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CONTENTS

Physical Maps of the Mouse and Human Immunoglobulin-like Loci

ERIC LAI, RICHARD K. WILSON, AND LEROY E. HOOD

I.	Introduction	1
II.	Methods for the Generation of Physical Maps	3
III.	Chromosomal Locations of the Immunoglobulin-like Loci	6
IV.	Structure and Organization of Immunoglobulin Loci	7
V.	Structure and Organization of the Major Histocompatibility Complexes	28
VI.	Structure and Organization of T-Cell Receptor Gene Loci	34
VII.	Mechanisms for the Evolution, Recombination, and Diversification of the Immunoglobulin-like Loci	45
	References	47

Molecular Genetics of Murine Lupus Models

ARGYRIOS N. THEOFILOPOULOS, REINHARD KOFLER, PAUL A. SINGER, AND FRANK J. DIXON

I.	Introduction	61
II.	The Lupus Strains	61
III.	Immunoglobulin Germ Line and Autoantibody Genes	65
IV.	Genomic and Expressed T Cell Antigen Receptor Repertoire	78
V.	Conclusions	99
	References	100

Heterogeneity of Cytokine Secretion Patterns and Functions of Helper T Cells

TIM R. MOSMANN AND ROBERT L. COFFMAN

I.	Introduction	111
II.	T Cell Cytokine Secretion Patterns	111
III.	Functions of Thl and Th2 Cytokines	114
IV.	Functions of Thl and Th2 Cells	117
V.	Precursors of Thl and Th2 Differentiation States	122
VI.	Differential Induction of Thl and Th2	127
VII.	Cross-Regulation of Thl and Th2 Differentiation States	130
VIII.	Contributions of Thl and Th2 to Immune Responses	133
IX.	Human Helper T Cell Subsets	138
	References	139

The Leukocyte Integrins

Takashi K. Kishimoto, Richard S. Larson, Angel L. Corbi, Michael L. Dustin, Donald E. Staunton, and Timothy A. Springer

I. Introduction 149
II. Structural Features of the Leukocyte Integrins 153
III. Chromosomal Localization 159
IV. The Leukocyte Integrins in Inflammation 160
V. Ligand Molecules for the Leukocyte Integrins 169
VI. Molecular Basis of Leukocyte Adhesion Deficiency Disease 173
VII. Future Avenues of Research 176
References 177

Structure and Function of the Complement Receptors, CR1 (CD35) and CR2 (CD21)

Joseph M. Ahearn and Douglas T. Fearon

I. Introduction 183
II. Complement Receptor Type 1 183
III. Complement Receptor Type 2 201
References 213
Note Added in Proof 219

The Cellular and Subcellular Bases of Immunosenescence

Marilyn L. Thoman and William O. Weigle

I. Introduction 221
II. B and T Lymphocyte Activation 221
III. Age-Associated Alterations in Cell Subset Distribution and Marker Density 236
IV. Hematopoietic Stem Cells and Differentiation of Precursors 240
V. Regulatory Changes Occurring as a Function of Age 247
VI. Mucosal Immunity 251
VII. Conclusions 253
References 254

Immune Mechanisms in Autoimmune Thyroiditis

Jeannine Charreire

I. Introduction 263
II. Thyroid Antigens Involved in Thyroiditis 266
III. Experimental Models 273
IV. Genetic Control of Thyroiditis 282
V. Cellular Immune Responses during Thyroiditis 287
VI. Humoral Response(s) in Thyroiditis 298
VII. Prevention and Treatment of Thyroiditis 308

VIII. Concluding Remarks: Toward a New Conception of Autoimmune Reactivity 317
References 319

INDEX 335

CONTENTS OF RECENT VOLUMES 351

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ADVANCES IN IMMUNOLOGY, VOL. 46

Physical Maps of the Mouse and Human Immunoglobulin-like Loci

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I. Introduction

The vertebrate immune system protects the host organism from disease-causing microorganisms, such as bacteria and viruses, and from cancer cells. The immune system is highly complex and consists of many different cell types each carrying out their unique function. The primary task of the immune response is to distinguish between self and foreign antigens. This function is mediated by cell-surface receptors located on two major types of lymphocytes, T cells and B cells. B cells constitute the humoral pathway of the immune system, which defends primarily against acute bacterial and viral infections. The cell-surface antigen receptor of B cells is a membrane-bound immunoglobulin (Ig) of the IgM class. Immunoglobulins are composed of two chains, heavy (H) and light (L). Following protein synthesis, the heavy chain combines with one of two types of light chain, λ or κ. The cellular pathway of the immune system is mediated by T cells, which are most effective against fungi, parasites, cancer cells, and foreign tissue as well as cells which have been infected by viruses. The cell-surface antigen receptor of T cells may be composed of α and β chains or, perhaps less commonly, γ and δ chains. Like immunoglobulins, the α and β or γ and δ chains associate following protein synthesis to form a heterodimeric receptor molecule. The molecules encoded by the genes of the Major Histocompatibility Complex (MHC) also play an important role in the vertebrate immune response. These proteins present foreign peptide antigens to the α/β T cell receptors, thus facilitating the ability of T cells to identify and respond to foreign antigens. Furthermore, MHC-encoded antigens allow T cells to distinguish between self-antigens and non-self-antigens.

The immunoglobulins, T cell receptors, and MHC-encoded proteins all are members of a large family of structurally related molecules,

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constructed from an immunoglobulin homology unit. In this review, we will group these gene families as immunoglobulin-like loci, since they are molecular families which are constructed based on the immunoglobulin homology unit. Each immunoglobulin homology unit is approximately 110 amino acids in size, and has several conserved amino acids and a centrally placed cysteine disulfide bridge usually spanning 60–75 amino acids. The tertiary structure of the homology unit is highly conserved and composed of two sheets of three to four antiparallel β-pleated strands (Amzel and Poljak, 1979). The tertiary structure of one homology unit appears to facilitate interactions with a second homology unit to form a functional domain. This is illustrated by the interactions of the immunoglobulin light and heavy chains, the T cell receptor α and β or γ and δ chains, and the membrane proximal region of the MHC class I polypeptide with β_2-microglobulin.

In addition to their structural similarities, these molecules all are encoded by discontinuous gene segments which are organized on the chromosomes as discrete loci. During the course of cellular differentiation and development, gene segments in these loci are rearranged and joined to produce the final coding sequence. Since most of the discontinuous gene segments exist as multiple-member pools, the combination of different sequences gives rise to a high degree of diversity in the final protein product. Since all of the immunoglobulin-like loci share similar patterns of organization and mechanisms of rearrangement and expression, it is likely that they all arose from a common primordial genetic ancestor. The initial molecular characterization of these loci has revealed many of these mechanisms, and has presented clues as to the evolutionary pathways of immunoglobulin-like loci. In order to fully understand the evolution of the antibody and T-cell receptor repertoires, a detailed characterization of all of the immunoglobulin-like loci will be necessary. These characterizations will include physical mapping of each locus using a combination of cytogenetic and molecular techniques.

Physical maps represent the actual distances between genes along a chromosome. There are many techniques currently available for constructing physical maps, with each technique yielding a different level of resolution. Inherent in each mapping method is a trade off between the level of detail, or resolution, in the map and the extent to which the map provides a convenient overview of the chromosome or DNA region of interest. Low-resolution physical maps can be obtained from the distinctive pattern of bands that are observed along each chromosome after staining (George, 1970). Genes can then be assigned to particular bands of a specific chromosome by *in situ* hybridization experiments. The banding technique is important because it allows the average chromosome to be divided into 10 to 20 distinct regions to which genes

may be assigned. This provides an excellent starting point for the generation of a physical map of a chromosome and allows approximate localization of any gene on a chromosome. However, using *in situ* hybridization, the exact physical location of a given gene can only be estimated to within a few thousand kilobases. Physical maps of higher resolution are obtained by DNA cloning and restriction enzyme analysis. This latter method provides detailed maps of smaller regions, but connectivity (linkage) between the smaller regions is often difficult, and it is impossible to distinguish between nonoverlapping clones that are close to another on the same chromosome and clones derived from different chromosomes. In this article we will summarize the methods that are currently available for generating physical maps and discuss the advantages and disadvantages of each method.

II. Methods for the Generation of Physical Maps

The chromosomal location of a given gene can be determined by a number of techniques. One of the most commonly used methods involves the use of somatic hybrid cell lines from one species (i.e., hamster) that contain a single chromosome from a different species (i.e., human). With a panel of cell lines that each contain a different human chromosome, the assignment of any given gene can be made by the consistent coexistence of the gene and presence of a particular chromosome. The most direct method for chromosomal assignment is *in situ* hybridization of condensed chromosomes using a DNA probe specific for the gene of interest. Chromosomal location also can be determined if chromosomal translocation involving the gene of interest has occurred.

The relative order of a number of genes along a region of the chromosome can be determined by a number of techniques, including the use of deletion mutants, and the analysis of chromosomal recombination points and translocations. In the immunoglobulin and T-cell receptor gene families, synthesis of a functional messenger RNA may require the joining of as many as three distinct gene segments. These gene segments, variable (V), joining (J), and diversity (D), are discontinuous in the germ line DNA and are brought together upstream from a constant (C) region gene by DNA rearrangement during development (Sakano *et al.*, 1979, 1980; Chien *et al.*, 1984a; Siu *et al.*, 1984a,b; Hayday *et al.*, 1985a,b; Winoto *et al.*, 1985). This DNA rearrangement is mediated by specific rearrangement signals located directly 5′ and 3′ to the gene segments (see below). The mechanisms of rearrangement include looping-out and chromosomal deletion, sister chromatid exchange, and inversion (Kronenberg *et al.*, 1985; Malissen *et al.*, 1986; Lai *et al.*, 1987; Linsten *et al.*, 1987). Looping-out and chromosomal

deletion have been found to be the most frequently employed mechanisms (Kronenberg *et al.*, 1985). When two gene segments are brought together by the looping-out mechanism, all of the gene segments that are located between the two rearranging gene segments will be deleted and lost from the genome. Therefore, the examination of many cell lines with different rearranged gene segments allows the generation of an ordered deletion map.

The fine structure and organization of a gene family is determined by analysis of cloned DNA using a battery of restriction enzymes coupled with Southern blot hybridization. There are a number of cloning vectors available for this purpose, and each vector system has advantages and disadvantages. The most widely used cloning vectors for genomic DNA are derivatives of bacteriophage λ (Murray and Murray, 1974; Thomas *et al.*, 1974; Blattner *et al.*, 1977). This is due mainly to the high cloning efficiency of these vectors and the relative ease with which the recombinant clones may be stored, transported, and screened. The most significant advantage of λ vectors is the stability of the recombinants, since any alteration in the size of the insert will render the recombinant molecule unable to be packaged and replicated. The only limit of this class of vectors is the relatively small maximum insert size of 20 kilobases (kb).

Inserts larger than 20 kb in size routinely have been cloned in cosmid vectors (Hohn and Murray, 1977). Cosmids are plasmids that contain bacteriophage λ cos site sequences, thus enabling *in vitro* packaging of recombinant DNA molecules. Depending on the size of the vector, DNA inserts in the range of 30 to 44 kb can be packaged and cloned. This represents a twofold decrease in the number of clones required to span a given region of the genome as compared to cloning with λ vectors. However, because of the larger insert size and the absence of size selection during subsequent replication, genomic regions containing recombination-prone sequences are not stably maintained. Thus, it is not uncommon to use λ clones to fill gaps between regions which have been cloned in cosmids.

