulating antibody to the conjugates to form large aggregates that are removed before the conjugates reach the targeted lymphocytes and which enhance nonspecific toxicity of the conjugates for tissues in which the aggregates collect. Also, even if recognized by the appropriate target, the conjugate may not be internalized or may not cross the lysosomal membrane if processed through the endosomal system, or may be inactivated in the process. Toxicity of some of these agents may also limit their usefulness. Some of the conjugates are potentially effective, as evidenced by studies in tissue culture and pretreatment of intact rats; however, much work

FIG. 2. Toxin-AChR conjugates cause cell destruction by two different mechanisms. Daunomycin (DM), an anthracycline antibiotic used in cancer chemotherapy, can be conjugated to AChR by an acid-sensitive linkage. Immunoglobulins (Igs) on the surface of B lymphocytes recognize and bind AChR in the conjugate. The complex is internalized into the endosomal system, and at the acid pH in the lysosome DM is freed and passes through the lysosomal and nuclear membranes to intercalate into the DNA. The ribosomal inactivating proteins including gelonin, ricin, and saporin act by a different mechanism. Following coupling to AChR, gelonin-AChR conjugates are also recognized by Igs on the B lymphocyte surface and internalized into the lysosomal system. The gelonin in the conjugate is freed in the lysosome and passes into the cell cytoplasm blocking protein synthesis at the level of the ribosomes.

remains to be done to optimize this approach to treatment of ongoing EAMG.

The concept of "radioactive antigen suicide" in which ^{125}I -labeled antigen of high activity specifically inhibits lymphocytes responsive to that antigen has been tested in animals in several models of autoimmune disease. ^{125}I -labeled AChR pretreatment was effective in preventing the onset of EAMG (Sterz *et al.*, 1985), ^{125}I -labeled myelin basic protein significantly suppressed the development of experimental autoimmune encephalomyelitis (Filipp *et al.*, 1981), and autoimmune hepatitis was inhibited by pretreatment with ^{125}I -labeled liver specific membrane antigen (Feher *et al.*, 1984). Effectiveness in ongoing autoimmune disease has not yet been described. It is presumed that in EAMG, with high titers of circulating autoantibodies, any injected radioactive antigen would be rapidly removed.

Another approach to specific targeting of immunotherapy is the use of antiidiotypic antibodies. Reports of the efficacy of this therapy in EAMG are conflicting. In one study, exposure of AChR-reactive lymph node cultures to antiidiotypic-racine A chain conjugates resulted in the inability of the cells to respond to an AChR challenge (De Shambo and Krolick, 1986). Antiidiotypic therapy using xenogenic antibodies has been reported to be successful in preventing the onset of EAMG (Dwyer and Schonbeck, 1986; Souroujon *et al.*, 1985), lowering antibody titers during ongoing EAMG (Agius and Richman, 1986) and mitigating passively transferred EAMG (Souroujon *et al.*, 1986b). Other groups have found that antiidiotypes prepared against monoclonal anti-AChR antibodies were unsuccessful in preventing the development of EAMG in rats (Lennon and Lambert, 1981). Also, rat monoclonal antibodies to the MIR were used to immunize rats and induce antiidiotypic antibodies (Killen *et al.*, 1985). In this study, when rats producing antiidiotypic antibodies were immunized with AChR, they showed no net decrease in anti-AChR antibody production. Also, there was little cross-reaction between the antiidiotypic antibodies and the various anti-MIR monoclonal antibodies. In another study, antiidiotypic sera raised in rabbits against anti-AChR purified from the sera of three MG patients inhibited binding of homologous idiotype to the AChR by up to 80%. However, substantial idiotype sharing between individual patients could not be demonstrated (Lang *et al.*, 1985). While naturally occurring antiidiotypic antibodies have been reported in the sera of myasthenic patients (Dwyer *et al.*, 1983b), their significance in MG is not clear. Given the polyclonality of the antibody response in MG and EAMG, it is doubtful that antiidiotypic antibodies will play a future role in MG therapy.

In cytotoxic models of T cell-mediated autoimmune diseases, immunization against pathologically significant idiotypes with disrupted T cells has been reported to suppress the disease (Lider *et al.*, 1987). Effective therapy through immunization with a patient's own lymphocytes is an appealing concept. However, EAMG depends on T helper cells rather than cytotoxic T cells. Also, it has been reported that this approach was not effective on EAMG (Drachman, 1988).

Several groups have tried to suppress EAMG with suppressor T cells or suppressor T cell factors (Bogen *et al.*, 1984; Pachner and Kantor, 1984; Sinigaglia *et al.*, 1984; McIntosh and Drachman, 1986). In a murine EAMG model, AChR-specific suppressor T cells from sensitized lymph nodes were cultured as T cell lines. These cell lines were reported to be capable of suppressing some of the antibody production to AChR and clinical signs of EAMG when injected intravenously prior to immunization with AChR (Pachner and Kantor, 1984); however, some nonspecific suppression was apparent. Also, AChR-specific suppressor cells were induced in sensitized lymphocytes obtained from rats with EAMG by culturing in the presence of cyclosporin A and purified AChR (McIntosh and Drachman, 1986). When these cells were cultured with lymphocytes from rats with EAMG, there was suppression of the response to AChR. The efficacy of this approach has not yet been tested in ongoing EAMG.

B. MG

Methods of treatment currently available for MG are providing most patients with an acceptable quality of life. However, the treatments are palliative, not a cure, and are not without adverse side effects. Ideally, treatment should be aimed at eliminating only the abnormal autoimmune response while leaving the ability to respond to other antigens intact. Also, the treatment should have a permanent effect without relapse following discontinuation of the treatment. To date, these goals have not been achieved. Generally accepted methods of treatment currently include (1) inhibitors of acetylcholinesterase, (2) immunosuppressive drugs, (3) surgical thymectomy, and (4) plasma exchange.

Anticholinesterase drugs, including neostigmine and pyridostigmine, are the cornerstone of therapy for MG. Because these drugs inhibit enzymatic hydrolysis of ACh at the neuromuscular junction, there is prolonged interaction of ACh released at the nerve terminus with remaining AChRs, thereby increasing the effective concentration and duration of effect of the ACh in the synaptic cleft. The dosage must be titrated to each individual's needs because requirements vary from day to day in response to activity levels and stress. Underdosage

or overdosage may result in muscular weakness and possibly death due to paralysis of intercostal muscles. Gastrointestinal side effects may occur as a result of accumulation of ACh at muscarinic receptors on cholinergic synapses in smooth muscle and secretory glands. With prolonged exposure to high concentrations of ACh, AChRs are desensitized and nonfunctional. High doses of anticholinesterase drugs have been shown in laboratory animals to result in pathological changes at the neuromuscular junction similar to those seen in MG, including simplification of postsynaptic membrane structure and loss of AChR. These changes have been shown to be mediated by calcium influx due to prolonged agonist action (Leonard and Salpeter, 1979).

Early thymectomy is currently recommended for adult MG patients with generalized weakness of moderate to severe degree and in some studies was shown to speed the remission rate and improve the clinical course of MG (Buckingham *et al.*, 1976; Rodriguez *et al.*, 1983). In long-term follow-up studies, serum antibody levels against AChR correlated with clinical improvement after thymectomy (Vincent *et al.*, 1983; Kagotani *et al.*, 1985). Most MG patients have thymic abnormalities, either thymic hyperplasia or thymoma (Bofill *et al.*, 1985; Castleman and Norris, 1966). The thymic medullary epithelium in MG is frequently hyperplastic, and lymphoid follicles with active germinal centers have been described in the thymic medulla (Bofill *et al.*, 1985). Both normal and MG patient thymus glands contain muscle-like "myoid" cells that have been shown to contain extrajunctional AChRs (Kao and Drachman, 1977; Schlupe *et al.*, 1987). Theoretically, this could be a primary source of antigen in MG, however, the most recent and detailed histological study of thymic myoid cells concludes that myoid cells do not appear to be the focus of immune stimulation in the thymus (Schlupe *et al.*, 1987). Also, when lymphocytes from myasthenic thymus glands are cultured *in vitro*, they produce significant amounts of anti-AChR antibody (Scadding *et al.*, 1981), although the amount of anti-AChR antibody produced in the thymus represents only a very minor fraction of the total antibody production.

Adrenal corticosteroids are the most commonly used immunosuppressive agents in patients who do not respond to anticholinesterase drugs or thymectomy. While often resulting in clinical improvement, their precise mode of action in MG is not yet fully defined. They may result in decreased serum anti-AChR antibody concentrations (Seybold and Lindstrom, 1981). However, chronic glucocorticoid treatment can independently produce muscle weakness and wasting due to impaired muscle protein and carbohydrate metabolism

(Goldberg *et al.*, 1980; Afifi and Bergman, 1969). Also, resistance to infection is decreased because the immunosuppressive effects are generalized. In addition to immunological effects, steroid treatment can produce features of Cushing's syndrome. Alternate day dosage may minimize some of the adverse side effects. Other immunosuppressive drugs such as azathioprine and cyclophosphamide have been used with some success (Mertens *et al.*, 1981) although relapse may occur following discontinuation of therapy (Hohlfeld *et al.*, 1985b). The side effects of bone marrow depression, potential neoplastic changes, and decreased resistance to infection limit their application to resistant cases.

Plasmapheresis, while effective in lowering antibody titers and often resulting in clinical improvement, is expensive and reserved as a rescue procedure for severely affected patients that are resistant to other forms of therapy (Newsom-Davis *et al.*, 1978, 1979; Dau *et al.*, 1977; Dau, 1980, 1984). The benefits from the procedure are temporary, requiring the patient to undergo multiple courses of plasmapheresis or to receive a concurrent course of immunosuppressive drugs.

In summary, therapy of MG can be directed at one or more of the following levels; (1) minimizing the effects of the autoimmune response on neuromuscular transmission, (2) removing the autoantibodies which mediate the immune assault, (3) preventing synthesis of the autoantibodies, or (4) eliminating the source of immunogen. Treatment with acetylcholinesterase inhibitors compensates in part for the antibody-induced loss of functional AChRs. Although it is only a symptomatic therapy, it is probably the single most important approach to reducing mortality and improving the quality of life for MG patients. Correct doses are important because overdosing can cause a potentially fatal cholinergic crisis, and remissions and exacerbations characteristic of MG require constant vigilance to ensure proper dosage. Practical therapies to inhibit antibody binding *in vivo*, reducing antibody-induced AChR internalization, or minimizing focal lysis have not been developed and seem unlikely approaches. Plasmapheresis provides a method for directly removing the pathological autoantibodies. However, other soluble blood components are removed, the process is expensive and not without risk, and the benefits are transient. Plasmapheresis is useful in severe cases resistant to other forms of therapy. Improved procedures which remove only the Ig fraction of plasma or only antibodies to AChR could improve this approach, but would still leave the basic chronic immunologic disease process untouched. Synthesis of antibodies to AChR could, in principle, be prevented at the level of the B cell, the T cell, or the

antigen-presenting cell. Prednisone and azathioprine are frequently used to treat MG. However, both nonspecifically suppress immune responses, neither eliminates the immune response to AChR, and both have a high incidence of serious side effects after constant use. Monoclonal antibodies to antigen-presenting proteins which bind AChR epitopes might be used to suppress the function of these proteins, but this is neither an antigen-specific nor a permanent solution to the disease process. Using antibodies to T helper cells would be even less antigen-specific. Antiidiotypic therapies have the theoretical potential for specifically eliminating only autoimmune B or T lymphocytes. Despite reports of shared idiotypes of anti-AChR antibodies and prevention of EAMG by pretreatment with antiidiotypic sera, the preponderance of evidence suggests that there is not extensive sharing of idiotypes. Thus, it seems unlikely that a practical antiidiotypic therapy for MG will be developed. Conjugates of AChR with toxins might in the future provide magic bullets for specifically killing only B or T lymphocytes which are specific for AChR, but there are many practical barriers to designing a conjugate with suitable antigenicity, toxicity, and solubility, ability to survive high levels of patient antibodies to reach the targeted cells without accumulating unacceptable nonspecific toxicity. Thymectomy may be beneficial for MG patients. Although the mechanism of this effect is unclear, it may, in part, be due to the removal of immunogen from thymic myoid cells which is in intimate association with autoimmune lymphocytes. There are almost certainly other sources of immunogen. If the initial immunizing event which provokes the autoimmune response to AChR were known, it might become practical to think of treating MG by eliminating the source of sustaining immunogen, perhaps by eliminating infection of a muscle-like cell which provokes the autoimmune response in the first place.

VI. Prospects

The cause or causes of the autoimmune response to AChR in MG may be difficult to discover. Thus far, no evidence of viral involvement has been found (Klavinskis *et al.*, 1986) and arguments for bacterial involvement (Dwyer *et al.*, 1983a) are not compelling. Nevertheless, it seems likely that an environmental factor is involved. Penicillamine is clearly one such factor (Bever *et al.*, 1982). The association of thymoma with MG (Compston *et al.*, 1980) and the presence of autoantibodies to other muscle components (Peers *et al.*, 1977; Williams and Lennon, 1986) suggest that the initiating mecha-

nism or mechanisms involve more than the AChR per se. It is interesting that the rabies virus seems capable of using the AChR as a tissue-specific recognition site (Lentz *et al.*, 1986). It is appealing to think that a viral infection of one or more cell types might lead to the source of initiating and sustaining immunogen and, occasionally, to thymomatous cells, but no proof of this exists. It is clear that genetic proclivity for the antibody-mediated autoimmune response to AChR is important, as reflected in the HLA grouping in these patients (Compston *et al.*, 1980) and in the increased frequency of other autoimmune diseases (Simpson, 1960). The application of molecular genetic techniques to this problem may provide further clarification (Bell *et al.*, 1986).

Diagnosis of MG has improved with the availability of assays for antibodies to AChR. The ease and availability of these assays will likely increase, helped in part by improvement in the source of AChRs through tissue culture and molecular genetic techniques, and it should become possible to routinely measure not only total anti-AChR concentration, but also the fraction of these antibodies to the MIR and ACh-binding site, as well as the subclass composition of these antibodies. However, whether this will dramatically improve prognosis or therapy remains to be determined.

Some of the pathological mechanisms in MG are well known, some are beginning to be studied, while other aspects remain obscure. The pathological mechanisms of MG at the endplate are moderately well understood, but can certainly benefit from improvement in detail. It seems unlikely that major revelations will occur here. Advances in molecular studies of AChRs should permit more detailed understanding of the AChR as an immunogen and antigen. In the next couple of years, much should be learned about the specificities and activities of T cells in EAMG and MG. However, the mechanisms associated with initiating and sustaining the autoimmune response in MG are *terra incognita*.

The first important advance in therapy of MG was the use of ACh esterase inhibitors (Walker, 1934), which occurred when the site of the lesion was suspected but its mechanisms not known. With the realization of the autoimmune nature of MG, therapy has improved through the use of generally immunosuppressive drugs, plasmapheresis, and increased reliance on thymectomy. However, no specific cure is available, and current therapies have unfortunate side effects. Even without understanding what initiates and sustains the autoimmune response to AChR in MG, it may be possible to develop specific approaches for suppressing this response. Advances in molecular

studies of AChRs should permit manipulations to provide useful antigens and immunotoxins for human studies. EAMG and naturally occurring canine MG provide systems for developing and testing such specific therapeutic strategies. Some might prove pragmatically useful in MG patients, while others might be only academically interesting.

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Alterations of the Immune System in Ulcerative Colitis and Crohn's Disease

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I. Introduction

Crohn's disease and ulcerative colitis are chronic inflammatory diseases whose primary clinical and pathologic manifestations are gastrointestinal. Their most prominent clinical characteristics are abdominal pain and diarrhea. Ulcerative colitis involves only the colon; Crohn's disease can involve either the colon, or the small bowel, or both. In ulcerative colitis, there are mucosal ulcers and infiltration of the mucosa and submucosa with neutrophils, macrophages, and lymphocytes. In Crohn's disease, the inflammatory infiltrate frequently contains granulomas and extends through all layers of the bowel wall rather than being confined to the mucosa and submucosa as in ulcerative colitis. Both diseases have been viewed as "chronic" inflammatory diseases because of their prolonged clinical courses and because their inflammatory infiltrates contain lymphocytes and macrophages, a histologic picture that is characteristic of chronic inflammation. However, both diseases also have a less well-recognized, but equally prominent "acute" component marked by a constant flux of neutrophils out of the circulation, into the inflamed mucosa, and then through the epithelium and into the intestinal lumen.

The etiologic agents for ulcerative colitis and Crohn's disease have not been identified. A large number of microbial and dietary agents have been put forth as candidates for etiologic roles in these diseases, but none has proven out. The mechanisms for the spontaneous exacerbations and remissions, characteristic of these diseases, are also undefined. Although the etiologic agents are unknown, recent studies have given some insights into the mechanism for the amplification of the inflammatory response that results in the histologic and clinical

changes characteristic of these diseases. The immediate causes of the functional and histologic changes seen in inflammatory bowel disease (IBD) appear to be soluble mediators of inflammation (1,2). Neutrophil and macrophage infiltration suggest the presence of soluble chemotactic agents that cause neutrophils and monocytes in the circulation to migrate into the mucosa. Mucosal edema and hyperemia reflect the presence of soluble mediators that induce enhanced vascular permeability and vasodilation. Inflammatory responses in the intestine can be induced by disorders of immunoregulation (e.g., systemic lupus erythematosus), infectious colitis (e.g., shigellosis), impaired circulation (e.g., ischemic colitis), or toxic compounds (e.g., acetic acid colitis). There are striking similarities in clinical and morphologic manifestations among the diseases marked by intestinal inflammation (3,4). The likely explanation for these similarities is that the clinical and histologic manifestations of each of these diseases arise, not from the etiologic agents, but from the soluble mediators of inflammation that are generated in the inflammatory process.

A number of the immunologic perturbations associated with IBD in the past have been found to be effects rather than causes, phenomena secondary to the sequelae of the diseases (inflammation and malnutrition, for example) or to the immunosuppressive consequences of steroid therapy (Table I). Nevertheless, as advances have been made in our understanding of the immune system and as investigative techniques have become increasingly sophisticated, we have been able to achieve a growing confidence that recent studies are yielding new information on the immunopathogenesis of IBD (Fig. 1); moreover, this information is beginning to translate into promising therapeutic leads. In short, a balanced review of the subject of alterations of the immune system in IBD must incorporate both the frustrations of the past and the promise provided by current progress.

TABLE I
COMMON PITFALLS IN THE STUDY OF ULCERATIVE COLITIS AND CROHN'S DISEASE

Initial observed abnormalities subsequently found to be secondary to malnutrition, disease duration, and/or therapy with steroids and immunosuppressive agents
Alterations supposedly specific for IBD actually present in numerous other gastrointestinal, infectious, and systemic autoimmune disorders
Immune function changes, thought to be primary abnormalities, shown to be due to secondary events related to the acute inflammatory process or sensitization of the immune system to mucosal antigens

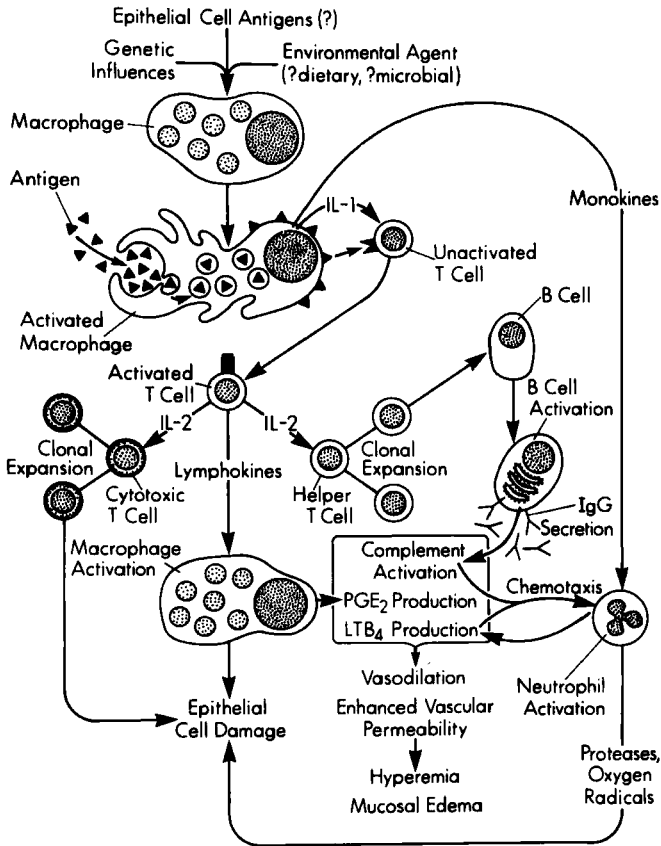


FIG. 1. Sequence of immunologic events which may account for the initiation and perpetuation of inflammatory responses in ulcerative colitis and Crohn's disease. After sensitization to environmental agents and/or epithelial cell antigens, possibly facilitated by undefined genetic influences, the first cell population involved would be the intestinal macrophage. Upon phagocytosis, antigen processing, and presentation to a T lymphocyte, in combination with IL-1, a population of sensitized and activated T cells capable of IL-2 secretion would stimulate clonal expansion of specific cytolytic T cells and a helper T cell/B cell collaboration resulting in increased antibody secretion (particularly IgG subclasses). At the same time, monokines would activate neutrophils, and T cell lymphokines would induce further macrophage activation. The release of inflammatory mediators would not only have direct biologic effects, but would also serve via chemotactic effects to amplify and perpetuate the immune response.

II. Genetic Markers

The frequent association of IBD in more than one family member and certain ethnic groups has led to a search for a genetic marker. Studies of IBD in family members have shown incidences ranging from 10% to 35% (5), a rate many times higher than would be predicted by the prevalence of IBD in the general population. However, examination of the standard HLA-A, -B, -C, and -DR antigens (6–10) has not shown a clear or uniform correlation with the occurrence of IBD. Even within families, patients with the disease do not necessarily have the same HLA type. On the other hand, HLA-B27 is more frequent in the subset of patients with IBD who have ankylosing spondylitis. Furthermore, extensive analysis and summation of all available data (11) have demonstrated an increased association of Crohn's disease with HLA-2, and ulcerative colitis with HLA-B27 and HLA-Bw35 in caucasians; and ulcerative colitis with HLA-B5 in Japanese. In addition, IBD is quantitatively associated with several genetic disorders, such as Turner's syndrome.

A genetic defect may be responsible for the patient's inability to mount an adequate immune response or for the inability to shut down the intestinal immune response. It may also affect the transition from immune activation to inflammation—a step in the pathogenesis of inflammatory bowel disease that has not been well characterized. Polygenic influences, variability of penetrance, and incomplete recognition of mild or subclinical disease states could all tend to obscure correlation of tissue typing results with patterns of inheritance in IBD. Nevertheless, some patient groups with restricted genetic heterogeneity, such as Japanese or Israeli Jewish populations, have been found to have definite, but different HLA phenotypes associated with IBD. There also is a second genetic locus associated with regulation of the immune response: the immunoglobulin heavy chain complex, which is on chromosome 14 in humans. Crohn's disease has been shown (12) to be associated with certain heavy chain allotypes. In future studies, when competing genetic effects are screened out or when genetic markers using molecular biology techniques such as restriction fragment length polymorphism are applied, the immunogenetic factors that play a role in IBD will be better understood.

III. Alterations of Peripheral Blood Lymphocyte Function in IBD

Because of easy accessibility, peripheral blood lymphocytes have been the starting point for many studies of immune function. During

the 1960s and 1970s, a concerted effort was made to delineate the changes in peripheral blood lymphocyte populations that occur in inflammatory bowel disease. However, immunologic abnormalities exhibited by peripheral blood mononuclear cells (MNC) in patients with IBD have most often proven to be nonspecific and secondary in nature and to not represent primary abnormalities related to disease pathogenesis. The duration and severity of IBD, adequacy of nutrition, and amount and types of therapy all have been proven to have a major influence on tests of cell-mediated immune function and have all too often been the cause of reported abnormalities. These past lessons (Table I) are critical to remember in assessing or planning studies on the immunology of IBD.

Early studies (13,14) disagreed as to the quantitative peripheral blood lymphocyte subpopulation changes seen in Crohn's disease. The conflicting results from many studies were clarified when Auer and co-workers (15,16) divided their patients with Crohn's disease into two groups: (1) patients in whom the disease was newly diagnosed and who had never been treated with steroids, and (2) patients in whom the disease was of long duration and who had been treated with sulfasalazine and/or steroids. It was the second group, the patients who had had long-term disease and who had been treated, in whom significant total lymphocytopenia and a decrease in T cells occurred (15). In contrast, minimal changes were seen in the group of untreated patients with recent onset Crohn's disease, because secondary influences on the immune system were minimized. In a follow-up study, Auer and co-workers (16) presented evidence that the secondary influences causing the total and T cell lymphocytopenia were related to the chronicity and activity of the Crohn's disease, rather than the effect of therapy. Thus, total lymphocyte numbers as well as T cell numbers are decreased in a subset of patients with Crohn's disease who have had active disease of long duration; this is a secondary rather than a primary event, since it is not observed in patients with recent-onset disease who have not been treated. Furthermore, in studies using monoclonal antibodies, Selby and Jewell (17) and Yuan *et al.* (18) found no significant differences between IBD patients and controls with regard to the proportions of circulating T lymphocytes or their subsets. Early studies of delayed hypersensitivity using skin test antigens (19,20) demonstrated an anergic state in Crohn's disease. Subsequently, these results also were found to be due to the effects of malnutrition in severely ill patients (21,22). Similarly, defective responsiveness to mitogenic lectins (23) was subsequently found to be due to secondary events in severely ill, malnourished IBD patients.