Burke *et al.* (1987) have reported the use of yeast artificial chromosome (YAC) cloning vectors for cloning DNA inserts as large as 500 kb. In addition, their data suggest that these cloned fragments are relatively stable despite their large size, and no cloning artifacts have been noted so far. The current limitation of the YAC cloning system is the difficulty associated with the screening procedure wherein each clone must be picked individually before screening. Presently, YAC libraries containing only a few thousand clones have been constructed and not many of them have been well characterized. From the limited data that are available on the YAC clones, it appears that this system might provide

a method of cloning genomic regions that are underrepresented in cosmid libraries. Undoubtedly, the YAC system will represent an important cloning method in the future when the difficulties associated with screening can be diminished. Recently, a cloning system based on bacteriophage Pl also has been reported (N. Sternberg, personal communication). This system has the advantage of being able to package and clone DNA inserts as large as 100 kb.

As discussed above, chromosomal mapping using cytogenetic techniques provides broader information but at low resolution (10,000 kb). In contrast, high-resolution mapping using cloned DNA can yield data only from a small region (50 to 500 kb). Recent developments in DNA separation methods such as pulsed-field gel electrophoresis (Schwartz and Cantor, 1984) have provided the means of bridging the gap between cytogenetic data and detailed physical maps. Recently, a restriction map of the *Escherichia coli* 4.7-megabased (Mb) genome has been constructed using infrequently cutting enzymes, followed by separation of DNA fragments using pulsed-field gel electrophoresis (Smith *et al.*, 1987). Using this method, DNA molecules as large as 12.5 Mb, which is approximately one-fourth the size of the smallest human chromosome, can be resolved (Orbach *et al.*, 1988). Using pulsed-field gel electrophoresis, separation of *Schizosaccharomyces pombe* chromosomes (3, 5, and 6.5 Mb) recently has been achieved in 24 hours (Clark *et al.*, 1988). This suggests that further improvements in pulsed-field technology may fill the gap in providing physical maps which can be used to relate cloned DNA regions and chromosomes.

There are a number of problems inherent to DNA cloning that are independent of the choice of a cloning vector. One of the fundamental problems often is due to the source of starting DNA. In mice, there are many different strains with very diverse genetic backgrounds. For example, in SJL, C58, and C57L mice, almost half of the T cell receptor β-chain variable region gene segments are missing (Behlke *et al.*, 1986). Genomic libraries made from these mice thus will contain only half of the variable gene segments. Genetic variations are evident even in substrains of mice that have been separated for no more than 20 years. This problem is even more complicated in humans, in which genetic polymorphism is common and each gene locus is potentially heterogeneous due to the nature of the diploid genome. Haploid cell lines prepared from hydatid mole tissue have been used as a source of human DNA in an effort to simplify the complexities of the library (Berman *et al.*, 1988). However, the use of DNA prepared from mole or other continuous-growing, transformed cell lines raises the question of whether the resulting library is indeed representative of a "normal" human

genome because of the deletions and chromosomal translocations that can be associated with permanent cell lines. Therefore, the safest course of action is to construct human genomic libraries from DNA prepared from sperm or nontransformed primary fibroblast cells.

III. Chromosomal Locations of the Immunoglobulin-like Loci

The chromosomal locations of immunoglobulin-like loci have been determined by the cytogenetic techniques discussed above. The results of these studies are summarized in Table I. In both the mouse and human immunoglobulin and T cell receptor gene families, all of the data are consistent with chromosomal linkage of V and C genes. A number of immunoglobulin-like V genes have been localized on a chromosome different from the one with the corresponding C genes, but all have been shown to be processed pseudogenes and thus could not be functionally rearranged. In both mice and humans, the functionally related T cell receptor α- and δ-chain gene families are located on the same chromosome (see below), although such close linkage of gene loci does not always imply a functional relationship. Although the immunoglobulin κ light chain and the T cell receptor β-chain gene families both are located on mouse chromosome 6, this is not an essential feature of the organization of these loci. In humans, these loci are located on chromosome 2 and chromosome 7, respectively. In the human genome, two sets of two unlinked loci are located on the same chromosome. The immunoglobulin heavy chain and the T-cell receptor α- and δ-chain loci both are found on chromosome 14. There is no simple explanation for the association of these loci, since the homologous genes are found on different chromosomes in the mouse. However, the relatively close

TABLE I
CHROMOSOMAL LOCATIONS OF THE IMMUNOGLOBULIN-LIKE LOCI

Loci	Mouse	Human
Immunoglobulin		
Heavy (H) chain	12	14q32
Kappa (κ) chain	6	2p12
Lambda (λ) chain	16	22q11
T cell receptor		
Alpha (α) and Delta (δ) chains	14C–D	14q11–12
Beta (β) chain	6B	7q32–35
Gamma (γ) chain	13A2–3	7p15
Major Histocompatibility Complex	17	6p21

linkage of these loci and their similar rearrangement mechanism may lead to the frequent observation of chromosome 14 inversion in leukemias. Similarly, the human T cell receptor β and γ chains both are located on chromosome 7. Since the β- and γ-chain loci are located on separate chromosomes in the mouse, it appears that chromosomal proximity of the various immunoglobulin-like loci is not an essential feature and does not have any functional significance. Chromosomal rearrangements with breakpoints that are close to the immunoglobulin-like loci are commonly observed in leukemias. In leukemias for which the breakpoints have been characterized, most of the rearrangements actually occur within one of the immunoglobulin-like loci. This suggests that immunoglobulin-like loci may be especially prone to chromosomal translocations due to the frequency of normal gene rearrangements in these loci.

IV. Structure and Organization of Immunoglobulin Loci

Immunoglobulin molecules are made up of two identical heavy and two identical light polypeptide chains. Each chain is composed of an N-terminal variable region and a C-terminal constant region. The variable (V) regions of both the heavy (H) and light (L) chains are assembled from discrete gene segments (Sakano *et al.*, 1979, 1980, 1981; Early *et al.*, 1980). The heavy chain variable region is assembled from three different gene segments: the variable segment (V_H), which encodes for approximately 98 N-terminal amino acids; the diversity segment (D_H), which encodes the next 1 to 15 amino acids; and the joining segment (J_H), which encodes the final 12 to 17 amino acids. These gene segments are located in separated clusters along the chromosome. A functional heavy chain variable region gene is generated by the joining of the V_H, D_H, and J_H during B cell development. There are two types of light chains, κ and λ. The variable regions of the light chain are encoded by the $V_{(\kappa,\lambda)}$ and $J_{(\kappa,\lambda)}$ gene segments. No D gene segments are involved in the assembly of the light chain variable region.

The variable regions of immunoglobulins have been shown to contain hypervariable segments in their light (Milstein, 1967; Kabat, 1967; Wu and Kabat, 1970) and heavy (Kabat and Wu, 1971; Kehoe and Capra, 1971) chains. Three hypervariable segments were delineated from a statistical examination of amino acid sequences and these segments were hypothesized (Wu and Kabat, 1970; Kabat and Wu, 1971) to be the complementarity-determining regions (i.e., CDR1, CDR2, and CDR3) containing amino acid residues which make contact with cognate antigens. The rest of the V region constitutes the framework (i.e., FR1, FR2, FR3, and FR4), with the three CDRs separating the four FRs. This has been verified by X-ray diffraction studies (Davies and Metzger, 1983).

A. MOUSE λ CHAIN

In the mouse, 5% of immunoglobulin light chains is encoded by the genes of the λ-chain locus (Hood *et al.*, 1967; Cotner and Eisen, 1978). A functional λ light chain variable region is encoded by two gene segments, V and J. No D gene segments have been found in the λ-chain locus.

Amino acid analysis of λ light chain proteins isolated from myeloma tumors has indicated that there are three λ subtypes: λ1 (Weigert *et al.*, 1970; Appella, 1971; Cesari and Weigert, 1973), λ2 (Schulenberg *et al.*, 1971; Dugan *et al.*, 1973), and λ3 (Azuma *et al.*, 1981). Each subtype is defined by a distinctive constant (C) region amino acid sequence and is encoded by separate J-C pairs (see Fig. 1). The nomenclature of the J-C gene segments follows the numbering system of the λ subtype proteins. C1 differs from C2 and C3 by 39 and 40 amino acid residues out of 104, respectively. C2 and C3 are more homologous to each other, differing by only 5 out of 104 amino acids. The J4C4 gene segment pair appears to be a pseudogene and is not expressed due to the lack of a proper RNA splice site in J4 and a 2-basepair (bp) deletion from the 5′ heptamer sequence of J4 that is required for V-J rearrangement (Miller *et al.*, 1982; Blomberg and Tonegawa, 1982). In most mouse strains, λ1 accounts for about 80-90% of all λ chains (Cotner and Eisen, 1978).

Three V region gene segments, V1, V2 (Brack *et al.*, 1978), and VX (Sanchez *et al.*, 1987; Dildrop *et al.*, 1987), have been found. The V1 and V2 gene segments encode the first 97 amino acids of the λ chain and the VX gene segment is 6 amino acids longer due to an insertion in the third hypervariable (CDR3) region. There are four J gene segments with a single J located 5′ to each of the four C region gene segments. The J gene segments encode 11 amino acids. In addition, there is another constant region gene segment (C5), which encodes a putative

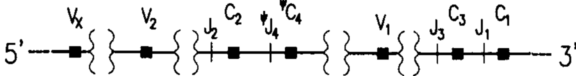


FIG. 1. Physical map of the mouse λ-chain locus; ψ denotes pseudogenes.

nonantibody protein that is selectively expressed in pre-B cells (Sakaguchi and Melchers, 1986; Sakaguchi *et al.*, 1986; Kudo *et al.*, 1987; Mami *et al.*, 1988).

Organization of the Mouse λ-Chain Locus

The mouse λ-chain genes have been found to occur in clusters: VX(?)-V2-J2C2J4C4-V1-J3C3J1C1′ (Miller *et al.*, 1981; Blomberg *et al.*, 1981; Selsing *et al.*, 1982; Sanches *et al.*, 1987). These gene segments are all located on chromosome 16 (D'Eustachio *et al.*, 1981); however, their order is unknown and the two J-C clusters have not been physically linked to each other or to the V gene segments. The chromosomal location of C5 is not known.

The observation that both the rearranged and germ line forms of the V1 and V2 gene segments can occur in the same cell line suggests that V1 is associated with the J3C3J1C1 cluster and that V2 is upstream of the J2C2J4C4 cluster (Blomberg *et al.*, 1981). In addition, the association pattern of the V and V-C gene segments appears to be highly restricted; when a V gene segment rearranges, it recombines almost exclusively with a particular J-C gene segment pair (Reilly *et al.*, 1984). Analysis of the rearrangement pattern of 74 λ chains reveals 39 V1-J1C1 rearrangements, 19 V2-J2C2 rearrangements and 13 V1-J3C3 rearrangements. There were only three unusual rearrangements observed, all involving the V2 gene segment; two of these rearrangements were V2-J1C1 and one was V2-J3C3. Assuming that looping out and deletion is the rearrangement mechanism used in the λ-chain locus, these data strongly suggest the gene order shown in Fig. 1. This indicates that a V gene segment will almost always rearrange with a J gene segment in the nearest downstream J-C cluster. The location of VX is based on the finding that expressed λ chains containing VX utilize the J2C2 gene segment pair. Since rearrangement of a downstream V to an upstream constant region has not been observed in the λ-chain locus, it is assumed that VX is located 5′ of the J2C2 cluster. However, the relative order of V2 and VX is not known.