With regard to antigen-specific activation of lymphocytes, Bull and Ignaczak (24) and Bartnik and co-workers (25) demonstrated that enterobacterial common antigen could induce production of migration inhibition factor (MIF) by peripheral blood lymphocytes from patients with ulcerative colitis and, to a lesser degree, from patients with Crohn's disease. Interestingly, to date these data appear not to be nonspecifically influenced by the disease processes and suggest that cross-reactive bacterial or intestinal antigens could secondarily sensitize and activate peripheral blood lymphocytes.

Raedler and co-workers (26) have found that high numbers of activated T9 positive ($T9^+$) lymphocytes are present in patients with active Crohn's disease (26), with 19.7% of peripheral blood lymphocytes and 24.3% of peripheral blood T cells demonstrating a positive phenotype for T9, whereas patients with inactive Crohn's disease exhibited only 8.5% $T9^+$ peripheral blood lymphocytes and 9.9% positive peripheral blood T cells (26). In a follow-up study of active Crohn's disease, Raedler and co-workers found a significantly increased number of Fc α receptor positive cells within the subset of $T9^+$ cells (27). Elevated percentages of Fc α -bearing T cells were uniquely found in inflammatory bowel disease and were not found in patients with systemic lupus erythematosus, rheumatoid arthritis, sarcoidosis, or infectious gastroenteritis (27). Although further studies will be needed to support the author's contention that the $T9^+$ Fc α receptor-bearing cell subset may be considered to be pathognomonic for inflammatory bowel disease, the studies to date by Raedler and co-workers (26,27) provide evidence for the activation of discrete mononuclear cell subpopulations as part of the alterations in the immune response of inflammatory bowel disease patients.

IV. Serum Antibodies and DR Antigens

Broberger, Hammarstrom, Lagercrantz, Perlmann, and co-workers (28-32) demonstrated anticolon antibodies which were (1) directed against epithelial cells, (2) cross-reactive with *Escherichia coli* 014, and (3) present in sera from 56% of patients with ulcerative colitis (UC) and 67% of patients with Crohn's disease. On the basis of these and other confirmatory studies (28-35), it was hypothesized that anticolon antibodies could be produced by sensitization with *E. coli* 014 and then function as an initiating or perpetuating factor in IBD. Subsequent studies, however, provided evidence against a role for anticolon antibodies in IBD. Broberger and Perlmann (36) observed that sera from patients with ulcerative colitis, in the presence of

complement, did not kill fetal colonic cells. Rabin and Rogers (37) immunized rabbits with intestinal tissue, resulting in circulating anticolon antibodies, but did not observe any pathologic tissue damage in the intestine. Deodhar *et al.* (38) first provided evidence for the lack of disease specificity of anticolon antibodies by observing that 28 of 54 patients with autoimmune diseases had anticolon antibodies, but that none of these patients had evidence of IBD. The lack of disease specificity of anticolon antibodies was conclusively demonstrated by Carlsson and co-workers (39), who examined 1310 sera samples and found that although 60% of ulcerative colitis patients and 61% of Crohn's disease patients had serum titers of anticolon antibodies greater than 1:16, so too did 69% of patients with urinary tract infections, 53% of patients with multiple polyposis, 62% of patients with cirrhosis, 50% of patients with salmonellosis, 47% of patients with gastroenteritis, and 47% of patients with irritable bowel syndrome. Finally, Heddle and Shearman (40) measured serum antibody titers against enterobacterial common antigen, *E. coli* 014, and five other *E. coli* serotypes, and found that patients with ulcerative colitis had the same levels of antibodies as did control subjects. Thus, the lack of disease specificity and the lack of pathogenic capability argue strongly against a primary role for anticolon antibodies in IBD and suggest that they arise secondary to the disease process. Moreover, the use of appropriate disease specificity controls in these experiments serves as an important model for the design of all IBD clinical studies.

Korsmeyer and co-workers (41,42) found that lymphocytotoxic antibodies occur in approximately 40% of IBD patients, 40% of household contacts of patients with IBD, 50% of spouses of patients with IBD, and 34% of relatives of patients with IBD. Furthermore, the lymphocytotoxic antibodies were found to be directed against synthetic double-stranded RNA, both in patients with IBD and family members, providing indirect evidence for the presence of RNA viruses in patients with IBD, with transmission to family members (42). These data (41,42) raise the possibility that antilymphocyte antibodies are directed against viral components initially, and against lymphocytes as a secondary, cross-reactive phenomenon. Strickland and co-workers (43) characterized the lymphocytotoxic antibodies as being predominantly cold reactive, with 60% of the antibodies being directed against both T and B cells, while only 20% were directed against T cells alone and 20% against B cells alone.

Although the pathogenic role of lymphocytotoxic antibodies in IBD is unclear, lymphocytotoxic antibodies in the sera of some patients

with systemic lupus erythematosus (SLE) have been shown to be directed against suppressor T cells, to be capable of inactivating suppressor cell generation (44–46), and to be directed against a presuppressor T cell, necessary for the generation of suppressor cells (47,48). Another possible pathogenic role of lymphocytotoxic antibodies in IBD was suggested by Matuchansky and co-workers (49), in their report of a patient with chronic ulcerative colitis and primary hypogammaglobulinemia who had low numbers of B cells and a lymphocytotoxic antibody directed against B cells. These observations (44–49) suggest that patients with IBD, who have lymphocytotoxic antibodies, could have alterations of antibody synthesis and secretion as a result.

We found cold-reactive antilymphocyte antibodies in 40% of patients with ulcerative colitis and 41% of patients with Crohn's disease, as opposed to only 10% of control patients. Antilymphocyte antibodies against B cells were present at 37°C, as well as 20° and 4°C, indicating the presence of IgG warm-reactive, as well as IgM cold-reactive antilymphocyte antibodies. The sera were extensively absorbed with platelets, after which the lymphocytotoxic antibodies were found to react exclusively with B cells and macrophages. The lymphocytotoxic antibodies were not directed against any particular HLA antigens, but a number did react to DRW types. However, the DR specificities were broad, with most of the lymphocytotoxic antibodies reacting against two, or sometimes three, DR specificities, perhaps representing supertypic determinants. Reactivity was commonly seen against combinations of DRW types (i.e., 3, 5, plus 6, or 4 plus 7). In our experience, therefore, IgM and IgG lymphocytotoxic antibodies in patients with IBD were found to be directed primarily against B cells and macrophages. Our studies also showed that reactivity against T cells was removed after absorption with platelets and that IBD lymphocytotoxic antibodies are directed against a wide variety of DRW antigen types. Our findings, therefore, implicate sensitization to DR antigens in the development of antilymphocyte antibodies.

Hirata and co-workers (50) have used immunohistological techniques to carry out an extensive analysis of a variety of different cell types in normal and IBD intestine. Surface membrane staining using antihuman HLA-DR antibodies revealed staining of a number of different cell types in normal intestine, including lamina propria mononuclear cells, dendritic cells, fibroblasts, as well as both vascular and lymphatic endothelial cells (50). Mononuclear cells with the morphology of macrophages showed intense surface staining for HLA-DR antigens in control specimens. The most marked change

noted in inflammatory bowel disease was the intense staining by anti-HLA-DR antibodies of epithelial cells from diseased tissue, indicating the induction of large amounts of HLA-DR antigens on epithelial cells from inflammatory bowel disease patients (50). Lymphatic and vascular endothelial cells also demonstrated HLA-DR antigen positivity in inflammatory bowel disease specimens.

The role of HLA-DR antigens in the generation of the normal immune response, as well as possible implications for roles in autoimmune diseases has received considerable attention in recent studies. It has been known for many years that the expression of HLA-DR antigens is prominent on B cells, macrophages, dendritic cells, and thymic epithelium. Activation of cells of a variety of types will lead to the induction of HLA-DR antigens on their surfaces. Helper T cells are capable of recognizing foreign antigens in the presence of HLA-DR molecules (51). Polymorphism of the major histocompatibility complex provides the potential for presenting antigen in different contexts and may allow either the establishment of self-tolerance or the expansion of autoreactive T cells. The recognition of antigens in conjunction with self-HLA-DR molecules by T cells results in a population of T cells activated to recognize the same antigen presented by self-HLA-DR components at future times (52).

Therefore, the immunohistological studies by Hirata and co-workers (50) demonstrate a variety of different cell types within the intestine that may be capable of functionally interacting with and presenting antigen, including macrophages and dendritic cells, as well as HLA-DR antigens on vascular and lymphatic endothelium in the intestine. Furthermore, in IBD intestinal specimens, mucosal epithelial cells also may have the potential for significantly enhanced antigen presentation. It has been proposed that cytotoxic T lymphocytes, sensitized by defined antigens in combination with cell surface HLA-DR antigens, could result in an expansion of cells bearing T cell receptor idiotypes with cross-reactive potential for normal tissue components (53). It is possible that only certain HLA-DR antigens may result in generation of cross-reactive cytotoxic T lymphocytes, thus explaining the linkage of certain autoimmune disorders with HLA-DR types. Furthermore, in recent studies, Mayer and Shlien (54) have demonstrated that intestinal epithelial cells, obtained from resected specimens, are capable of processing and presenting antigens *in vitro*.

DR determinants on intestinal epithelial cells that are amplified during the inflammatory process could result in both antilymphocyte and anticolon antibodies. It is, therefore, possible that the antilym-

phocyte antibodies arise as part of a "networking" phenomenon that also involves anticolon antibodies. Formation of anticolon antibodies directed against DR antigens on epithelial cells would lead to antibodies capable of reacting with DR antigens on B cells and monocytes and thereby may modulate immune function. Sensitized T cells capable of recognizing DR antigens on intestinal epithelial cells may be induced and mobilized at the same time. The role of DR antigens in initiating and regulating cell-mediated immune mechanisms in the intestine is a very important area and will receive a great deal of attention in future studies.

V. Cell-Mediated Cytotoxicity

Perlmann and Broberger (55) observed that exposure of fetal human colon cells to peripheral blood MNC from children with ulcerative colitis, but not control subjects, led to target cell death. Watson and co-workers (56) and Shorter and co-workers (57) demonstrated that peripheral blood mononuclear cells from patients with Crohn's disease, as well as ulcerative colitis were capable of killing adult allogeneic human colon epithelial target cells in the absence of complement. Shorter and co-workers (58-60) observed that the cytotoxic effector cells were T cells, that the cytotoxicity diminished 10 days after intestinal resection, that a 4-day incubation with either *E. coli* 0119:B14, LPS, or the IgM fraction of sera from patients with IBD could induce normal peripheral blood MNC to become cytotoxic for colon cells, and that lymphotoxins were released by IBD MNC. Stobo and co-workers (61) showed that the cytotoxic capabilities of UC peripheral blood MNC were present in the non-T, non-B, Fc receptor-bearing class of lymphocytes (K cells). It should be pointed out, however, that controlled and blinded cytotoxicity experiments using peripheral blood MNC from patients with autoimmune disorders, chronic liver diseases, infectious diarrheal illnesses, and other intestinal diseases have not yet been performed. If, indeed, anticolon antibodies are prearming cells, patients without IBD, who have anticolon antibodies (as discussed in Section IV) could have prearmed cytotoxic K cells or, alternatively, could exhibit altered natural killer (NK) activity against colonic epithelial cell targets. Nevertheless, these studies (55-61) led to the proposal that antigen-antibody complexes containing anticolon antibodies arm Fc receptor-bearing K cells in patients with IBD and lead to killing via antibody-dependent cytotoxicity (ADCC) mechanisms.

A number of experimental observations have led investigators to

question cell-mediated cytotoxicity as the primary cause of IBD. First, in contrast to peripheral blood MNC, control and intestinal MNC from patients with IBD are poor mediators of cytotoxicity against cell line targets in either ADCC or spontaneous cell-mediated cytotoxicity (SCMC) assays (62,63). Second, Auer and co-workers (64,65) have demonstrated that peripheral blood MNC from patient's with Crohn's disease have decreased cytotoxic capabilities against noncolonic cell line targets, in both SCMC and ADCC systems and that patients with infectious diarrheal illnesses exhibit similar cytotoxicity defects. Beeken and co-workers (66) also found that cytotoxicity by MNC from Crohn's disease patients was consistently lower than healthy control subjects. Ginsburg and co-workers (67) further demonstrated by cytofluorometry that IBD patients with decreased NK cell activity had normal numbers of circulating NK cells indicating that the decreased NK cell activity is not due to diminished numbers of NK cells. We have observed decreased cytotoxicity by peripheral blood MNC from patients with both active and inactive Crohn's disease and found that peripheral blood cell-mediated cytotoxicity was partially restored after incubation with lectins or interferon in patients with inactive but not in those with active disease (68). These findings (64-68) establish the fact that there is generalized defective K cell and NK cell function in patients with active Crohn's disease.