As indicated in Fig. 1, the distances between the J and C gene segments are almost identical in all four constant regions, and the distance between C2J4 and C3J1 is similar. In addition, examination of the J-C introns and comparison of the C2 and C3 gene segments showed high homology between the two constant region clusters. These relationships suggest that the λ locus may have arisen from a primordial JC gene segment pair which duplicated to an ancestral JC*x*-JC*y* gene cluster at about the time of the first mammal. The data further suggest that the JC*x*-JC*y* gene cluster duplicated after the mammalian radiation that separated

mouse and man, with JCx the precursor of J2C2 and J3C3, and Cy the precursor of J1C1 and J4C4. The pseudogene, C4, probably became inactive at about the time of duplication of the ancestral JCx-JCy gene cluster.

The percentage of amino acid replacement is approximately the same for V1-V2 as for C3-C2, implying that the ancestral V gene segment was duplicated at the same time as, and possibly together with, the JCx-JCy gene cluster. The third λ V gene segment, VX, is only 30-33% homologous to V1 and V2 at the amino acid level and about 50% homologous at the nucleotide level. Thus, it appears that the VX gene segment diverged long ago from the ancestral λ-chain gene segment that eventually gave rise to V1 and V2.

B. The Human λ-Chain Gene Family

In humans the ratio of immunoglobulin κ to λ light chains is approximately 2 : 1 (Hood *et al.*, 1967; Fu *et al.*, 1979). This is in contrast to the mouse, for which the κ and λ ratio is 20 : 1 and thus indicates that the λ chain contributes extensively to the diversity of human immunoglobulin light chains. This therefore suggests the existence of a much larger repertoire of human V_λ gene segments. As found with the mouse λ chain, a functional human λ light chain V region is encoded by two gene segments, V_λ, and J_λ. No D gene segment has been found for the λ chain. The λ locus has been mapped to the long arm of chromosome 22 at band 11 by examining somatic cell hybrids (Erikson *et al.*, 1981; McBride *et al.*, 1982a) and chromosomal translocations in leukemias (Goyns *et al.*, 1984), and by *in situ* hybridization (Emanuel *et al.*, 1984). The chromosomal orientation of the λ locus was found to be centromere-V-J-C (Emanuel *et al.*, 1985).

The finding that large amounts of human immunoglobulin light chains can be obtained as homogeneous Bence-Jones proteins aided in the early characterization of the λ chain. Immunochemical and amino acid sequence analysis showed that there are three nonallelic forms of human λ chains which can be localized structurally to the constant region. One form, designated Oz (Ein and Fahey, 1967; Ein, 1968), is characterized by the presence of a lysyl residue (Oz^+), instead of an arginyl residue (Oz^-), at position 190 (Appella and Ein, 1967). The second form, designated Kern (Ponstingl *et al.*, 1968; Hess *et al.*, 1971), is associated with the substitution of a glycyl ($Kern^+$) for a seryl ($Kern^-$) residue at position 156. The third form was based on amino acid sequencing of the λ Bence-Jones protein Mcg (Fett and Deutsch, 1974, 1975). Mcg-type proteins differ from non-Mcg proteins by the substitution of asparagine for alanine at position 116, threonine for serine at

position 118, and lysine for threonine at position 167. The demonstration that all of these forms can be found within an individual's light chain pool (Ein, 1968; Gibson *et al.*, 1971; Fett and Deutsch, 1975) indicated that the nature of these factors is isotypic rather than allotypic. There are four known isotypes based on the classification of the factors discussed above, Mcg, $Kern^-Oz^-$, $Kern^-Oz^+$, and $Kern^+Oz^-$. Each of these isotypes has been found to be encoded by a separate constant region gene segment (see below).

The structure of the V_λ gene segments was obtained mainly from amino acid sequence analysis. Kabat *et al.* (1987) characterized the λ V regions into six subgroups. Very little is known about the structure of the V_λ gene segments, since DNA clones containing V_λ gene segments were not isolated until 1984 (Anderson *et al.*, 1984). Since then, a number of genomic clones corresponding to some of the existing subgroups have been isolated (Tsujimoto and Croce, 1984; Anderson *et al.*, 1985) and a new V_λ subgroup has been found (Anderson *et al.*, 1984).

Organization of the Human λ-Chain Locus

The constant region of the human λ locus contains a cluster of six C gene segments (Hieter *et al.*, 1981a) arranged within a 40-kb fragment, all of which have been sequenced (Hieter *et al.*, 1981a; Dariavach *et al.*, 1987). The six C gene segments were found to be regularly spaced along the chromosome at approximately 5-kb intervals (Fig. 2). Nucleotide sequencing of the six C gene segments has shown that they are extremely homologous and suggests that they are the result of recent gene duplications. Duplication of the C2–C3 region also has been observed in some individuals (Taub *et al.*, 1983). Two of the gene segments, C4 and C5, were shown to be pseudogenes and the remaining gene segments, C1, C2, C3, and C6, have been shown to encode

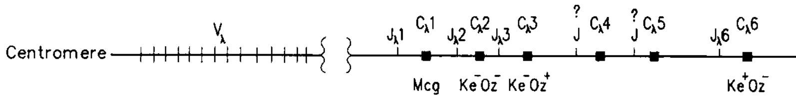


FIG. 2. Physical map of the human λ-chain locus. $C_\lambda 4$ and $C_\lambda 5$ are pseudogenes. No J has been isolated from $C_\lambda 4$ and $C_\lambda 5$ so far, although hybridization experiments suggest that there are J-like sequences contained in these regions. The isotypic forms of λ chains are labeled below the C gene.

the Mcg, Kern$^-$Oz$^-$, Kern$^-$Oz$^+$, and Kern$^+$Oz$^-$ isotypes, respectively (Heiter *et al.*, 1981b; Dariavach *et al.*, 1987). C4 is a pseudogene, has a stop codon in the third exon, and has three deletions that correspond to codons 5 through 7, 21, and 64. C5 is nonfunctional because of an 11-bp deletion (codons 41–44), which results in a frameshift. There is at least one functional J_λ gene segment located 1.3 or 1.5 kb upstream of each functional C_λ gene segment (Chang *et al.*, 1986; Sun *et al.*, 1985; Dariavach *et al.*, 1987; Udey and Blomberg, 1987). Additionally, Southern blot hybridization experiments using a mouse J_λ gene segment as a probe suggested that there is at least one J_λ gene segment located upstream of $C_\lambda4$ and $C_\lambda5$ (Udey and Blomberg, 1987). However, sequence analysis of these regions did not reveal the presence of any residues indicative of J_λ gene segments (Dariavach *et al.*, 1987). The organization of the human immunoglobulin λ-chain locus therefore is very similar to the organization of the mouse λ locus in that each C_λ gene segment is preceded by a single J_λ gene segment.

Very little is known about the organization of the V_λ gene segments corresponding to the protein subgroups which have been described (Tsujimoto and Croce, 1984; Anderson *et al.*, 1984; Dariavach *et al.*, 1987). From Southern blot experiments, it was found that DNA probes specific for each subgroup hybridized to an average of 10 bands. This suggests that there are about 70 V_λ gene segments, consistent with other estimates of a total repertoire of 50 to 100 human V_λ gene segments.

Several groups have reported the presence of a number of C_λ-like genes in man. A pseudogene which appears to have been derived from processed mRNA has been reported by Hollis and co-workers (1982) and is not located on chromosome 22, where $C_\lambda1$–$C_\lambda6$ are located. Chang *et al.* (1986) reported three C_λ-like gene segments outside the cluster discussed above. One of these C_λ-like genes appears to be a pseudogene, since it does not have an associated J_λ gene segment, and has accumulated multiple mutations which render it nonfunctional. The other two C_λ-like genes have associated J_λ gene segments and open reading frames, and are 86–89% homologous to the known λ genes. However, it is not known if these new C_λ-like genes are expressed, since they do not encode for any constant region described at the protein level and are not located on chromosome 22.

C. The Mouse κ-Chain Gene Family

The κ chain represents 95% of the immunoglobulin light chain found in most inbred mouse strains (Hood *et al.*, 1967; Cotner and Eisen, 1978). Therefore, initial amino acid sequence analysis of immunoglobulin light chains focused on κ-chain polypeptides (Hood *et al.*, 1970, 1973). Amino

acid sequencing of κ chains revealed that there are many V_κ sequences, but only a single C_κ sequence, in accord with the later finding that there are many V_κ gene segments and only a single C_κ gene segment (Seidman *et al.*, 1978). Like the λ chain, a functional immunoglobulin κ light chain V region is encoded by two gene segments, V_κ and J_κ, which become linked by a rearrangement that joins V_κ to J_κ during B cell development. No D gene segments have been found in the κ chain locus. The κ gene segments have been mapped to chromosome 6 using somatic cell hybrids (Hengartner *et al.*, 1978; Swan *et al.*, 1979).

Five J_κ gene segments have been found in the κ locus based on nucleotide sequence analysis. Four of these J_κ gene segments correspond to amino acid sequences found in myeloma light chains. $J_\kappa 3$ appears to be a pseudogene, since it contains alterations in three invariant amino acid positions (i.e., 99, 103, and 108) that are found in all expressed κ chains. In addition, $J_\kappa 3$ lacks a functional RNA splicing signal and has been shown to be incapable of recombining with a V_κ gene segment (Nishi *et al.*, 1985). The four functional J_κ gene segments encode the 13 carboxyl-terminal amino acids of the variable region.

Since immunoglobulin light chain diversity occurs mainly in the κ chain, the number of V_κ gene segments in the locus must be very high. Using the amino acid sequences obtained from myeloma κ chain proteins, Potter (1977) classified κ chains into subgroups based on partial sequence to the first invariant cysteine (Cys23). All of the available κ chain sequences were compared to a common hypothetical sequence, and any sequences that differed from the others by three or more residues were arbitrarily assigned to a new subgroup. Using this system, Potter (1977) proposed that there were at least 26 κ subgroups. As more κ proteins were sequenced, the classification was revised to the first invariant tryptophan (Trp35). Twelve substitutions were used as a basis for defining subgroups based on the known number of substitutions that are found in $V_\kappa 21$ proteins (Potter *et al.*, 1982). $V_\kappa 21$ proteins were used as a model because these proteins had been extensively studied and several complete amino acid sequences had been determined (McKean *et al.*, 1978; Weigert *et al.*, 1978). By this criteria, there are 18 subgroups in the Trp35 dendrogram. A number of Cys23 subgroups were not included in the Trp35 dendrogram because of incomplete sequences (i.e., 6, 7, 14, 16, 17, and 18), and seven Cys23 subgroups were condensed into three Trp35 subgroups: $V_\kappa 1$, $V_\kappa 3$, and $V_\kappa 26$ became $V_\kappa 1$; $V_\kappa 12$ and $V_\kappa 13$ were grouped as $V_\kappa 12$; $V_\kappa 15$ and $V_\kappa 19$ were condensed as $V_\kappa 15$. This classification system seems to correlate reasonably well with molecular cloning studies in which DNA probes from different subgroups do not cross-hybridize under normal stringency conditions (i.e., less than 80% homology).

Organization of the Mouse $\varkappa$-Chain Locus

As discussed above, there are five $J_\varkappa$ gene segments and a single $C_\varkappa$ gene segment in the $\varkappa$-chain constant region (see Fig. 3a). All five $J_\varkappa$ gene segments are located upstream from the $C_\varkappa$ region gene (Sakano *et al.*, 1979; Max *et al.*, 1979), and the most distal $J_\varkappa 5$ gene segment is only 2.4 kb from $C_\varkappa$. The $J_\varkappa$ gene segments are regularly and closely spaced with the following distances: $J_\varkappa 1$-310 bp-$J_\varkappa 2$-245 bp-$J_\varkappa 3$-268 bp-$J_\varkappa 4$299-$J_\varkappa 5$ (see Fig. 3a).

Deletion analysis of myeloma cell lines suggested that the $V_\varkappa$ gene segments are located upstream of the constant region (Seidman *et al.*, 1980; Selsing and Storb, 1981). The exact number of $V_\varkappa$ gene segments is not known but it has been estimated that there are 100 to 300 $V_\varkappa$ gene segments (Rabbitts, 1977; Cory *et al.*, 1981; Zeelon *et al.*, 1981; Briles and Carrol, 1981; Gibson, 1984; Nishi *et al.*, 1985). The organization of the $V_\varkappa$ gene segments has been examined using recombinant mouse strains and restriction fragment length polymorphism in inbred mouse strains. Existing data allow the $\varkappa$ locus to be divided into eight clusters—seven $V_\varkappa$ clusters and the constant region (D'Hoostelaere and Gibson, 1986; D'Hoostelaere *et al.*, 1988) (see Fig. 3c). The gene order within a cluster is not known, and interspersion of subgroups within a region is possible since human $V_\varkappa$ subgroups have been found to be interspersed (see below).

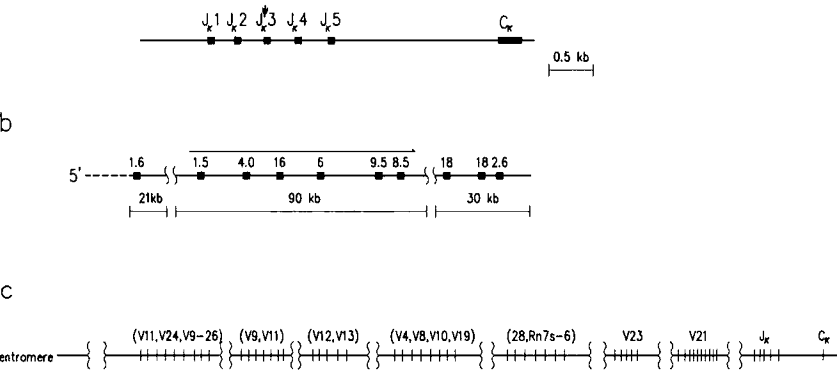


FIG. 3. Physical maps of the mouse $\varkappa$-chain locus. (a) Organization of the mouse $\varkappa$ genes; ψ indicates a joining pseudogene segment. (b) Organization of the mouse $V_\varkappa 21$ gene segment genomic clusters. (c) Organization of the mouse $V_\varkappa$ locus. The variable gene segments are represented by their subgroup numbers. The relative order of $V_\varkappa$ gene segments shown within parentheses has not been determined.

The $V_\kappa 21$ subgroup has been studied extensively by both amino acid sequencing and genomic DNA cloning. Heinrich *et al.* (1984) isolated 10 $V_\kappa 21$ gene segments and linked the gene segments into three regions by chromosomal walking (see Fig. 3b). Southern blot hybridization analysis of two cell lines provided evidence for the tentative order of the regions. The 21-kb region containing the 1.6-kb V_κ was placed at the 5′-most end. A cluster of six $V_\kappa 21$ gene segments is located in a 90-kb region downstream from the 21-kb region. All six gene segments are in the same transcriptional orientation, and the order of these gene segments is shown in Fig. 3b. A 30-kb region containing three more $V_\kappa 21$ gene segments is located further downstream. The three regions have not been linked to each other. Based on these genomic data, the average distance between V_κ gene segments was calculated to be 10 to 12 kb. If the V_κ gene segments are equally spaced on the chromosome, this would imply the size of the κ locus to be 1000 to 3000 kb. Recent mapping experiments using pulsed-field and two-dimensional gel electrophoresis (Woolf *et al.*, 1988) suggested that most V_κ subgroups are clustered (E. Lai, unpublished results).

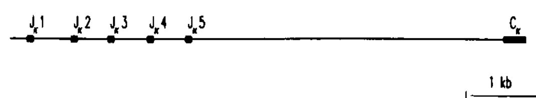
D. THE HUMAN κ-CHAIN GENE FAMILY

Immunoglobulin κ light chains constitute about 60% of the light chains found in human serum (Milstein, 1965; Hood *et al.*, 1967). Over 180 human κ proteins have been sequenced (Kabat *et al.*, 1987) and may be divided into six distinct subgroups on the basis of amino acid sequence. In contrast, mouse κ chains comprise at least 18 subgroups (see above).

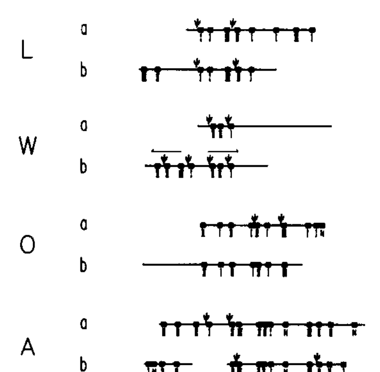
This probably reflects a greater usage of κ-chain genes in the mouse, since κ chains constitute almost the entire mouse light chain repertoire. Similar to mouse and human λ light chains, an active κ light chain is formed by the joining one of a number of V_κ gene segments to a J_κ gene segment located 5′ to the constant region during B cell differentiation. No D gene segments have been observed for the κ chain. The κ-chain locus has been mapped to the short arm of chromosome 2 in *in situ* hybridization (Malcolm *et al.*, 1982) and somatic cell hybrids (McBride *et al.*, 1982b). The chromosomal order of the human κ-chain locus was found to be centromere-V-J-C.

Five functional J_κ gene segments have been found in the human κ locus based on nucleotide sequence analysis (Hieter *et al.*, 1982) (Fig. 4a). This is in contrast to the mouse J_κ region, where one of the J_κ gene segments, $J_\kappa 3$, is a pseudogene. Heteroduplex and DNA sequence analyses indicated that the mouse $J_\kappa 3$ pseudogene segment is not present in humans. In addition, the human $J_\kappa 5$ does not appear to have a counterpart in the mouse (Konkel *et al.*, 1979). As in the mouse, there is only a single human C_κ gene segment.

a



b



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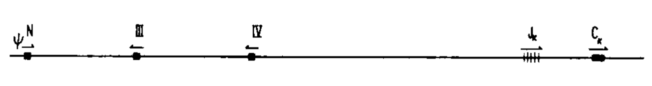
c



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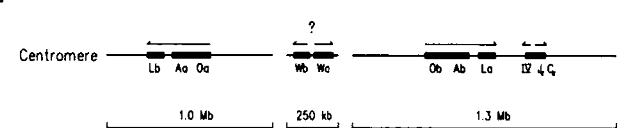
d



e

Chromosome 1

Chromosome 5

Chromosome 22

Due to the presence of large amounts of homogeneous light chains from Bence-Jones proteins, much of the characterization of the V_κ region has been obtained from amino acid sequence. Genomic clones containing V_κ gene segments specific for each subgroup have been isolated and it has been shown that protein subgroup classifications correlate well with the division of gene segments into subfamilies based on nucleotide homology of greater than 75%. The human V_κ gene segments which had been isolated initially by cross-hybridization with mouse V_κ probes represent subgroup V_κI (Bentley and Rabbitts, 1980; Jaenichen *et al.*, 1984). Human V_κ gene segments corresponding to subgroups V_κII (Klobeck *et al.*, 1984, 1985a; Weir and Leder, 1986), and V_κIII (Bentley, 1984; Klobeck *et al.*, 1985b; Marsh *et al.*, 1985) also have been isolated. In addition, a V_κ gene segment that does not correlate with any of the four known subgroups has been reported (Stavnezer *et al.*, 1985). The V_κ gene segments are distinguished from gene segments in the other light chain loci by a much longer intron within the leader sequence—over 400 bp as compared to an average of 120–200 bp. Southern blot hybridization experiments indicated that there could be as many as 25 bands for each of the V_κI, V_κII, and V_κIII subgroups (Bentley and Rabbitts, 1981; Klobeck *et al.*, 1985a). However, V_κI gene segment probes appear to cross-hybridize with members of the V_κIII and V_κIV subfamilies under nonstringent hybridization conditions. Subgroup V_κIV contains only a single member at stringent hybridization conditions (Klobeck *et al.*, 1985b; Marsh *et al.*, 1985). This suggested that the number of human V_κ gene segments is at least 50, i.e., significantly less than the estimated number of the mouse V_κ genes (see above). Presently, over 70 human V_κ gene segments have been identified on genomic clones (see below).

Organization of the Human κ-Chain Locus

The human κ-chain region consists of five functional J_κ gene segments located approximately 2.5 kb 5′ to a single C_κ gene segment (see Fig. 4a). The five J_κ gene segments are closely and regularly spaced at about 300-bp intervals (Hieter *et al.*, 1982).

Zachau and co-workers have extensively characterized the organization of the human κ-chain locus by cosmid cloning. Over the past 4 years,

FIG. 4. Physical maps of the human κ-chain locus. (a) Organization of the region containing the J_κ gene segments and the C_κ gene. (b) Physical maps of the eight V_κ regions. Subgroup numbers are indicated for each V_κ gene segment. Pseudogenes are designated by ψ. (c) Physical linkage of V_κ gene segments with the C_κ gene. Transcriptional orientation (arrows) is indicated for each gene element. (d) Organization of the human κ-chain locus. Three regions representing at least 2.5 Mb have been mapped. (e) Dispersion of V_κ-like gene segments on other chromosomes.

they have cloned over 1200 kb of genomic DNA in cosmids and characterized 70 germ line V_κ gene segments (see Fig. 4b). A number of important features of the κ-chain locus have been deduced from this data. First, the V_κ subgroups are highly interspersed in the locus. Second, 23% (16 out of 70) of the genes are pseudogenes and these are spread throughout the locus, interspersed with functional gene segments. This is in contrast with the existing deletion data on the organization of the mouse κ-chain locus, which suggested that the subgroups appear to be clustered on the chromosome. Similarly, V_H gene segments are interspersed in the human, whereas they are clustered in the mouse. Third, almost all V_κ-containing regions have been duplicated. These duplications involve large sections of DNA from 30 to 50 kb. Fourth, in most cases, all of the V_κ gene segments are in the same transcriptional orientation in the same cluster. However, other gene segments have been observed to be in different transcriptional orientations. This suggests that both deletion and inversion rearrangement mechanisms are utilized in the κ-chain locus. Fifth, the distances between gene segments range from 1 to 15 kb. These results indicate that estimations of the number of gene segments and the size of an immunoglobulin-like locus by methods based on hybridization can be regarded only as a guideline.