In comparison with control peripheral blood MNC, we found that intestinal MNC from both control and IBD specimens are relatively poor mediators of both SCMC and ADCC with cell lines as targets (63,68). Although lectins and interferon (62,63,68-71) can induce cell-mediated cytotoxicity by intestinal MNC, IBD intestinal MNC are hyporesponsive to activation by lectins or interferon (68). Our findings with regard to the cytotoxic capabilities of intestinal MNC were one piece of evidence that led us to conclude that lymphocytes in a solid organ compartment, such as the intestine, differ from peripheral blood lymphocytes and have their own unique biological and functional capabilities (63,68,70). Inactive cytotoxic effector cells may serve an important role as a pool of cells that would be able to participate in host mucosal defense mechanisms without nonspecifically causing damage to the surrounding tissue. Intestinal MNC need to be activated to become efficient effector cells (63,68,70). Thus, determination of the physiologically relevant mechanisms that either prevent intestinal MNC from mediating cytotoxic damage *in vivo* or, conversely, enable cytotoxic intestinal MNC activation will be of particular interest for future studies.

Fiocchi and co-workers (72) and Hogan and co-workers (73) have

observed that interleukin 2 (IL-2) will induce control, as well as IBD intestinal MNC to lyse cell line targets. In their studies (72,73), intestinal lamina propria mononuclear cells were found to be unable to mediate spontaneous cell-mediated cytotoxicity and to be relatively devoid of NK cells, but after incubation in culture with IL-2 for 3–4 days, lymphokine-activated killer cells were observed in mononuclear cell preparations isolated from both control and inflammatory bowel disease specimens. In addition to agent-induced activation by lectins, interferon, and IL-2, incubation for 3 days or longer in culture media will also activate intestinal MNC to mediate cytotoxicity (74).

Lymphokine-activated killer activity may be an important, cytotoxic mechanism relevant to intestinal mucosal immunity or antitumor cell control mechanisms. Before lectin or lymphokine-activated cytotoxicity is invoked in the immunopathologic processes of IBD, however, it should be noted that no significant increase in the levels of cytokine or agent-induced cytotoxicity has been observed by IBD intestinal MNC compared with control intestinal MNC (68,72,74). Interestingly, culture supernatants from intestinal MNC from patients with IBD produce decreased levels of IL-2 (75), making it less likely that IL-2 is activating IBD intestinal MNC to mediate cytotoxic damage in IBD. Furthermore, our recent observation (74,76) that sulfasalazine inhibits cell-mediated cytotoxicity by virtue of the effects of sulfapyridine, but not 5-aminosalicylic acid, argues more strongly against, rather than for a role for cytotoxicity as a cause of IBD.

Shanahan and co-workers (77) have demonstrated the presence of discrete subpopulations of cytotoxic effector cells in human intestine and have shown that spontaneous and lymphokine-activated killer cells exist as phenotypically distinct subpopulations. Wilders and co-workers (78,79) have found that increased numbers of “veiled cells” (Langerhan’s cells, dendritic cells) are found in IBD intestinal MNC preparations. These antigen-presenting cells (78) were observed to undergo peripoleosis followed by lysis of the veiled cells by autologous intestinal MNC (79).

Even though general cell-mediated cytotoxic activity is diminished in IBD patients, there is evidence that antigen-specific, sensitized T cells are able to lyse intestinal epithelial cell targets. During the inflammatory process, these cells may be secondarily sensitized by DR antigens or other cell surface components associated with epithelial cells and may then exert primary effector functions. Additionally, the isolation of discrete subpopulations (77) of intestinal mononuclear cells with functionally active cytotoxic NK cells underscores the need for further investigation of cytotoxic effector mechanisms by mucosal

lymphocytes in IBD. Shorter and co-workers (80) have found that intestinal Fc receptor-bearing, trypsin-sensitive MNC from patients with IBD are specifically cytotoxic against autologous epithelial cell targets. Roche and co-workers (81) have observed that IBD lamina propria MNC were able to lyse chicken red blood cells (RBC) coated with antigens isolated from colonic epithelial cells. Characterization of the cytotoxic cells isolated from IBD lamina propria revealed that they were OKT11⁺ and OKT3⁺ and could be depleted by rosetting with sheep RBC (81). The finding that in patients with IBD, specific antigen-sensitized cells are able to lyse intestinal antigen-containing targets (71,80,81), allows reconciliation of the two differing schools of thought concerning cytotoxicity in IBD in that, although there is decreased general cell-mediated cytotoxic activity (62-70), simultaneously there may be specifically sensitized T and/or Fc receptor-bearing cells present in patients with IBD (55-61,71,80,81). However, because these colonic antigen-specific, cytotoxic effector cells may have been sensitized to epithelial cell-associated components during the disease process, it is possible that their generation may be secondary rather than primary in nature.

VI. Immunopathology of IBD

Longstanding IBD is characterized by a mixed cellular infiltrate composed predominantly of B cells and T cells. The B cells are arrayed in follicles or in areas subjacent to ulcerations, while the T cells are found around granulomas, in perifollicular zones, and in deep areas of lesions. IgG-containing cells are increased more than other plasma cell types and are present in deeper tissue layers. Other inflammatory cells present in lesions of IBD include neutrophils, eosinophils, mast cells, and macrophages. The latter are a large component of the granulomas in Crohn's disease. The cellular infiltrate in IBD does not explain the origin of the lesion and may be similar to that found in any longstanding intestinal infection.

Brandtzaeg and co-workers (82) and Baklien and Brandtzaeg (83) demonstrated that the total lymphocyte number was four times greater than normal in intestinal specimens from patients with both ulcerative colitis and Crohn's disease, with the major increase occurring in IgG-containing cells. Compared with normal control specimens, numbers of IgG-containing cells were 30 times greater, while numbers of IgA-containing cells were two times greater and those of IgM-containing cells five times greater than normal. The diffuse, increased infiltration of IgG-containing cells was observed in the submucosa

and the lamina propria of involved ulcerative colitis as well as Crohn's disease specimens. Consistent with this observation, other investigators (84-86) have found results very similar to those of Brandtzaeg with regard to the increased numbers of IgG-, IgA-, and IgM-containing cells in IBD. Van Spreeuwel *et al.* (87) have found that biopsies of patients with acute infectious colitis had markedly increased numbers of IgA and slightly increased numbers of IgM but not IgG-containing cells. In comparison with IBD biopsies, significantly fewer IgG-containing cells were found in biopsies of patients with acute infectious colitis (87). The status of IgG-containing cells in the mucosa (87) may, thus, provide a significant marker in the delineation of differences between IBD and acute infectious colitis. Therefore, in severely inflamed areas of specimens from patients with both ulcerative colitis and Crohn's disease, the major cell type in the mucosal inflammatory infiltrate is IgG-containing B cells (82-86,88,89), whereas in the submucosa of Crohn's disease specimens, T cells constitute the major cell type (82-86,88,89). Studies by Selby and co-workers (90) of the T cell subsets present in IBD lesions demonstrated that T4/T8 ratios are similar to those in control intestinal tissue, leading them to conclude that an imbalance of mucosal immunoregulatory T cells as defined by monoclonal antibodies does not occur in IBD.

Das and co-workers (91) examined ulcerative proctitis mucosa and observed a significant decrease in both secretory component and IgA levels. Engstrom and co-workers (92) investigated a family with ulcerative colitis, and observed that three of four affected family members, plus two of four unrelated patients with ulcerative colitis, had either low levels of or no free secretory component, with normal IgA levels. Therefore, in a subset of patients with IBD, the lack of secretory component (91,92) may allow increased intraluminal destruction of secretory IgA by proteolytic enzymes, which in turn could compromise local host defense mechanisms and lead to repeated entry of bacteria into susceptible mucosa.

A potentially disease-specific IgG κ chain antibody from the colons of patients with ulcerative colitis, termed colitis colon-bound antibody, has been shown to bind primarily to the basement membrane and periphery of colonic epithelial cells in diseased tissue (93). Takahashi and Das (94) have shown that quantities of tissue-bound IgG were significantly higher in patients with UC than in Crohn's or control patients (94) and that the colitis colon-bound IgG recognizes a 40-kDa protein in both IBD and control epithelial cells.

VII. Immunoregulatory Alterations

The intestine contains numerous antigenic substances that are immunogenic, to which the mucosal immune system must respond and mount a protective response. The normal ongoing intestinal immune response must be effective and yet controlled. In IBD, on the other hand, the mucosal immune system may evidence heightened immune responses because of defective or insufficient immunoregulation. It has been proposed by a number of groups that IBD may be caused or influenced by altered regulation of the immune response to antigens and/or agents normally present in the intestinal lumen. Hodgson and co-workers (95) found a loss of normal Con A-induced suppressor cell activity by IBD peripheral blood MNC. In subsequent studies, Victorino and Hodgson (96) demonstrated the lack of both spontaneous and Con A-induced suppressor cell function with no effect due to steroid therapy. Furthermore, loss of suppressor cell function was correlated with increasing activity of disease (96). Auer and co-workers (97) demonstrated a deficiency of suppression of proliferation of autologous lymphocytes in patients with active Crohn's disease, but not in patients whose Crohn's disease was in remission. The status of suppressor cell activity in IBD intestinal MNC has been less clear: increased suppressor cell activity was observed by Fiocchi *et al.* (98), while decreased suppressor cell activity was found by Goodacre and Bienenstock (99).

Ginsburg *et al.* (100) examined the type and quantity of light chain expressed on peripheral blood B cells from patients with IBD and found increased numbers of "monoclonal" lymphocytes, which could represent either clones of proliferating B cells to particular antigens, or an abnormality of immune regulation. Stevens and co-workers demonstrated diminished tetanus-specific immunoglobulin G production *in vitro* by peripheral blood mononuclear cells from patients with Crohn's disease, 7 days after immunization with tetanus toxoid (101), which correlated with a lack of increase in serum antitetanus IgG titers. The majority of IBD patients who did not produce an IgG antitetanus toxoid antibody response also failed to produce an IgG antidiphtheria antibody response after immunization. Therefore, the inability of lymphocytes from Crohn's disease and ulcerative colitis patients to produce normal quantities of antibodies after *in vivo* immunization indicates that secondary humoral immune responses to certain antigens may be decreased in inflammatory bowel disease patients. Stevens *et al.* (101) proposed that in IBD the inability to effectively generate a normal humoral response to potential path-

ogens could lead to persistent infection with chronic inflammation resulting.

Brogan *et al.* have further examined NK cell-mediated cytolytic and antibody suppression capabilities in Crohn's disease patients on and off therapy with 6-mercaptopurine (6-MP) (102). Crohn's disease patients on 6-MP therapy had further reductions in peripheral blood natural killer cell activity against K-562 targets compared to Crohn's disease patients not on 6-MP. Natural killer cell suppression of lymphoblastoid B cell antibody production was likewise decreased in 6-MP-treated patients. In contrast, the *in vivo* generated lymphoblastoid B cell antibody response of Crohn's disease patients on 6-MP therapy was not decreased compared to normal, while Crohn's disease patients not on 6-MP therapy had significantly impaired IgG antitetanus antibody as noted previously by Stevens *et al.* (101). These findings suggest that 6-MP therapy in Crohn's disease effects several lymphoid subpopulations, resulting in (1) decreased natural killer cell cytotoxic activity against K-562 target cells, (2) a decreased natural killer cell ability to suppress lymphoblastoid B cell antibody production, and (3) an improved humoral immune response following tetanus toxoid booster immunization.

Elson *et al.* (103) found that pokeweed mitogen (PWM)-stimulated peripheral blood MNC from Crohn's disease patients secreted normal amounts of IgM. However, in half of the patients studied, *in vitro* culture at optimal ratios of isolated T and B cells in the presence of PWM revealed "covert" suppressor T cells capable of marked IgM suppression (103). Elson and co-workers (104) subsequently described two patients in whom the development of common variable hypogammaglobulinemia followed the development of Crohn's disease; both of these patients had suppressor T cells capable of suppressing normal antibody synthesis and secretion. James and co-workers (105) characterized the covert suppressor T cells in Crohn's disease patients as having a unique (LEU-2a⁺, HNK-1⁺) cell surface phenotype.