The single-member subgroup V_κIV was found to be located in the *B* region together with a V_κIII gene segment (Lorenz *et al.*, 1987) (Fig. 4c). Klobeck *et al.* (1985b) have observed that this gene segment was frequently deleted or aberrantly rearranged in a large number of lymphoid cell lines and they suggested that this may be a consequence of specialized function and/or novel structural organization. Pulsed-field gel electrophoresis data showed that the V_κIV gene segment and the constant region gene segments both hybridized to a 220-kb SalI fragment, and the distance between these genes was estimated to be less than 150 kb. This result suggests that chromosomal walking would be a feasible method to link the two regions. Klobeck *et al.* (1987) found that the distance between the V_κIV gene segment and the J_κ gene segments was 23 kb. The transcriptional orientation of the V_κIV gene segment was found to be opposite of that of the constant region. This finding confirms the notion that inversion is a required rearrangement mechanism for the assembly of some V_κ genes in the human κ-chain locus.

The arrangement and the distances between the cloned κ-chain regions was investigated by pulsed-field gel electrophoresis (Lorenz *et al.*, 1987) (Fig. 4d). Regions *A* and *O* were found to be physically linked. A 1-Mb NotI fragment containing regions *Lb*, *Aa*, and *Oa* (a total of 31 gene segments) was located at the 5′ end of the locus. This fragment is followed by a 250-kb NotI fragment containing regions *Wb* and *Wa* (a total of

10 gene segments). A 1.3-MB NotI fragment containing regions *Ob, Ab, La, B*, and the constant region (a total of 32 V_{κ} gene segments) was mapped to the telomeric side of the locus. No V_{κ} gene segments were found on the 3′ side of the constant region (Klobeck *et al.*, 1987). All of the V_{κ} gene segments located on the 1.3-Mb NotI fragment, except for those immediately adjacent to the J_{κ} gene segments, are arranged in the same transcriptional orientation as the constant region genes. All of the V_{κ} gene segments on the 1-Mb fragment also are in the same transcriptional orientation, although their orientation is opposite of the 3′V_{κ} gene segments. Since the V_{κ} gene segments on the 1-Mb fragment have been shown to rearrange by inversion, their transcriptional polarity must be opposite to that of the constant region. The location and the orientation of the *Wa* and *Wb* region (250-kb NotI fragment) is hypothetical since it has not been linked to the other two regions. Six of the nine V_{κ} gene segments located on the 250-kb NotI fragment have been sequenced and all appear to be pseudogenes. The size of the human κ-chain locus is at least 2.5 Mb. This is based on the assumption that the 1- and 1.3-Mb fragments are contiguous, and that within these fragments the mapped regions are arranged in maximal proximity. If the *W* regions are located as shown in Fig. 4d, the size of the κ-chain locus would be much larger. The number of human V_{κ} gene segments is now estimated to be slightly more than 80 members (Lorenz *et al.*, 1987).

In addition to the κ-chain gene segments on chromosome 2, there are a number of V_{κ} gene segments that have been found dispersed on other chromosomes. Lotscher *et al.* (1986) have mapped two solitary V_{κ} gene segments and a cluster of three V_{κ} gene segments to chromosomes 1, 15, and 22, respectively (Fig. 4e). Three of these gene segments were sequenced and determined to be nonprocessed pseudogenes. The dispersed V_{κ} gene segments on chromosome 22 are located at 22q11, the same chromosomal band as the human λ locus, and are centromeric to the λ genes (Adolph *et al.*, 1988). This finding is relevant to the estimation of the size of the repertoire of any immunoglobulin-like locus, since Lotscher *et al.* (1986) estimate about 10% of the V_{κ} gene segments are located outside of chromosome 2. Other examples of dispersed genes include those of the major histocompatibility complex and the human heavy and light chain constant region gene segments.

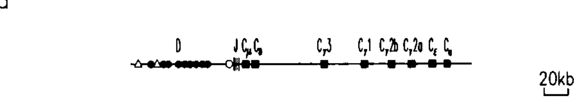
E. The Mouse Heavy Chain Gene Family

There are eight classes of heavy chains (μ, δ, $\gamma 1$, $\gamma 2b$, $\gamma 2a$, $\gamma 3$, ϵ, α) that are defined by their constant region. Two types of recombination are required for the production of the heavy chain protein. During B

cell development, the first recombination creates a complete V_H region by joining a germ line V_H, a D_H, and a J_H gene segment. The completed V_H chain then can be expressed as functional polypeptide because the C_H gene segment is located immediately 3′ to the J_H gene segments (see below). Later in the course of differentiation, the same rearranged V_H gene segment is recombined and expressed with a different C_H gene segment. This mechanism, called heavy chain class switching, is mediated by nucleotide sequences located at the 5′ side (switch region) of every C_H gene segment except C_δ.

There are four functional and one pseudo-J_H gene segments located 5′ to the C_μ gene segment (Newell *et al.*, 1980; Sakano *et al.*, 1980) (Fig. 5a), and three subfamilies of D_H gene segments have been identified in the mouse (Kurosawa and Tonegawa, 1982). The first D_H subfamily, DSP2, consists of nine members, each 17 bp long. The second subfamily, DFL16, consists of two members, one 23 bp long and the other 17 bp long. The third subfamily, DQ52, contains a single 10-bp member.

a



b

V_H10

3609–J558–(J606,VGAM3-8,S107)–3660–(X24,Q52,7183)–D_H–J–C------Centromere

C57BL/6: 3609–J558–3609–Q52 --

A/J: ----------------------↑---------------------- 3609

BALB/c: 3609,J558 --

NFS/N: NIH Swiss ---------------------------------- Q52,7183 ------------------

FIG. 5. Physical maps of the mouse heavy chain locus. (a) Organization of the D_H, J_H, and C region genes. The D_HQ52 gene segment is represented by an open circle. D_HSP2 subfamily members are represented by solid circles and D_HFL16 subfamily members are represented by open triangles. (b) Organization of the heavy chain locus is shown for several mouse strains. Dashed lines indicate identity with the general map (top). The brackets under the gene segments represent interspersion of subfamilies. V_H10 has been mapped 5′ to Q52 and 7183.

The number of V_H gene segments has been estimated to be between 100 (Brodeur and Riblet, 1984) and 1000 (Livant *et al.*, 1986). The V_H gene segments have been grouped into subgroups based on amino acid sequences (Dildrop, 1984; Dildrop *et al.*, 1985; Kabat *et al.*, 1987) or into subfamilies based on nucleotide sequence homology (Brodeur and Riblet, 1984; Brodeur *et al.*, 1984). Ten V_H subfamilies have been classified and are summarized in Table II. One important feature of the heavy chain locus is the high percentage of pseudogenes found. Several studies have indicated that as many as 40% of V_H gene segments may be pseudogenes (Bothwell *et al.*, 1981; Huang *et al.*, 1981; Givol *et al.*, 1981; Rechavi *et al.*, 1983; Loh *et al.*, 1983; Schiff *et al.*, 1985). J558 is the most extensively characterized V_H subfamily and 16 of the 52 known germ line J558 members appear to be pseudogenes (Blankenstein *et al.*, 1987). This may be a consequence of extensive duplication of gene segments and the price paid for maintaining such a large repertoire of genes.

Organization of the Mouse Heavy Chain Locus

The finding that thousands of genetic crosses (Herzenberg, 1964; Potter and Lieberman, 1967) did not give rise to a recombinant between the eight C_H gene segments suggested that they are tightly linked on chromosome 12 (Hengartner *et al.*, 1978). The chromosomal orientation of the heavy chain was found to be centromere-C-J-D-V (Erikson *et al.*, 1985). Hybridization kinetic analysis using DNA probes has shown that the order of the constant region is C_μ-C_δ-$C_\gamma3$-$C_\gamma1$-$C_\gamma2b$-$C_\gamma2a$-C_ϵ-C_α (Honjo and Kataoka, 1978). This order was confirmed by physical mapping of the locus. Initial cloning experiments succeeded in linking $C_\gamma3$ to $C_\gamma1$, $C_\gamma2b$, $C_\gamma2a$, C_ϵ, and C_δ (Nishida *et al.*, 1981; Shimizu *et al.*, 1981; Roeder *et al.*, 1981) and J_H to C_μ and C_δ (Liu *et al.*, 1980; Moore *et al.*, 1981). In 1982, Shimizu *et al.* reported the complete linkage of the constant region gene segments. The distances of the gene segments were found to be 5′-J_H-6.5-kb-C_μ-4.5-kb-C_δ-55-kb-$C_\gamma3$-34-kb-$C_\gamma1$-21-kb-$C_\gamma2b$-15-kb-$C_\gamma2a$-14-kb-C_ϵ-12-kb-C_α (Fig. 5a). The locations of the 12 known D_H gene segments were mapped by Wood and Tonegawa (1983) and they are found to be within 80 kb of the 5′-most J_H gene segments with the closest D_H, DQ52, only about 700 bp from the J_H gene segments (Sakano *et al.*, 1981). The 5′-most D_H gene segment belongs to the two-member V_DFL16 subfamily. The other member of the D_HFL16 subfamily was found to be interspersed with the D_HSP2 subfamily. All of the gene segments in the constant region are in the same transcriptional orientation, and the

TABLE II
V_H SUBFAMILIES

Subfamily	Kabat *et al.* (1987)	Dildrop (1984); Dildrop *et al.* (1985)	Brodeur and Riblet (1984)	Winter *et al.* (1985)	Kofler (1988)
3609(V31)	IB	8	V_H3609	V31	—
J558	IIA, IIIB, IVD, VB	1	V_HJ558	—	—
J606	IIC, IIIC	6	V_HJ606	—	—
VGAM3-8	IIA	9	—	GAM3-8	—
S107	IIIA	7	V_HS107	—	—
3660	IA	3	V_H36-60	—	—
X24	IIIB	4	V_HX24	—	—
Q52	IB	2	V_HQ52	—	—
7183	—	5	V_H7183	—	—
V_H10	—	—	—	—	MRL-DNA4

distance from the 5′-most D_H gene segment to C_μ is about 260 kb (see Fig. 5a). Unlike the organization of the λ light chain, where there is a J gene segment 5′ of each C gene segment, the four J_H gene segments located 5′ of C_μ are the only J gene segments that exist in the heavy chain constant region (Shimizu *et al.*, 1982). In addition, no C_H gene duplication or pseudogenes have been found in the mouse heavy chain locus. This is in sharp contrast to the human heavy chain locus, where extensive gene duplications, pseudogenes, and dispersed C_H gene segments have been found (see below).

The organization of the mouse immunoglobulin heavy chain was determined by analysis of V_H gene segment deletions in B cell lines (Rathbun *et al.*, 1987), and by restriction fragment length polymorphism analysis of recombinant-inbred strains (Brodeur *et al.*, 1984; Blankenstein *et al.*, 1987). Both of these techniques have shown that subfamilies seem to be arranged in discrete clusters on the chromosome. This is supported by genomic cloning experiments which suggest that two V_H gene segments found linked on the same clone belong to the same subfamily (Schiff *et al.*, 1985). However, details of the relative order of V_H gene segment subfamilies differ between deletion and recombination analysis. By recombinant-inbred strain analysis, the map order of subfamilies within the locus was found to be (3609, J606, 3660, X24)-J558-S107-Q52-7183-constant region. The organization of the heavy chain locus produced by deletion mapping is shown in Fig. 5b. The J558 subfamily maps 5′ of J606, and 3660 maps 3′ of S107. The deletion map data, shown in Fig. 5b, are more consistent with V_H rearrangement frequencies, thereby suggesting preferential utilization of D_H-proximal V_H gene segments.

The organization of the heavy chain locus also differs among inbred mouse strains, and interspersion of some subfamilies is observed in some strains. Analysis of cell lines from NIH/Swiss and NFS/N inbred strains indicated that several members of the Q52 subfamily lie 3′ to the majority of the 7183 subfamily members, with some members of the two subfamilies actually interspersed (Kleinfield *et al.*, 1986; Reth *et al.*, 1986). In C57BL/6 mice, the majority of the relatively small 3609 subfamily is situated 3′ of the J558 subfamily, and a Q52 gene segment is interspersed into the 5′ J558 cluster. Deletional data also indicated that at least one 3609 gene segment is interspersed and located 3′ of the 3660 subfamily in A/J mice, and that the 3609 and J558 subfamilies may overlap in BALB/c mice (Rathbun *et al.*, 1987). These results suggest that at least some V_H subfamilies are organized into irregular clusters. These data better correlate with the organization of the human heavy chain locus, where subfamilies are totally interspersed throughout the

locus (see below). The exact size of the heavy chain locus is not known. Pulsed-field gel analysis indicated that the most proximal V_H gene segment (81X) is at least 300 kb away from the J_H gene segments (Taussig, 1987).

F. The Human Heavy Chain Gene Family

In humans, nine immunoglobulin heavy chain isotypes can be detected in normal serum. Eleven C_H gene segments, including two pseudogenes, have been cloned in the human heavy chain locus (Takahashi *et al.*, 1982; Krawinkel and Rabbitts, 1982; Max *et al.*, 1982) (see Fig. 6a). This is in contrast to the mouse, in which only eight C_H gene segments are found, with no pseudogenes present. In addition, two C_α gene segments are present in the human. Most of the genomic C_H gene segments have been sequenced: $C_\gamma1$ (Ellison *et al.*, 1982), $C_\gamma2$ (Ellison and Hood, 1982), $C_\gamma3$ (Huck *et al.*, 1986), $C_\gamma4$ (Ellison *et al.*, 1981), $C_\epsilon1$ (Flanagan and Rabbitts, 1982a; Max *et al.*, 1982; Nishida *et al.*, 1982), $C_\epsilon2$ (Max *et al.*, 1982), and $C_\alpha1$ and $C_\alpha2$ (Flanagan *et al.*, 1982). The constant region is encoded by four separate exons which correspond to functional domains C_H1, hinge, C_H2, and C_H3 (Takahashi *et al.*,

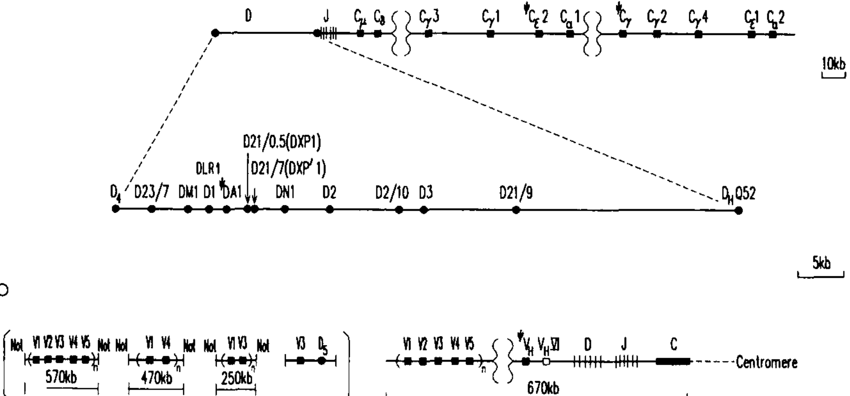


FIG. 6. Physical map of the human heavy chain locus. (a) Organization of the D_H, J_H, and C region genes. The D_H subregion is shown enlarged below; ψ indicates a pseudogene. DLR1 and D1, D21/0.5 (DXP1), and D21/7 (DXP'1) represent identical gene segments isolated from different laboratories. (b) Organization of the heavy chain locus is shown in five noncontiguous blocks. The relative order of the gene segments within the brackets has not been determined.

1982; Krawinkel and Rabbitts, 1982). The $C_\gamma 3$ gene is unique in that it possesses four separate hinge-coding segments (Takahashi *et al.*, 1982; Krawinkel and Rabbitts, 1982; Huck *et al.*, 1986). The four C_γ gene segments were found to have a high degree of homology (greater than 95% at the nucleotide level), and there is a C_γ pseudogene which lacks an acceptor RNA splice site.

There are nine human J_H gene segments located 5′ to the constant gene segments (Ravetch *et al.*, 1981). Six of the J_H gene segments are functional and three are pseudogene segments (only functional J_H gene segments are shown in Fig. 6a). The pseudo-J_H gene segments are interspersed with active J_H gene segments. The pseudo-J_H gene segment found in the mouse corresponds to one of the pseudo-J_H gene segments observed in the human locus, and its nucleotide sequence has drifted far from an active J_H gene segment. This suggests that this pseudo-J_H gene segment is the oldest pseudogene and may have become inactive before mammalian radiation exposure. The other two human pseudo-J_H gene segments have no homologues in the mouse.

Nucleotide sequence comparison of the most distal mouse D_H gene segment (D_HQ52) to the region 5′ of the human J_H region revealed one homologous human D_H gene segment (Ravetch *et al.*, 1981). Presently, 12 functional and one pseudo-D_H gene segments have been identified by cloning and sequencing (Siebenlist *et al.*, 1981; Ichihara *et al.*, 1988; Buluwela *et al.*, 1988) (see Fig. 6a).

The human V_H gene segments were initially divided into three subgroups (I, II, and III) based on amino acid sequences (Kabat *et al.*, 1987). Genomic clones containing nucleotide sequences corresponding to each of these subgroups have been reported. Genes from subgroups I and III have been isolated using homologous mouse V_H probes (Matthyssens and Rabbitts, 1980; Rechavi *et al.*, 1982, 1983), and a V_H gene segment belonging to subgroup II was isolated from an EBV-transformed human cell line (Takahashi *et al.*, 1984). Since 1987, V_H gene segments that are less than 65% homologous to members of the three known subgroups have been isolated from DNA libraries. These novel V_H gene segments were designated as V_HIV (Lee *et al.*, 1987; Berman *et al.*, 1988), V_HV (Shen *et al.*, 1987; Humphries *et al.*, 1988; Berman *et al.*, 1988), and V_HVI (Schroeder *et al.*, 1987; Berman *et al.*, 1988), consistent with the existing classification system.

Organization of the Human Heavy Chain

The constant region gene segments of the human heavy chain locus were determined to be closely linked on chromosome 14 by analysis of somatic cell hybrids (Croce *et al.*, 1979). Further studies on somatic cell hybrids and *in situ* hybridization experiments showed that the locus is

localized to chromosome band 14q32.3 (Cox *et al.*, 1982; Kirsch *et al.*, 1982; McBride *et al.*, 1982b). The human heavy chain locus was found to have the same chromosomal order as that of the mouse: centromere-C_H-J_H-D_H-V_H (Erikson *et al.*, 1982). Linkage analysis using restriction fragment length polymorphisms indicated that the V_H and C_H gene regions are located within 4000 kb of each other (Johnson *et al.*, 1984).

Figure 6a summarizes the organization of the C_H region as derived by deletion analysis (Migone *et al.*, 1984) and molecular cloning (Takahashi *et al.*, 1982; Flanagan and Rabbitts, 1982b). The order of the human C_H gene segments is similar to that in the mouse, in which the C_μ, C_δ, and $C_\gamma3$ gene segments are located on the 5′ side and the C_ϵ and C_α gene segments reside on the 3′ end (see Figs. 5a and 6a). However, it appears that a large multigene segment of the human constant region has been duplicated. Two gene clusters of similar organization can be observed. One cluster containing $C_\gamma3$-$C_\gamma1$-$C_\epsilon2$-$C_\alpha1$ is located 5′ to the second cluster, which contains $C_\gamma2$-$C_\gamma4$-$C_\epsilon1$-$C_\alpha2$ (Flanagan and Rabbitts, 1982b). Duplication of $C_\gamma2$ is also observed at a relatively high frequency in normal individuals (Bech-Hansen and Cox, 1986). In addition to the two C_ϵ gene segments located on chromosome 14, a third C_ϵ-like gene has been found and located on chromosome 9 (Battey *et al.*, 1982). The C_ϵ-like gene segment appears to be a processed pseudogene because it contains sequences homologous to the functional $C_\epsilon1$ gene segment but lacks introns.

The J_H cluster is located 5′ of the constant gene segments, with the most distal functional J_H gene segment about 6 kb upstream of the C_μ gene segment. A D_H gene segment homologous to the mouse DQ52 gene segment is located just upstream of the J_H region. Siebenlist *et al.* (1981) isolated a family of D_H gene segments (Dl, D2, D3, and D4) (Fig. 6a) and found that D_H gene segments are tandemly arranged in repeating units of 9 kb. Ishihara *et al.* (1988) sequenced one of the 9-kb repeating units and identified five D_H gene segments (DMl, DAl, DXPl, DXP′1, and DNl). DAl may be a pseudogene because the rearrangement heptamer and nonamer sequences are poorly recognizable. Additional D_H gene segments have been identified and mapped by analysis of chromosomal translocation of the heavy chain in Burkitt's lymphoma cell lines (Buluwela *et al.*, 1988). Overlapping cosmid clones containing the genomic region from D4 to C_μ have been isolated (Matsuda *et al.*, 1988).

A D_H gene segment, D5, with several unusual features has been isolated (Buluwela *et al.*, 1988; Matsuda *et al.*, 1988). The D5 gene segment has been physically linked to a pseudo-V_HIII gene segment located within 18 kb on the chromosome, and rearrangement or deletion

of the D5 gene segment has not been observed in any B cell lines. This suggests that the D5 gene segment is located on the distal side of the V_H locus and is interspersed with the V_H gene segments (Fig. 6b).

In the mouse, V_κ and V_H gene subfamilies are thought to be organized in clusters along the chromosomes (see above). However, the organization of the human V_κ gene subfamilies is interspersed (see above), and a similar interspersed organization is found for the human heavy chain locus (Kodaira *et al.*, 1986; Berman *et al.*, 1988; Humphries *et al.*, 1988). Kodaira *et al.* (1986) isolated 23 cosmid clones containing 61 V_H gene segments which span approximately 1000 kb of genomic DNA. V_H gene segments of different subgroups were found to be present in the same clone. Detailed restriction enzyme analysis of these cosmid clones revealed that V_H subgroups are highly interspersed. One of these cosmid clones contained two V_HI, two V_HII, and three V_HIII gene segments (Kodaira *et al.*, 1986). Berman *et al.* (1988) confirmed these results and showed that V_HIV gene segments also are interspersed with the other subgroups. The transcriptional orientation of the V_H gene segments within one of the clusters has been determined by nucleotide sequencing and was found to be in the same direction (Kodaira *et al.*, 1986).