James and co-workers examined helper T cell function of lamina propria lymphocytes enriched for T cells and found that T cells from both peripheral blood and lamina propria of Crohn's disease patients were similar to normal peripheral blood T cells in their ability to provide help for IgM synthesis (106). With both Crohn's disease and control patients, intestinal lamina propria T cells provided more help for IgM synthesis than did peripheral blood T cells. When suppressor cell function was assessed, neither peripheral blood nor lamina propria T cells from patients with Crohn's disease or control patients

caused inhibition of IgA synthesis. These results, therefore, differed from their own previous studies (103,105) in which covert suppressor T cells were found in Crohn's disease patients. James and co-workers (106) came to the conclusion that patients with active Crohn's disease have no alteration of immunoregulatory T cell function for polyclonal mitogen-induced immunoglobulin synthesis at the gut mucosal level, despite the presence of an inflammatory process in the intestine.

Elson and co-workers examined the regulatory effects of T cells isolated from the lamina propria of patients with Crohn's disease (107). Helper T cell activity of lamina propria T cells from patients with Crohn's disease was comparable to that of control lamina propria T cells or normal allogeneic peripheral blood T cells (107). Helper T cell activity from intestinal mononuclear cells was not isotype restricted, but was roughly equivalent for IgM, IgG, and IgA and no significant suppressor activity was demonstrable from the lamina propria T cell fractions from either control or Crohn's disease specimens (107). Therefore, similar to the studies of James and co-workers (106), Elson and co-workers (107) also found that lamina propria of control and Crohn's disease intestines contain T cells which are able to exhibit excellent helper function, but do not exhibit suppressor cell capabilities. Furthermore, in contrast to their previous observations (103), Elson and co-workers (107) also did not find covert suppressor cells in the lamina propria of Crohn's disease patients, while James and co-workers (106) did not find covert suppressor cells in either the peripheral blood or the intestine of Crohn's disease patients.

VIII. Antibody Secretion

Recent research has begun to focus on the synthesis and secretion of immunoglobulins by intestinal mononuclear cells, because there are reproducible changes that may be relevant to IBD. It is now evident that in IBD there are major changes in spontaneous immunoglobulin secretion by intestinal mononuclear cells, particularly with regard to IgA and IgG subclasses. There is an increase in cytoplasmic, surface, and secreted antibodies from IBD intestinal lymphocytes, due mainly to enhanced expression and production of IgG (82,83). We have found that immunoglobulin secretion patterns by peripheral blood and intestinal MNC from IBD patients are altered (108-111). Our studies have demonstrated that the peripheral blood mononuclear cells of patients with new-onset, untreated IBD display a strikingly high level of spontaneous synthesis and secretion of IgA (108,110). This pattern of IgA secretion is not unique to IBD; we have also observed it with

peripheral blood MNC of patients with systemic lupus erythematosus and Henoch–Schönlein purpura, as well as with normal human rib bone marrow MNC (109,112,113). Intestinal MNC from control specimens also spontaneously secrete large amounts of IgA, which is suppressed by PWM (108,109). In IBD patients, intestinal MNC exhibit decreased spontaneous IgA secretion, but have increased IgG secretion compared with control intestinal MNC (62,110,111). The changes in antibody secretion observed in IBD could be due to (1) preferential proliferation of subpopulations of cells due to immunoregulatory alterations, (2) switching of the isotype and/or subclass of antibody secreted by the lymphocytes themselves, or (3) changes in the homing and trafficking patterns of the lymphocytes due to the inflammatory process. Our studies have demonstrated that intestinal MNC have unique capabilities and differ in their functional characteristics when compared to peripheral blood MNC (63,68,70,108,109). We have, therefore, focused our studies on spontaneous antibody secretion by intestinal MNC, in particular. Furthermore, because PWM nonspecifically causes suppression of activated B cells (108–113), we feel studies utilizing PWM should be interpreted with great caution. Spontaneous antibody secretion *in vitro* may more closely reflect *in vivo* events than does overt, nonspecific PWM-induced suppression.

In comparison with control peripheral blood MNC (0.4 $\mu\text{g/ml}$), isolated control intestinal lamina propria MNC exhibit very high spontaneous secretion of IgA (25.1 $\mu\text{g/ml}$) (110). Compared to control intestinal MNC, secretion of IgA by ulcerative colitis intestinal MNC (9.0 $\mu\text{g/ml}$) and Crohn's disease intestinal MNC (7.4 $\mu\text{g/ml}$) is decreased (110). The IgA spontaneously secreted by human bone marrow MNC is predominantly monomeric (81%), while control peripheral blood MNC secrete 54% of their IgA in the monomeric form (110). Control intestinal MNC secrete predominantly dimeric IgA, with 31% of their IgA being monomeric IgA (110). Intestinal MNC from IBD specimens exhibit an increase in the proportion of IgA secreted as monomeric IgA, with 43% of IgA from Crohn's disease intestinal MNC and 53% of IgA from ulcerative colitis intestinal MNC being monomeric (110). IgA₁ is the predominant form of IgA secreted by human bone marrow (85%) and control peripheral blood MNC (88%), while control intestinal MNC secrete 61% of their IgA as IgA₁ (110). Upon examination of Crohn's disease specimens, intestinal lamina propria MNC were found to secrete 71% of their IgA as IgA₁, and intestinal MNC from ulcerative colitis specimens were found to secrete 74% of their IgA as IgA₁ (110). These changes are consistent

with the migration of monomeric IgA and IgA subclass 1-secreting cells from the peripheral blood into the diseased intestine and, as such, could represent a normal mucosal response to intestinal antigens or pathogens. It is also possible that local dimeric IgA and IgA₂ deficiency in diseased bowel could lead to local or homing B cells to secrete more monomeric IgA and IgA₁, either as a primary or secondary defense mechanism.

The increased spontaneous IgA secretion by control intestinal MNC is consistent with the intestinal lamina propria being a major source of IgA plasma cells which secrete dimeric IgA with J chain. Our findings (110) support the findings of Kutteh and co-workers (114,115), who demonstrated that human intestinal MNC secrete primarily dimeric IgA, that human bone marrow MNC secrete predominantly monomeric IgA, and that human peripheral blood MNC secrete large amounts of IgA₁. IgA has two subclasses: IgA₁ and IgA₂. IgA₁ is the major subclass in the serum (79–82%), while IgA₂ is significantly increased in secretions (26–41% IgA₂ in secretions compared to 18–21% in serum) (116). Our findings (110), therefore, are consistent with previous observations (114,115) in that peripheral blood MNC secreted 88% of its IgA as IgA₁, while isolated intestinal MNC secreted 61% of its IgA as IgA₁.

Control intestinal MNC spontaneously secrete more IgG (3.0 $\mu\text{g/ml}$) than do control peripheral blood MNC (0.4 $\mu\text{g/ml}$) (111). When compared with control intestinal MNC, a further increase in spontaneous secretion of IgG is observed with IBD MNC (111). The most marked alteration in IgG synthesis is seen with ulcerative colitis intestinal MNC which exhibited a significant ($p < 0.01$) increase in spontaneous IgG secretion (15.7 $\mu\text{g/ml}$) in comparison with control intestinal MNC (111). Crohn's disease intestinal MNC have moderately increased spontaneous secretion of IgG (7.6 $\mu\text{g/ml}$) (111). Examination of IgG subclass secretion (111) has demonstrated that control intestinal MNC synthesize and secrete appreciable amounts of IgG₁ (1.8 $\mu\text{g/ml}$) and IgG₂ (1.3 $\mu\text{g/ml}$), and modest amounts of IgG₃ (0.2 $\mu\text{g/ml}$). The most marked change in IgG subclass secretion (111) is with ulcerative colitis intestinal MNC which spontaneously secrete large amounts of IgG₁ (13.4 $\mu\text{g/ml}$: 7.5 times that of control intestinal MNC, $p < 0.001$, and 3.7 times that of Crohn's disease intestinal MNC, $p < 0.05$). IgG₂ secretion by ulcerative colitis intestinal MNC (1.9 $\mu\text{g/ml}$) is not significantly different from control intestinal MNC, while IgG₃ secretion (0.8 $\mu\text{g/ml}$) demonstrates a 4-fold increase over control ($p < 0.01$). Crohn's disease intestinal MNC samples exhibit a different pattern (111), with increased IgG secretion involving all

three subclasses measured, with the greatest increase in IgG₂. Crohn's disease intestinal MNC IgG₁ secretion (3.6 $\mu\text{g/ml}$) is increased 2-fold over control, IgG₂ secretion (2.9 $\mu\text{g/ml}$) is increased 2.2 times that of the control value, and 1.5 times that of UC IgG₂ secretion, and IgG₃ secretion (0.5 $\mu\text{g/ml}$) is increased 2.5 times that of control (111). Therefore, there are marked alterations in the secretion of IgG subclasses in IBD patients. Ulcerative colitis intestinal B cells secrete enormously increased amounts of IgG₁ in comparison to intestinal mononuclear cells from healthy subjects. Spontaneous secretion of IgG₃ is also somewhat heightened, whereas IgG₂ is not elevated. In patients with Crohn's disease, on the other hand, intestinal B cells show a moderate increase in secretion of all IgG subclasses, particularly IgG₂.

That IBD MNC should exhibit increased IgG and IgG subclass secretion *in vitro* is not surprising in the light of previous observations (82,83) of increased numbers and altered ratios of intestinal lymphocytes and plasma cells in IBD. The increased secretion of IgG and IgG subclasses from IBD intestinal MNC, therefore, is most likely related to the increased percentage of IgG-containing cells present *in vivo* in inflamed mucosa. The 4-fold increase in total intestinal lymphocytes in IBD with only a 2-fold increase in IgA-containing cells could produce a dilutional effect leading to the apparent decrease in total IgA secretion that we have observed. However, Danis and co-workers (117) investigated intestinal mucosal immunoglobulin secretion by culturing colonoscopic biopsies and observed significant reductions in IgA secretion from both ulcerative colitis and Crohn's disease patients. These results (117), therefore, are consistent with our findings in that we also have found (108–110) decreased secretion of IgA from both CD and UC intestinal MNC, in comparison to control intestinal MNC.

The clinical importance of IgG subclasses and their regulation is demonstrated by reports of selectively altered subclass levels in immunodeficiencies and autoimmune diseases (118–123). Serum levels of IgG₁ are elevated in patients with rheumatoid arthritis, systemic lupus erythematosus, and other immune complex diseases. Therefore, because elevated levels of serum IgG₁ have been associated with a number of potentially autoimmune disorders, it could be speculated that IgG₁ contributes locally to tissue autoimmune events in patients with ulcerative colitis. Different antigens and mitogens induce antibody responses restricted to particular IgG subclasses in both murine and human systems (119,124–127). Recent studies by Scott and Nahm (128) have shown human IgG₁ and IgG₃ to be

preferentially and coordinately stimulated in *in vitro* responses to mitogens, which may in part account for the elevation of IgG₃ secretion in conjunction with the increase in IgG₁ secretion by UC intestinal MNC. IgG subclasses are not randomly expressed. Subclass expression is complex and depends on the nature of the antigenic signal, as well as on regulatory factors and B cell subpopulations. The finding of differences between Crohn's disease and ulcerative colitis in the distribution of IgG subclasses secreted by intestinal mononuclear cells also is of particular interest. IgG₁ and IgG₃ antibodies account for the predominant IgG response to proteins and T cell-dependent antigens. Both IgG₁ and IgG₃ are better complement pathways activators and opsonins than IgG₂ and IgG₄. Delineation of the stimuli and antigens which induce the increased secretion of IgG subclasses in IBD may provide insight into possible etiologic and immunopathogenic aspects. The inducing signal in UC might preferentially induce IgG₁, leading to the production of IgG₁ antibodies capable of complement fixation and chemotaxis of accessory cells to the pathologic site. It would be useful to determine which IgG subclass constitutes the anticolon and antilymphocyte antibodies in patients with ulcerative colitis.

IgG₂ provides the predominant IgG response to carbohydrates and many bacterial antigens. IgG₂ and IgG₄ deficiencies have been detected in patients with recurrent bacterial infections (e.g., otitis media, pneumococcal respiratory tract infections, pericarditis, meningococcal infections) and in some inherited immunodeficiency disorders (such as ataxia telangiectasia and Wiskott-Aldrich syndrome). In patients with Crohn's disease, the moderate elevations of all IgG subclasses, and of IgG₂ in particular, are probably induced by an infectious agent. The increased production of IgG₂ would then be an appropriate immune response that might partially contain, but not eradicate, a chronic infectious process.

Therefore, our studies (63,68,70,108-111) have demonstrated that (1) major alterations occur with regard to spontaneous antibody secretion in IBD, (2) PWM-induced suppressor cell generation is a nonspecific phenomenon, (3) the peripheral blood compartment reflects mainly secondary phenomena in IBD, (4) intestinal MNC comprise a unique immunologic compartment with separate immunobiologic capabilities, and (5) it is within the intestine involved with disease that major alterations in antibody secretion are occurring, particularly with regard to the IgA and IgG subclasses.

The finding of increased secretion of total IgG by IBD intestinal mononuclear cells—with differences between Crohn's disease and

ulcerative colitis in the secretion of IgG subclasses—has a number of significant implications. Traditionally, IgA has been viewed as the major protective mucosal immunoglobulin of the intestine. Now it appears that, for inflammatory bowel disease at least, IgG and its subclasses also need to be carefully examined. This is not surprising, if the effector capabilities of the two immunoglobulins are examined. IgA mounts its defense in a relatively nonspecific manner; it adheres to a foreign antigen or substance and prevents bacteria from colonizing and toxins or antigens from being absorbed. IgG, in contrast, has more aggressive capabilities: it can activate complement components, initiate phagocytosis, and induce cell-mediated cytotoxicity. It therefore makes sense that IgG, rather than IgA, may be particularly involved in IBD, either by mounting a normal defense mechanism or by abnormal destruction of normal bowel components.

On the basis of our studies of IgA and IgG secretion and synthesis, we postulate the following sequence of events (Table II). In the inflamed intestine, the constant stimulation of the secretory IgA system results in the priming and activation of B cells for accelerated production of IgA₁ and polymeric IgA. In IBD patients, a break in the mucosal barrier and/or failure of IgA₂ and dimeric IgA to control pathogens result in increased synthesis and secretion of IgG—with a preferential increase of IgG₁ and IgG₃ in ulcerative colitis and an increase of all IgG subclasses, particularly IgG₂, in Crohn's disease. IgG antibodies, either by autoimmune cross-reactivity or local immune complex formation, may contribute in a primary or secondary

TABLE II
INTERPRETATIONS OF OBSERVED ALTERATIONS OF ANTIBODY SECRETION IN IBD

Observations

Total IgA secretion is decreased and total IgG secretion is increased by isolated intestinal MNC in both ulcerative colitis (UC) and Crohn's disease (CD)
The percentages of IgA₁ and monomeric IgA are increased in both UC and CD
IgG₁ levels are markedly elevated in UC with a moderate increase in IgG₃; IgG₂ levels are moderately elevated in CD with a slight increase in IgG₁ and IgG₃

Interpretations

Mucosal defense deficiency due to decreased dimeric IgA and IgA₂ may occur in both UC and CD
In both UC and CD increased monomeric IgA and IgA₁ secretion may occur due to homing of B cells from the peripheral blood as part of a compensatory protective mechanism to make up for the loss of normal "gatekeeper" mucosal IgA function
In UC an IgG₁-mediated immune response and in CD an IgG₂-mediated immune response may result in either a normal immune protective response to specific agents or destructive immune effector responses directed against intestinal cells

fashion to the pathogenesis of the disease. It is, therefore, likely that altered secretion patterns of IgA and IgG (and their subclasses) reflect a normal mucosal response to infectious agents or stimulatory molecules, which augment or perpetuate the immune response. Identification of the antigens against which antibodies of IgG and IgA subclasses are directed in IBD may provide important information regarding the altered immunobiology that underlies ulcerative colitis and Crohn's disease.

IX. Granulocyte Function

Inflammatory bowel disease has long been viewed as a disorder of chronic inflammation—a view that is reinforced by the large numbers of lymphocytes and histiocytes in the diseased mucosa and submucosa. It recently became apparent that the disease also has histologic characteristics of acute inflammation: there is intense infiltration of the mucosa and submucosa with neutrophils. Large numbers of neutrophils leave the bloodstream and enter the inflamed mucosa and submucosa of the bowel. Some neutrophils migrate across the epithelium into the lumen and are passed in the stool, while others are destroyed in the inflamed tissue before they have a chance to migrate into the lumen. This constant flux of neutrophils implies the presence of chemotactic agents in the inflamed mucosa. As markers of acute inflammation, the abundant neutrophils indicate that there is a large acute component to inflammatory bowel disease that coexists with a chronic disease state. Investigators have examined polymorphonuclear leukocyte (PMN) function, including migration, chemotaxis, adherence, and phagocytosis, in IBD. Rhodes *et al.* (129) demonstrated decreased random migration and decreased chemotaxis of normal peripheral blood leukocytes exposed *in vitro* to drugs frequently used in the treatment of IBD (prednisone, sulfasalazine, and 5-aminosalicylic acid). By the skin window chamber technique, random migration of PMNs was found to be suppressed in Crohn's disease *in vivo* (130). Inhibitors directed against both chemotactic factors and leukocytes in sera from patients with Crohn's disease and ulcerative colitis were described by Rhodes *et al.* (131).

On the other hand, chemotaxis induced by zymosan-activated serum or casein has been found to be normal in patients with IBD, and phagocytosis by PMNs from patients with IBD has been shown to be no different from control subjects (130–133). Furthermore, Saverymattu *et al.* have recently reported studies on the *in vivo* assessment of granulocyte migration to diseased bowel (134,135).

Patient's peripheral blood granulocytes were isolated *in vitro* and radiolabeled by incubation with ^{111}In tropolonate. The radiolabeled granulocytes were injected intravenously with the patient positioned beneath a gamma camera. In 20 of 22 Crohn's disease patients the radiolabeled granulocytes accumulated rapidly in the inflamed bowel (134). A similar study done in ulcerative colitis showed no delayed migration in any of the 15 patients tested (135). In addition to establishing the absence of a defect of migration in IBD, these studies emphasize the importance of granulocytes in the mediation of inflammation in IBD (134,135).

X. Products of the Complement Pathway

Although complement activation is one of the major effector mechanisms of the immune system, the role of complement pathway products as soluble mediators of inflammation has received surprisingly little study in the context of IBD. Investigators have proposed pathogenic roles for some of those molecules, but the nature and extent of their involvement in the inflammatory response remain largely undefined. Some of the disease's clinical and histological changes are consistent with the biological activities of C3b and C5a, but the scant data obtained in studies to date are conflicting. A number of investigators have proposed roles for products of the complement pathway in the pathogenesis of IBD, but the nature and extent of their roles remain largely undefined. Two products of the complement pathway that may play roles in the inflammatory response in IBD are C3b and C5a. Opsonization by complement leading to particle coating with C3b is an important step in phagocytosis, enabling the necessary contact between phagocyte (neutrophil or macrophage) and particle through specific cellular receptors. Phagocytosis of bacteria and cellular debris occurs at an accelerated rate in intestinal inflammation, irrespective of the cause. Abnormalities in phagocytosis could influence the intensity of the inflammatory response. C5a, like LTB₄, is a potent chemotactic agent for neutrophils and increases vascular permeability (136). The large numbers of neutrophils in IBD mucosa suggest the presence of a chemotactic agent in IBD and the presence of mucosal edema suggests the presence of a soluble mediator that enhances vascular permeability. Thus, some of the functional and histologic changes seen in IBD are consistent with the biologic activities of C3b and C5a.

The attempts to define the role of the complement system in IBD have focused on the levels of complement components in the circulation and their rates of turnover (137). Several studies have found normal levels of C3 and C4 in the circulation in IBD. Incubation of serum with zymosan or nylon fibers activates the alternative pathway with production of C3b and C5a. The state of activation of the alternative pathway in IBD has variously been described as decreased, normal, and increased. In support of decreased activation of the alternative pathway is a study in which depression of properidin and properidin convertase along with diminished consumption of C3-C9 after reaction with cobra venom were seen in sera from patients with Crohn's disease and ulcerative colitis (137). In another study supporting decreased activation of the alternative pathway in IBD, sera from normals and patients with Crohn's disease was incubated with zymosan (138) or nylon fibers (139) and the amount of C5a-related chemoattractant activity was assayed. C5a activity was diminished in Crohn's sera compared to normals. There was also decreased consumption of the major complement component C3 (139). The generation of C5a was more markedly reduced in Crohn's disease patients treated with steroids (138).

In a similar study with different results (139), the alternative pathway was activated by incubation of serum with zymosan and the opsonization of zymosan with C3b was quantitated. Binding of C3b to zymosan was greater in the Crohn's sera than in normals, whereas, consumption of C3, the precursor of C3b, was similar in the two groups (139). Thus, this study (139) suggests that the activation of the alternative pathway in Crohn's disease is normal but the degradation of C3b is impaired.

Finally, there are two studies indicating an increased level of activation of the complement pathways in IBD (140,141). In one (140), radioiodinated C3 was injected intravenously into normals and patients with IBD. Both the synthesis and catabolism of C3 were enhanced in patients with Crohn's disease and ulcerative colitis, suggesting an enhanced state of activation (141). In the second study, the levels of a C3 split product, C3c, were assayed in plasma from normals and untreated outpatients with Crohn's disease and ulcerative colitis (142). The levels of C3c in the Crohn's patients were 10-fold greater than those in the normals or ulcerative colitis patients. C3c levels did not correlate with disease activity in Crohn's disease. Elevated levels of C3c in Crohn's disease suggest hypercatabolism of C3 and activation of the complement cascade.

The apparently conflicting results of these various studies of the state of activation of the complement pathway may be due to different methods of activation and different assay techniques. Unfortunately, these studies, taken either individually or collectively, do not give a clear insight into the role of the complement pathways in the pathogenesis of inflammation in IBD.

XI. Prostaglandins and Leukotrienes

Some of the functional and macroscopic changes seen in IBD, including mucosal hyperemia and edema, are typical of changes seen in any inflammatory state, no matter what organ system is involved. These changes are the products of soluble mediators released in the process of inflammation (Table III). They cause tissue edema by increasing vascular (postcapillary venule) permeability to albumin and other macromolecules; hyperemia results from mediators that induce vasodilation. Other functional changes (including diminished salt and water absorption) that are characteristic of intestinal inflammation are probably also the result of soluble mediators, but their pathogenesis is less clear. Progress has been made in the characterization of the soluble mediators of inflammation and their role in the amplification of the immune response in IBD. Although study of mediators of inflammation is not likely to provide any insight into the events that initiate the disease, there are two reasons for defining their

TABLE III
INFLAMMATORY MEDIATORS IN IBD

Mediator	Origin	Biologic Effects
LTB ₄	Neutrophils	Neutrophil chemotaxis, increased vascular permeability
LTC ₄ , LTD ₄ , LTE ₄	Macrophages, mast cells	Smooth muscle contraction, increased vascular permeability
PGE ₂	Macrophages, epithelial cells	Vasodilation, increased vascular permeability, enhanced salt and water secretion
C5a	Antigen/antibody activation	Neutrophil chemotaxis, increased vascular permeability

pathogenic role in IBD. First, the soluble mediators appear to be largely responsible for the clinical and histologic changes seen in the disease. Second, the drugs that have proven to be beneficial for ulcerative colitis and Crohn's disease appear to exert their therapeutic effect by blocking the synthesis of these mediators. As will be expanded upon below, corticosteroids and sulfasalazine block the synthesis of prostaglandins and leukotrienes *in vivo* and *in vitro* (143,144). Furthermore, until the etiologic agents of IBD are identified, it is likely that advances in medical therapy for IBD will be in the area of regulation of the synthesis of soluble mediators of inflammation.

There are several groups of soluble mediators whose role in IBD is totally, or almost totally, unexplored. Among these soluble mediators are the kinins, the vasoactive amines (e.g., histamine and serotonin), and platelet-activating factor. There are, however, two groups of soluble mediators whose role in the pathogenesis of IBD have now been receiving considerable attention: prostaglandins and leukotrienes.

Arachidonic acid is metabolized through the cyclooxygenase pathway to prostacyclin, thromboxanes, and prostaglandins. Prostaglandins are produced by almost all mammalian cells, including intestinal epithelium and cells associated with inflammatory events (i.e., mast cells, macrophages, and platelets) (1,2). Prostaglandins, particularly those of the E series, have biologic properties that are proinflammatory (i.e., enhanced vascular permeability, vasodilation, and production of pain) (1,2). Prostaglandins also have specific functional effects on the intestine. They induce mucosal secretion of water and electrolytes in the small intestine (145). The effects of prostaglandins on secretory function in the colon are less well defined; pharmacologic doses may impair salt and water transport in the colon but physiologic doses have little effect. (146).