Pulsed-field gel electrophoresis has been used to estimate the distances between the heavy chain gene segments and the size of the entire locus. Surprisingly, V_HVI and C_μ probes were found to hybridize to the same 90-kb SpeI fragment. Further analysis using partial and double restriction enzyme digestions confirmed that the V_HVI gene segment is located within 90 kb of the C region (Berman *et al.*, 1988). Cosmid cloning has physically linked the V_HVI gene segment to 20 kb 5′ of the D4 gene segment, and the V_HVI gene segment has the same transcriptional orientation as the constant region (Sato *et al.*, 1988). Recently, a pseudo-V_HIII gene segment also has been isolated and mapped to a location 105 kb 5′ of the C_μ gene segment (Buluwela *et al.*, 1988). The size of the heavy chain locus has been estimated by cosmid cloning to be at least 1000 kb (Kodaira *et al.*, 1986). Estimation of pulsed-field gel electrophoresis with restriction enzymes NotI (Berman *et al.*, 1988) and MluI (Matsuda *et al.*, 1988) indicated that the size of the locus is approximately 2000 to 3000 kb, assuming that the restriction fragments are arranged contiguously. The organization of the human heavy chain locus is summarized in Fig. 6b. Since the detailed organizations of each subregion have not been determined, each restriction fragment is shown with the unordered V_H subgroup members which it contains.

It has been estimated that in the mouse heavy chain locus, up to 40% of the V_H gene segment repertoire may be pseudogenes (see above).

Nucleotide sequencing of germ line V_H gene segments reveals that 13 out of 38 (34%) V_H gene segments appeared to be nonfunctional (Kodaira *et al.*, 1986; Lee *et al.*, 1987; Berman *et al.*, 1988; Humphries *et al.*, 1988). This suggests that the percentage of pseudo-V_H gene segments is similar in mice and humans. However, the distribution of pseudogenes does not appear to be random among the subgroups, and no pseudogene has been found in the V_HIV subgroup (Lee *et al.*, 1987).

V. Structure and Organization of the Major Histocompatibility Complexes

The Major Histocompatibility Complex, the *H-2* locus in mouse and HLA locus in human, has been divided into class I, II, and III genes on the basis of structural and functional similarities (Klein, 1975; Klein *et al.*, 1983; Schwartz, 1985; Steinmetz and Hood, 1983). The class I and class II molecules are integral membrane proteins involved in the immune recognition reactions that permit T cells to distinguish between self and nonself. Class I molecules are present on the surface of all cell types (Klein, 1975), whereas class II molecules are expressed only on the surface of cells of lymphatic origin, such as B cells, macrophages, dentritic cells, and certain epithelial cells. Class III genes encode several components of the classical complement pathway, factor B of the alternate complement pathway, and an enzyme involved in steroid biosynthesis, 21-hydroxylase. The Major Histocompatibility Complexes have been divided into discrete regions and subregions on the chromosome. Each region contains one or more class I, II, or III genes. Regions which contain class I genes are *K, D, Qa*, and *Tla* in mice and HLA-A, HLA-B, HLA-C, and HLA-E in humans. Class II genes are located in the *I* region in mice and HLA-D region in humans. Class III genes are located in the *S* region in mice and between the HLA-D and HLA-B regions in humans. The structure and expression of these genes have been extensively reviewed (Steinmetz and Hood, 1983; Flavell *et al.*, 1985; Whitehead and Sackstein, 1985; Flavell *et al.*, 1986; Giles and Capra, 1985; Auffray and Strominger, 1986) and will be discussed only briefly in this review. Instead, we will summarize the physical map of the two loci with the latest data obtained from pulsed-field gel electrophoresis.

A. MOUSE *H-2* COMPLEX

The class I molecules that are expressed on the surfaces of all cell types are encoded by genes in the *K, D*, and *L* regions. These molecules are highly polymorphic and serve as the restriction elements for cytotoxic T cells (Klein, 1975). In contrast, protein products of the *Qa* and *Tla*

regions are less polymorphic (Yokoyama *et al.*, 1981) and their expression is limited to certain tissues (Flaherty, 1981; Coligan *et al.*, 1981). In addition, these molecules are probably not involved in recognition by T cells and their function is unknown. Other genes that have mapped telomeric to *Qa* and are similar in structure to the class I genes have also been described (Singer *et al.*, 1988).

The class II molecules I-A and I-E are encoded by genes A_α, A_β, E_α, and E_β of the *I* region (Jones *et al.*, 1978). These class II molecules function as presenting and restricting elements for the regulatory T cells (helper), and govern the immune responsiveness to certain foreign antigens (Benacerraf and McDevitt, 1972). There are two additional β-related sequences, $A_\beta 2$ and $E_\beta 2$, the functions of which are unknown (Braunstein and Germain, 1985; Larhammer *et al.*, 1985a). A pseudogene, $A_\beta 3$, is located aproximately 90 kb distal to the *K* gene (Widera and Flavell, 1985).

The murine *S* region codes for the following class III proteins: the C2 and C4 components of the classical complement pathway (Chaplin *et al.*, 1983), factor B of the alternate complement pathway (Chaplin *et al.*, 1983), sex-limited protein (SLP), a hemolytically inactive C4-like serum protein found in males of certain strains (Shreffler, 1982), and 21-hydroxylase (White *et al.*, 1984).

The organization of the *H-2* complex of BALB/c mice is shown in Fig. 7. Two class I genes have been found in the *K* region; the *K* gene encodes the class I H2-K protein. Distal to the *K* region lies the *I* region, which encodes the class II proteins. The *K* and *I* regions have been physically linked on cosmid clones (Steinmetz *et al.*, 1986). The distance

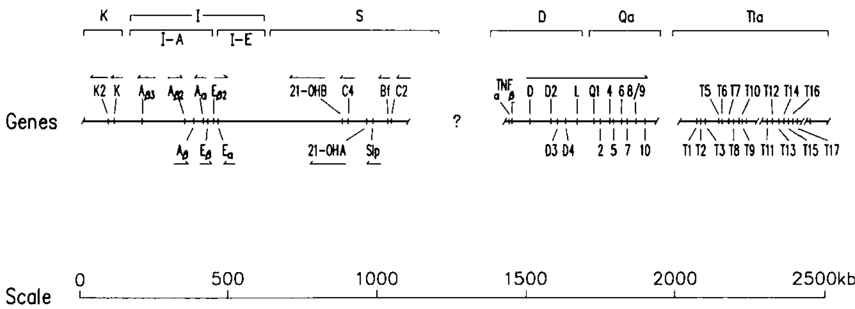


FIG. 7. Physical map of the mouse *H-2* Major Histocompatibility Complex. The orientations of the genes are shown as arrows above the genes. The distance between *S* and *D* has not been determined.

between the *I* and *S* regions was estimated by pulsed-field gel electrophoresis to be 430 kb (Müller *et al.*, 1987b). The transcriptional orientation of the *S* region genes are the same as the *K* region genes, but opposite to that of the *D* region. Müller *et al.* (1987a) have found that tumor necrosis factor (TNF-α) and lymphotoxin (TNF-β) are located within the *H-2* complex and map close to the *D* region (see Fig. 7). The transcriptional orientation of the TNF-α and -β genes is opposite to that of the class I *H2-D* genes. The exact distance between the *S* and *D* regions has been estimated to be at least 420 kb by pulsed-field gel electrophoretic experiments (Müller *et al.*, 1987b). The *D* and *Qa* regions have been cloned and mapped by Stephan *et al.* (1986). There are five *D* and eight *Qa* class I genes in this region. The *D* and *L* genes encode the H2-D and H2-L proteins, respectively. The *Tla* regions have been mapped distal to the *Qa* region by restriction fragment length polymorphism (Winoto *et al.*, 1983). There are two clusters of *Tla* genes which probably arose from duplication (Hammerling *et al.*, 1985). The distances between the *Tla* and *Qa* regions have not been determined; however, recent data suggested that the *Tla* cluster containing *T1* through *T11* is located within 500 kb of the *Qa* region, and the two *Tla* clusters are within 250 kb (E. Straus, S. Hunt, E. Lai, and L. Hood, unpublished results). These data suggested that the *H-2* complex spans at least 2500 kb on the chromosome.

It appears that the organization of the *H-2* complex is variable among different inbred mouse strains. While five *D* class I genes have been found in the BALB/c mouse, only one has been found in AKR and C57BL/10 mice (Weiss *et al.*, 1984). Thus, the *D2*, *D3*, and *D4* genes are not essential, and whether they are functional remains to be determined. There are only 8 *Qa* genes in BALB/c mice, in contrast to 10 genes in the C57BL/6 *Qa* region. The *Q3* gene is missing in the BALB/c strain, and the *Q8* and *Q9* genes have been fused into a *Q8/Q9* hybrid gene (Weiss *et al.*, 1984). The greatest differences among the C57BL/10 and BALB/c mice are in their *Tla* regions. The *T1* through *T10* cluster is present in both C57BL/10 and BALB/c, but the second cluster, *T11* through *T17*, is absent in C57BL/10 mice (Weiss *et al.*, 1984). It appears that part of the *Tla* region has been duplicated in the BALB/c genome.

B. Human HLA Complex

The human HLA complex has been localized to the short arm of chromosome 6 (Lamm *et al.*, 1974; van Someren *et al.*, 1974; Francke and Pellegrino, 1977). Morton *et al.* (1984) determined the chromosomal orientation of the HLA complex to be centromere–class II–class I by *in situ* hybridization, and located the HLA complex to band p21.3. In

addition to the HLA-linked genes, a number of structurally related molecules have been found which are not mapped to chromosome 6 (Calabi and Milstein, 1986).

The human class I molecules which have been detected serologically are encoded by genes located in the HLA-A,B,C regions which correspond to the murine H-2-K,D,L antigens. Southern blot hybridization and cloning experiments have shown that there are 20 to 30 class I genes in the HLA, and individual HLA-A,B,C genes have been identified by gene transfer experiments and nucleotide sequence analysis (Srivastava *et al.*, 1985, 1987). Recently, another class I gene, HLA-E, which is expressed in resting T cells, has been isolated and mapped between the HLA-A and HLA-C loci (Carroll *et al.*, 1987; Koller *et al.*, 1988) (Fig. 8a). Similar to the mouse *Qa* and *Tla* region class I genes, the function and expression of most of the non-HLA-A,B,C genes are not yet characterized.