There is no question that IBD is associated with increased levels of prostaglandin production. Elevated levels of prostaglandins (primarily PGE₂) are found in the stool, venous blood, and rectal mucosa in IBD (147-149). Elevated levels of prostaglandin metabolites are found in the urine (149). When incubated *in vitro*, pieces of rectal mucosa from patients with ulcerative colitis synthesize increased amounts of PGE₂ and thromboxane B₂ (147). An *in vivo* estimate of prostaglandin synthesis by rectal mucosa is achieved by use of bags of dialysis tubing, which are filled with buffer and placed in the patient's rectum. After a period of hours, the bag is removed and the concentra-

tion of prostaglandins in the bag measured by bioassay or radioimmunoassay (150). Studies using this technique reveal elevated levels of PGE₂ in the rectal dialysates of patients with ulcerative colitis. Prostaglandin levels in IBD, whether in mucosa, serum, or rectal dialysate, correlate with disease activity and successful medical management results in a reduction in prostaglandin levels. In inactive UC, prostaglandin levels are not significantly different from normal controls (151).

The source of the prostaglandins in colonic mucosa in IBD is not well defined. Both epithelial cells and mononuclear inflammatory cells produce prostaglandins. Definition of relative rates of synthesis by epithelial and mononuclear cells requires physical separation of the cell types with all the attendant artifacts of the separation system. Despite these difficulties, it appears that intestinal mononuclear cells are responsible for as much, or more, prostaglandin synthesis as are intestinal epithelial cells (152). This finding is consistent with studies of inflammation in other organ systems in which inflammatory cells were found to be the major source of prostaglandin synthesis (1,2).

IBD is clearly associated with elevated prostaglandin levels and enhanced prostaglandin synthesis. Furthermore, prostaglandins have proinflammatory biological properties that could account for some of the histologic and functional changes seen in IBD. Alternatively enhanced prostaglandin synthesis in IBD may be a nonspecific product of intestinal inflammation that has little to do with the pathogenesis of the disease. Some insight into this question comes from studies of the effects of drug therapy on prostaglandin levels in IBD. Sulfasalazine and its cleavage product, 5-aminosalicylate, inhibit cyclooxygenase *in vitro* (147). Moreover, when patients are treated with sulfasalazine, prostaglandin synthesis in the rectal mucosa diminishes. Corticosteroids induce the synthesis of a protein termed lipomodulin, which inhibits phospholipase A₂ (143). The rate-limiting step in the synthesis of prostaglandins and other arachidonic acid metabolites is the release of arachidonate from phospholipids by phospholipase A₂. Inhibition of phospholipase A₂ synthesis by lipomodulin results in diminished synthesis of prostaglandins. Measurement of PGE₂ levels in rectal dialysates from patients with UC reveals that even a brief course of therapy with oral prednisolone results in a marked decrease in PGE₂ production (150). Thus, data from both *in vitro* and *in vivo* studies with sulfasalazine and corticosteroids are consistent with the suggestion that prostaglandins are major mediators of inflammation in IBD and, moreover, that the therapeutic actions of sulfasalazine and corticosteroids are achieved

by inhibition of prostaglandin synthesis. However, there is substantial evidence against a significant role for prostaglandins as mediators of inflammation in IBD. This evidence comes from a few small clinical studies of nonsteroidal antiinflammatory drugs (e.g., NSAIDs), particularly indomethacin, in IBD (149,153,154). Indomethacin and other NSAIDs are potent inhibitors of cyclooxygenase. Use of these drugs in rheumatoid arthritis and other inflammatory diseases results in both decreased prostaglandin synthesis and diminished clinical activity. It is this clinical improvement in response to treatment with NSAIDs that helped establish a role for prostaglandins as important mediators of inflammation in rheumatoid arthritis. In contrast to their usefulness in rheumatoid arthritis, NSAIDs have no role in the medical management of IBD. Small trials of indomethacin administered orally (149) and rectally (153,154) revealed no improvement in ulcerative colitis. There is even some suggestion that indomethacin may cause clinical deterioration in ulcerative colitis, despite causing a decrease in prostaglandin production (149,154). Another NSAID, flurbiprofen, has also been shown to cause clinical deterioration in ulcerative colitis (155,156).

The failure of NSAIDs to induce clinical improvement in IBD, despite their inhibition of prostaglandin production, suggests that prostaglandins do not play important roles as mediators of inflammation in IBD. Moreover, these studies suggest that corticosteroids and sulfasalazine exert their therapeutic effects in IBD by a mechanism other than inhibition of prostaglandin synthesis. One interpretation of these data is that the elevated levels of prostaglandins in IBD are merely a nonspecific marker of inflammation, an epiphenomenon that has nothing to do with the pathogenesis of IBD.

Attention has now turned to lipoxygenase products. The cyclooxygenase pathway is present in effectively all mammalian cells but the 5-lipoxygenase pathway exists primarily in cells of bone marrow origin involved in the inflammatory process (i.e., mast cells, neutrophils, monocytes, and macrophages) (157). To date, there has been no evidence for the presence of the 5-lipoxygenase pathway in the epithelial cells of the intestine or colon. The major products of the 5-lipoxygenase pathway are 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and leukotrienes B₄, C₄, D₄, and E₄ (LTB₄, LTC₄, LTD₄, and LTE₄). Neutrophils metabolize arachidonate to 5-HETE and LTB₄ (151). The sulfidoleukotrienes (LTC₄, LTD₄, and LTE₄), along with 5-HETE and LTB₄, are products of mast cells and macrophages (157). LTB₄ and, to a lesser extent, 5-HETE are potent chemotactic agents for neutrophils. LTB₄, in the presence of neutrophils, also

induces enhanced vascular permeability. The sulfidoleukotrienes induce smooth muscle contraction in the lung, blood vessels, and the gastrointestinal tract (2, 157).

The lipoxygenase pathway was described much more recently than the cyclooxygenase pathway (158). As a result, there are fewer studies on the role of lipoxygenase products in IBD than on the role of prostaglandins. The studies on the role of the lipoxygenase pathway in IBD have focused on LTB₄. Normal colonic mucosa is not known to have an active lipoxygenase system and appears to make few or no leukotrienes. However, the observation of large numbers of neutrophils in the inflamed mucosa of IBD implies the presence of chemotactic factors that induce neutrophils to migrate out of the circulation into intestinal tissue. Leukotriene B₄ is present in IBD mucosa at concentrations high enough to activate neutrophils and induce chemotaxis. Even though leukotriene B₄ is a potent chemoattractant for neutrophils, other chemotactic agents, such as C5a (a product of the complement pathway), also are likely to be present in diseased mucosa. However, recent investigations point to leukotriene B₄ as a major mediator of neutrophil chemotaxis in both ulcerative colitis and Crohn's disease. Incubation of IBD mucosa with radiolabeled arachidonic acid results in the synthesis of large quantities of LTB₄ and 5-HETE and smaller quantities of PGE₂ and thromboxane B₂ (159). IBD mucosa produces larger quantities of lipoxygenase and cyclooxygenase products than normal mucosa. Patterns of arachidonate metabolism are similar for ulcerative colitis and Crohn's disease. Lipid extracts of IBD mucosa contain large amounts of LTB₄: 250 ng/g of IBD mucosa as compared to less than 5 ng/g of normal mucosa (156). A concentration of 250 ng/g of mucosa is the equivalent of a solution of 5×10^{-7} M, a concentration well within a range of biologic activity of LTB₄.

The results of this *in vitro* study were confirmed by an *in vivo* study using rectal dialysis. Lauritsen and co-workers measured the levels of PGE₂ and LTB₄ in rectal dialysates from normals and patients with ulcerative colitis. Levels of both PGE₂ and LTB₄ were markedly higher in rectal dialysates from the UC patients (150). Moreover, treatment of UC patients with a short course of prednisolone resulted in a decline in PGE₂ and LTB₄ almost to normal levels.

The presence of large numbers of neutrophils in IBD mucosa suggests that there is a chemotactic factor (or factors) present in IBD mucosa that induces neutrophils to migrate out of the circulation and into the tissue. LTB₄ is a potent chemoattractant for human neutrophils (160); there are, however, other chemotactic compounds that are likely to be present in IBD mucosa. Among these compounds are C5a,

a product of the complement pathway (161), and formylmethionylleucylphenylalanine (FMLP), a product of *E. coli* (162). In an attempt to sort out the contributions of various chemotactic factors to neutrophil infiltration in IBD, a study was done using homogenized IBD mucosa as the chemotactic stimulus for ^{51}Cr -labeled neutrophils in a Boyden chamber (163). IBD mucosa had far more chemotactic activity than normal mucosa. Moreover, lipid extracts of the IBD mucosa had 65–90% of the chemotactic activity of the whole mucosa, indicating that among the compounds contributing to the neutrophil chemotactic activity in IBD mucosa are one or more lipids. The lipid extract from IBD mucosa was fractionated by reverse-phase HPLC and the fractions assayed for chemotactic activity. The only fraction with significant chemotactic activity was the fraction coeluting with LTB_4 . Moreover, the chemotactic activity in the lipid extract of IBD mucosa was totally ablated with antiserum raised against LTB_4 . These data indicate that LTB_4 is a major neutrophil chemotactic agent in IBD mucosa. If the findings using the *in vitro* assay for neutrophil chemotaxis in the Boyden chamber can be extrapolated to the *in vivo* situation, one could argue that LTB_4 is the mediator that is primarily responsible for neutrophils leaving the circulation and entering the intestinal tissue in IBD. The generation of LTB_4 is certainly not the initiating event in IBD, nor is it specific for IBD. However, inhibition of the process of neutrophil migration into the mucosa and submucosa in IBD would diminish the intensity of the inflammatory response and, in turn, the degree of clinical disease activity. In this case, drugs that inhibit LTB_4 synthesis, or block LTB_4 binding to neutrophil receptors, or impair neutrophil responsiveness to LTB_4 might be expected to diminish the inflammatory response and disease activity in IBD.

Although the role of LTB_4 in IBD has been the most extensively studied there are preliminary reports of a possible role for the sulfidoleukotrienes (LTC_4 , LTD_4 , LTE_4). Colonic mucosa obtained at surgery was chopped and incubated in the presence and absence of the calcium ionophore, A23187. The sulfidoleukotrienes released into the media were assayed by radioimmunoassay. Crohn's colitis mucosa produced three times as much sulfidoleukotrienes as normal mucosa in both the stimulated and unstimulated conditions (164). The predominant sulfidoleukotriene found was LTE_4 , suggesting that released LTC_4 is rapidly degraded by peptidases. Sulfidoleukotrienes cause smooth muscle contraction, vasoconstriction, plasma exudation, and mucus release. Each of these biologic effects could be relevant to IBD: for example, effects on smooth muscle may be associated with increased intestinal motility.

XII. Immunopharmacology

The medications used to treat ulcerative colitis and Crohn's disease most likely exert their effects through the immune system (Fig. 2). Although drug therapy helps to control many of the signs and symptoms of inflammatory bowel disease, it is not curative. Corticosteroids have been the mainstay of therapy, markedly improving symptoms in the acute phases of the disease, but their long-term use can cause significant complications. Sulfasalazine, either alone or in combination with steroids, is beneficial for mild Crohn's colitis and is useful for inducing and maintaining remissions in ulcerative colitis. For the most part, the effects of these drugs on arachidonate metabo-

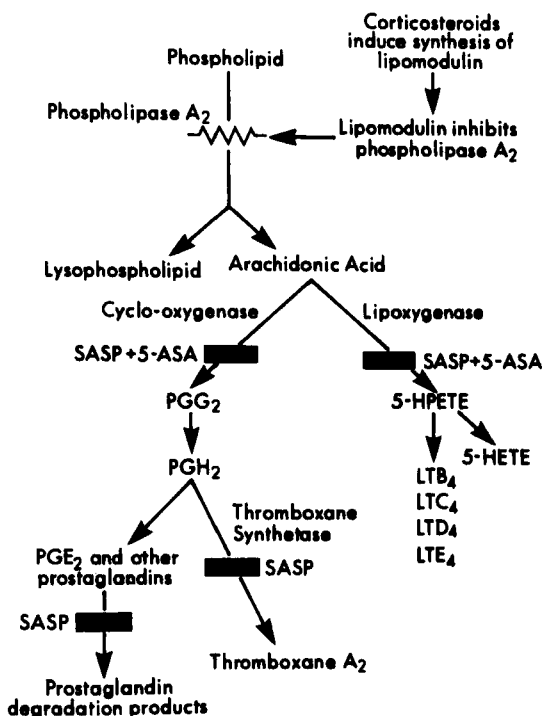


FIG. 2. Immunopharmacology of antiinflammatory therapeutic agents commonly used in IBD. Corticosteroids and sulfasalazine, two of the principal medications found useful for IBD therapy, probably exert their effects through inhibition of arachidonic acid metabolism. Corticosteroids, via lipomodulin, inhibit arachidonic acid release. Sulfasalazine (SASP) and its cleavage product, 5-aminosalicylate (5-ASA), interfere with prostaglandin production by blocking the cyclooxygenase pathway; they also block the lipoxygenase pathway, thus inhibiting the synthesis of leukotriene B₄ by neutrophils in the inflamed colon.

lism have been studied *in vitro* and many of these findings may not carry over into *in vivo* situations. As mentioned above, both sulfasalazine and 5-aminosalicylic acid (5-ASA) inhibit cyclooxygenase and, thus, prostaglandin synthesis. The ID_{50} for cyclooxygenase is about 1 mM for both compounds. In addition, sulfasalazine, but not 5-ASA, inhibits thromboxane synthetase in both platelets (165) and colon. Sulfasalazine has also been shown to inhibit 5-lipoxygenase and LTB_4 synthesis in human peripheral blood neutrophils and in inflamed colon (166). The ID_{50} of sulfasalazine for neutrophil 5-lipoxygenase is about 1 mM. 5-ASA also has an effect on the 5-lipoxygenase pathway, but the nature of that effect has not yet been elucidated (166).

Defining the mechanism of action of sulfasalazine is a particular problem. The stool concentrations of sulfasalazine and its cleavage product, 5-ASA, are extraordinarily high—i.e., 2×10^{-3} and 9×10^{-3} M, respectively. However, the serum concentration of sulfasalazine is 5×10^{-5} M and that of 5-ASA is 7×10^{-6} M (144). Thus, the colonic epithelial cells are exposed to a high drug concentration on the luminal surface and a much lower concentration on the basolateral surface. The concentrations of these drugs in the inflamed mucosa in IBD is not known. This is an important issue in that the inhibitory effects of sulfasalazine and 5-ASA on arachidonate metabolism described *in vitro* occur at concentrations around 1 mM, which is well within the range of concentrations in the colonic lumen but far higher than the concentrations in the circulation. Whether or not these concentrations are achieved in the inflamed mucosa is not known. Another difficulty in defining the mechanisms of action of sulfasalazine in IBD is the large number of *in vitro* effects of sulfasalazine that might be related to its therapeutic efficacy. The effects of sulfasalazine on arachidonate are enumerated above; however, sulfasalazine also has *in vitro* effects unrelated to arachidonate metabolism, including inhibition of chemotactic peptide binding to neutrophils (167), inhibition of neutrophil chemotaxis, and inhibition of NK cell cytotoxicity (74,76). Any or all of these biologic properties may contribute to the therapeutic efficacy of sulfasalazine.

The effects of sulfasalazine on NK cell cytotoxicity are of particular interest in view of the proposed role for cytotoxicity in the pathogenesis of IBD. Sulfasalazine and sulfapyridine inhibit spontaneous cell-mediated cytotoxicity by control and IBD peripheral blood MNC and intestinal MNC (74). Lectin-induced, antibody-dependent, and interleukin 2-induced cytotoxicity were all unaffected by sulfasalazine. Further studies investigated which step in the process of NK cytotoxicity is inhibited by sulfasalazine. These studies showed that

sulfasalazine inhibits the calcium-dependent events of the programming phase and also blocks the binding of NK cell cytotoxic factor to the target cell (76).

It is generally accepted that the therapeutic component of sulfasalazine is one of its cleavage products, 5-aminosalicylate. In its current formulation, sulfasalazine presents two problems. First, its other cleavage product, sulfapyridine, is largely responsible for the drug's undesirable side effects. Second, sulfasalazine is not cleaved until it encounters the bacteria of the colon and is, therefore, ineffective for patients with Crohn's disease of the small bowel. When 5-aminosalicylate alone is given orally, however, almost all of it is absorbed in the small intestine.

On the basis of those pharmacokinetic properties, several new drug formulations have been developed. One of these, sodium azodisalicylate, consists of two molecules of 5-aminosalicylate bound by an azo bond. Like sulfasalazine, azodisalicylate is poorly absorbed by the small intestine; when it is delivered to the colon, it is cleaved by colonic bacteria into two molecules of 5-aminosalicylate. In another new formulation, 5-aminosalicylate is encapsulated in an acrylic-based resin coating that is not digested in the small intestine but is degraded by colonic bacteria; 5-aminosalicylate has also been formulated as an enema, which has proven to be an effective treatment for ulcerative proctitis. None of these 5-aminosalicylate preparations is available in the United States except for investigational use, although they have had extensive and successful clinical use in many other countries.

The mechanism of action of corticosteroids in IBD is also unknown. Corticosteroids, like sulfasalazine, have a number of biologic properties which may be related to their therapeutic effects in IBD. These include effects on lymphocyte differentiation, lymphokine synthesis, and interferon production. Corticosteroids, like sulfasalazine, also have effects on arachidonic acid metabolism that may relate to their therapeutic effects in IBD. As noted earlier, corticosteroids promote the synthesis of a protein, lipomodulin (143), which inhibits phospholipase A₂ and, thus, blocks the release of arachidonic acid from phospholipids. The inhibition of arachidonic acid release would block the synthesis of both cyclooxygenase and lipoxygenase products. As mentioned above, Lauritsen and co-workers found markedly elevated levels of LTB₄ and PGE₂ in rectal dialysates of UC patients (150). When these patients were given a short course of oral prednisolone, the levels of both mediators fell almost to normal. One could interpret this as the result of the induction of lipomodulin synthesis by

corticosteroids. Alternatively, one could argue that corticosteroids diminished the inflammatory response by some other mechanism and the decline in LTB_4 and PGE_2 is a nonspecific manifestation of diminished inflammation.

XIII. Theories of Pathogenesis

Extensive investigation for more than two decades has failed to uncover the causes of ulcerative colitis and Crohn's disease. Moreover, no coherent underlying mechanism has emerged to elucidate the role of the immunologic abnormalities identified to date. There is enough convincing evidence, however, to conclude that immunologic processes are involved in the pathogenesis of these diseases. Among the questions being addressed in the pathogenesis of inflammatory bowel disease are the following:

1. How is the inflammatory response initiated? One theory suggests that there is an infectious agent against which an inflammatory response is elicited. The list of candidates for that role includes viruses, bacteria, and mycobacteria. A current candidate for the etiologic agent of Crohn's disease is a variant of *Mycobacterium paratuberculosis* (168–172). The infectious theory suggests that the host mounts an appropriate immune response against a pathogenic organism but is unable to eradicate the organism. Most of the candidate organisms have fastidious culture requirements, and it is suggested that our inability to isolate them is related to the difficulties of *in vitro* culture. A second possible theory is that the initiating agent is some common agent, either dietary or bacterial, against which a patient mounts an inappropriately vigorous and prolonged inflammatory response. According to this theory, the fundamental problem is a genetic defect in the patient's immune system.

2. Is there a defect in the regulation of the immune system? Both ulcerative colitis and Crohn's disease are largely confined to the gastrointestinal tract. There appear to be no problems with systemic immunity, which suggests that the immune defect, if there is one, is confined to the intestine. Several immune defects could potentially lead to the histologic and functional changes seen in IBD. One suggestion is that IBD is an autoimmune disease—i.e., the intestinal immune system is mounting a response against some normal component of the intestine, most likely the epithelial cells, which may have antigenic resemblance to some microbial pathogen. Alternatively, the epithelial cell may express new antigens (such as DR

antigens), perhaps in response to infection or inflammation, and the immune response is directed against one or more of the new antigens.

A second possible immune defect is a loss of tolerance. The intestinal tract is constantly exposed to large numbers of foreign antigens, both dietary and bacterial. If a vigorous immune response were mounted against each of those antigens, the gut would be constantly inflamed. One of the mechanisms for oral tolerance is the generation of intestinal suppressor cells that limit the immune response to the antigens. A failure of suppression of the intestinal immune response, could lead to chronic inflammation.

Our inability to identify specific antigens triggering the immune response in IBD may reflect the limitations of the methods used to look for the antigens or may reflect the transience of the antigens. That is, the antigen may be present only briefly and is then lost, although the immune response persists. It is also possible that there is no specific antigen, but that IBD is an inappropriate activation of the immune system in the intestine unrelated to a specific antigen. More likely, is the possibility that there is a specific antigen involved in the initiation of the immune response, but the antigen-specific clonal expansion is overwhelmed by the nonspecific lymphokine-mediated amplification of the immune response so that the specific component cannot be identified. Thus, IBD may be the result of an inability to mount an appropriate immune response to a specific antigen, such that the immune system is activated but fails to destroy the offending antigen and, as a result, we see the histologic and clinical manifestations of a prolonged but ineffective immune response. Alternatively, the defect in IBD may be a problem in turning off the immune response. In that case, what we see is an effective immune response that has destroyed the triggering antigen, but which has not been shut down at the appropriate time. Available data do not allow the choice of one of these explanations over the other.

Ulcerative colitis and Crohn's disease are largely, although not entirely, confined to the gastrointestinal tract. There is not the multiorgan involvement seen in some other chronic inflammatory diseases. The limitation to the intestinal tract may reflect an inappropriate response to an antigen that is present only in the intestinal lumen or in the intestinal mucosa. The suggestion that IBD is an inappropriate immune response to an antigen in the intestinal lumen is appealing, but the spottiness of the disease involvement—especially in Crohn's disease—makes that explanation seem less likely. One would think that a response to a luminal antigen would result in more uniform inflammation, as is seen in gluten-sensitive enteropa-

thy. Alternatively, the limitation to the intestine may reflect a defect in the regulation of the mucosal immune system but not the general immune system. The immune response seen in IBD is determined, in part, by the characteristics of the intestinal immune system, which is a unique functional immunologic compartment in comparison with peripheral blood MNC.

3. What effector mechanisms are responsible for the histologic and functional changes seen in IBD? The two most commonly proposed effector mechanisms are anticolon antibodies and lymphocyte-mediated cytotoxicity. The sera of many IBD patients contain antibodies directed against epithelial cells. However, those antibodies are also found in the sera of patients with other intestinal diseases. Recently, IgG antibodies bound to epithelial cells have been identified in the colon of ulcerative colitis patients. Whether these immunoglobulins participate in the pathogenesis of ulcerative colitis is unknown.

Within the involved intestine, there is a marked proliferation of IgG-containing cells and an IgG immune response, with preferential production of IgG₁ in ulcerative colitis and all IgG subclasses, with IgG₂ predominantly, in Crohn's disease. At the same time, an increased proportion of monomeric IgA and IgA₁ is secreted by both ulcerative colitis and Crohn's disease intestinal MNC. These alterations may represent the normal immune response to infectious agents or stimulatory molecules with preferential IgG and IgA subclass production, resulting in augmentation and perpetuation of the immune response. Delineation of the antigens, against which spontaneously secreted IgG and IgA subclasses from intestinal MNC are directed, may provide clues regarding etiologic factors in IBD.

The second proposed effector mechanism is lymphocyte cytotoxicity directed against epithelial cells. However, as previously noted, the general cytotoxic effector capabilities of intestinal lymphocytes are less than those of peripheral blood lymphocytes, and there is no evidence that intestinal lymphocytes from IBD patients have increased cytotoxic capabilities compared with controls. Although the basal cytotoxic potential of intestinal lymphocytes is low, it is possible that in IBD mucosa there is enhancement of lymphocyte cytotoxicity by lymphokines or clonal expansion of antigen-specific cytotoxic lymphocytes. Other cells in IBD mucosa, including neutrophils and activated macrophages, may be cytotoxic for epithelial cells, but their action as potential effector cells in intestinal cytotoxicity has not yet been adequately explored.

Another step in the pathogenesis of IBD that has not been well

characterized is the transition from immune activation to inflammation. Much recent progress in IBD research has come in the area of the characterization of mediators of inflammation in IBD and their role in the amplification of the immune response. Although studies in this area are not likely to give any insight into the initiating event in IBD, they are likely to give a better understanding of how the inflammatory response is promulgated and preserved.

The mechanism accounting for intestinal damage, therefore, most clearly operational in IBD is the generation of soluble mediators. Hyperemia, edema, and neutrophil infiltration are all products of soluble mediators. Which mediators are involved and their precise roles in the generation of the inflammatory response are less clear. Also unclear are the interactions between the soluble mediators and both immunoresponsive effector cells and antibodies. For example, does the binding of anticolon antibodies to epithelial cell antigens result in complement activation? Does the killing of epithelial cells by intestinal lymphocytes result in the release of factors that stimulate neutrophil infiltration?

Crohn's disease and ulcerative colitis are diseases marked by chronic immune activation of the intestinal tract. Insight into the pathogenesis of those diseases is most likely to come from a better understanding of normal immune regulation in the gastrointestinal tract—with particular attention to the ability of the intestinal immune system to suppress the generation of an inflammatory response to the multiple antigens normally present in the intestinal lumen. Progress in therapy is likely to come from a better understanding of the role of soluble mediators of inflammation in IBD.

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