The class II molecules are encoded by genes in the HLA-D region and, based on serological experiments, can be subdivided into three families: DR, DQ and DP. The DQ and DR molecules correspond to

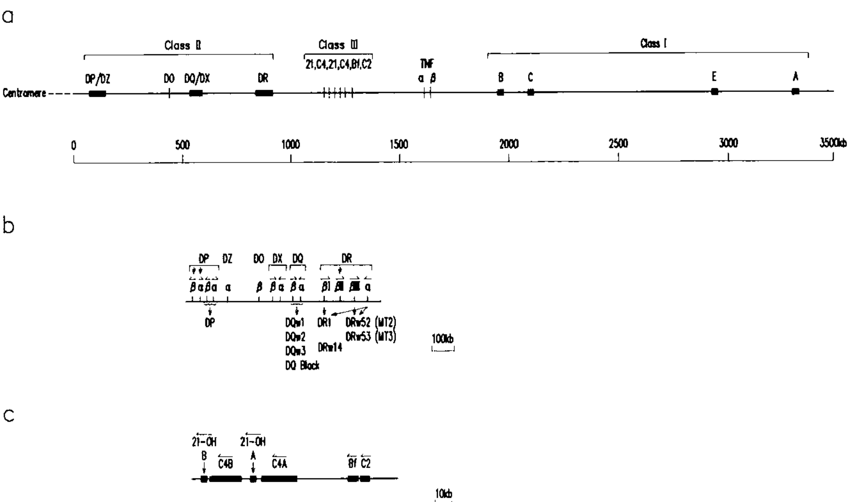


FIG. 8. Physical maps of the human HLA Major Histocompatibility Complex. (a) General organization of the locus. (b) Detail of the organization of the HLA-D subregion. The specificities of the gene products are shown below the genes. (c) Detail of the organization of the class III subregion. There are two copies of the C4 and 21-hydroxylase genes.

the murine I-A and I-E antigens, respectively. No known homologue of the DP molecule has been found in the mouse. Four genes have been cloned that belong to the DR family (Spies *et al.*, 1985). The DRα gene is characterized by an almost complete lack of sequence variability among humans (Rask *et al.*, 1985), and combines with one of the two functional DRβ genes to form the DR antigen. One of the DRβ genes appears to be a pseudogene (Larhammar *et al.*, 1985b; Spies *et al.*, 1985). An additional DRβ pseudogene has been isolated from an individual with the DR4 haplotype (Andersson *et al.*, 1987). The DQ region contains two α (DQ and DX) and two β (DQ and DX) genes. In contrast with the DRα gene, both DQα and DXα genes are extremely polymorphic (Chang *et al.*, 1983; Trowsdale *et al.*, 1983; Auffray *et al.*, 1984; Schenning *et al.*, 1984; Spielman *et al.*, 1984; LeGall *et al.*, 1985). The DQα and DQβ genes encode the DQ antigens that are detected by serological methods. Analysis of the nucleotide sequences of the DXα and DXβ genes has not revealed any pseudogene features that would prevent their expression (Auffray *et al.*, 1984; Okada *et al.*, 1985a; Jonsson *et al.*, 1987). However, neither mRNA nor protein products derived from the DX genes have been identified. Similar to the DQ region, there are two α and two β genes in the DP region (Auffray *et al.*, 1984; Trowsdale *et al.*, 1984; Okada *et al.*, 1985b). One set of α and β genes encodes the DP antigen (Roux-Dosseto *et al.*, 1983; Gustafsson *et al.*, 1984; Kappes *et al.*, 1984; Auffray *et al.*, 1984), and the second set of α and β consists of pseudogenes (Boss *et al.*, 1985; Servenius *et al.*, 1985). Two more genes, DZα (Trowsdale and Kelly, 1985) and DOβ (Inoko *et al.*, 1984; Tonelle *et al.*, 1985; Servenius *et al.*, 1987), have been described and mapped to the class II region (Fig. 8b).

Like that of the mouse, the human class III region contains genes which encode C2, C4, factor B (Carroll *et al.*, 1984), and 21-hydroxylase (Carroll *et al.*, 1985; White *et al.*, 1985), all of which are in the same orientation within the region (Fig. 8c). The class III region has been mapped in between the class I and class II regions by analysis of deletional cell lines (Whitehead *et al.*, 1985).

The organization of the human HLA locus is shown in Fig. 8a. The class I regions, A, B, C, and E, are located in the distal end of the locus. This is in contrast to that of the murine *H-2* locus, where the class I regions are found on both sides of the locus. The murine *H2-K* region may have been translocated to the 5′ side of the MHC locus after the mammalian radiation event. Clusters of cosmid clones containing class I genes have been isolated, but linkage of these clusters has not been determined (Barbosa *et al.*, 1982; Malissen *et al.*, 1982; Orr and DeMars,

1983). Analysis by pulsed-field gels has indicated that the class I genes may span over 1500 kb (Lawrance *et al.*, 1987; Carroll *et al.*, 1987).

The class II region has been mapped to the centromeric side of the locus by deletion analysis (Levine *et al.*, 1985) and *in situ* hybridization experiments (Morton *et al.*, 1984). The class II regions are present on the centromeric side of the locus and are arranged in the order 5′-DP-DQ-DR-3′ (Fig. 8a and b). Genes in the DR (Spies *et al.*, 1985; Rollini *et al.*, 1985; Andersson *et al.*, 1987), DQ (Auffray *et al.*, 1984; Okada *et al.*, 1985a; Jonsson *et al.*, 1987), and DP (Auffray *et al.*, 1984; Trowsdale *et al.*, 1984; Okada *et al.*, 1985b; Roux-Dosseto *et al.*, 1983; Gustafsson *et al.*, 1984, 1987; Boss *et al.*, 1985; Servenius *et al.*, 1985) subregions have been isolated as genomic clones. However, efforts to link these subregions have been unsuccessful because of the large size of the locus and the frequency of repeat sequences, both of which make chromosomal "walking" difficult. The development of pulsed-field gel electrophoresis has permitted the rapid mapping and ordering of these subregions on the chromosome. Hardy *et al.* (1986) reported the first successful use of the pulsed-field technology to map the order and distances of the human class II subregions (Fig. 8a and b). The DRα gene is located 3′ of the three DRβ genes, and the DR subregion is most telomeric (i.e., 3′). The DQ region is centromeric (i.e., 5′) to the DR subregion, with the DXα and DXβ genes 5′ to the DQα and DQβ pairs. DOβ is 5′ to DQ, and DZα is 5′ to DO but 3′ to DP. The DP pseudogenes form the centromeric ends of the HLA locus. These results have been confirmed by Carroll *et al.* (1987).

The class III region has been isolated as clusters of overlapping cosmid clones by a number of groups (Carroll *et al.*, 1984, 1985; White *et al.*, 1985; Dunham *et al.*, 1987). The region spans about 100 kb and contains the genes which encode the second (C2) component and two copies of the fourth (C4A and C4B) component of complement, factor B, and two copies of the gene for steroid 21-hydroxylase (Fig. 8c). The orientation of the class III region with respect to the DR region has been determined by pulsed-field gel electrophoresis. The 21-hydroxylase B gene was found to be on the centromeric side and is about 300 kb distal to the DRα gene (Dunham *et al.*, 1987; Lawrance *et al.*, 1987; Carroll *et al.*, 1987) (Fig. 8a).

Similar to what has been found in the mouse, genes for TNFα and TNFβ have been isolated and mapped to the HLA locus between the DR and HLA-A regions (Spies *et al.*, 1986). Long-range physical mapping has shown that TNFα and TNFβ genes are located between the class III and class I regions. The distance between the C2 gene and TNFα is

about 350 to 390 kb, and the HLA-B gene lies approximately 200 to 250 kb distal to the TNFβ gene (Dunham *et al.*, 1987; Carroll *et al.*, 1987; Ragoussis *et al.*, 1988) (Fig. 8a).

Taken together, these data indicate that the human Major Histocompatibility Complex spans about 3500 kb (Lawrance *et al.*, 1987; Dunham *et al.*, 1987; Carroll *et al.*, 1987) (see Fig. 8a). The estimated total size of the human HLA locus is thus almost twice that of the mouse *H-2* locus (see above). The most significant difference in size was between the class I regions, where the homologous region in the mouse was about one-third that of man, despite the fact that genes homologous to *Qa* and *Tla* have not yet been found in humans. The organization of the human Major Histocompatibility Complex is very similar to that of the mouse except for the translocation of the murine *H-2K* region. The location and transcriptional orientation of the class III genes also are the same in mouse and human.

VI. Structure and Organization of T-Cell Receptor Gene Loci

The T cell antigen receptor (TcR) is a 90-kDa disulfide-linked heterodimeric membrane-bound glycoprotein composed of α and β (Meuer *et al.*, 1983) or γ and δ chains (Brenner *et al.*, 1986, 1987). Like immunoglobulins, the T cell receptor is encoded by discontinuous variable, diversity, and joining gene segments which rearrange upstream to the constant gene during the course of T cell development, creating the immense diversity of receptor molecules required for T cells to respond to a virtually limitless universe of foreign antigens. The T cell receptor functions as an activating mechanism which triggers T cell response to cognate antigen only in the context of cell-surface molecules encoded by the genes of the MHC (Zinkernagel and Doherty, 1974; Rosenthal and Shevach, 1973; Katz *et al.*, 1973; Kindred and Screffler, 1972). The mechanism by which this dual specificity operates is still not clear. The α-β T cell receptor is present on the majority of mature $CD4^+$ and $CD8^+$ lymphocytes in association with the monomorphic CD3 complex. A second receptor, the γ-δ heterodimer, is present on $CD4^-$, $CD8^-$ T lymphocytes, some thymocytes, and dendritic epidermal cells (Brenner *et al.*, 1986, 1987; Weiss *et al.*, 1986; Lanier *et al.*, 1987; Lew *et al.*, 1986; Koning *et al.*, 1987).

Since the initial molecular characterization of T cell receptor-specific cDNAs (Yanagi *et al.*, 1984; Hedrick *et al.*, 1984; Saito *et al.*, 1984a,b; Chien *et al.*, 1984a), much has been learned about the germ line organization and repertoire of the α, β, and γ chain gene families in mice and humans (reviewed in Kronenberg *et al.*, 1986; Wilson *et al.*,

1988). More recently, gene segments encoding the mouse and human δ chains have been characterized and are located within the α-chain locus (Chien *et al.*, 1987a; Hata *et al.*, 1987; E. Y. Loh *et al.*, 1987).

A. β-Chain Gene Families

Functional mouse and human T cell receptor β-chain variable regions are encoded by V_β, D_β, and J_β gene segments. The V_β gene segments consist of two exons separated by an intron of 90 to 400 bp. The first exon encodes a small hydrophobic leader peptide of 16 to 29 amino acids. The second exon is approximately 250 to 350 bp and encodes the remainder of the variable domain. The D_β and J_β gene segments are quite small, encoding 4 to 6 and 15 to 16 amino acids, respectively. Unlike immunoglobulin D_H gene segments, the D_β gene segments may be translated in all three reading frames (Goverman *et al.*, 1985; Barth *et al.*, 1985; Toyonaga *et al.*, 1985). The mouse and human C_β region genes are composed of four exons that, unlike the immunoglobulin C genes, do not represent the four presumed functional domains of the constant region. The extracellular domain and the first few amino acids of the connecting peptide are encoded within the large first exon. The complete human C_β region contains 173 amino acids. The T cell receptor β-chain polypeptide contains two intrachain disulfide linkages; one spans 63 to 69 residues of the variable region, and a second spans 60 residues in the extracellular domain of the constant region. Furthermore, many of the amino acids thought to be important in the formation of the immunoglobulin homology unit and in stabilizing heavy and light chain interactions in immunoglobulins are conserved in the T cell receptor β chain.

1. *Organization of the Mouse β-Chain Locus*

The mouse β-chain gene family is located at chromosomal position 6B (Caccia *et al.*, 1984; Lee *et al.*, 1984) (Table I). As shown in Fig. 9, the mouse β-chain locus extends over 700 kb. The D_β and J_β gene segments and C_β genes are arranged in tandem clusters located downstream from the 3′-most V gene segment. Each cluster contains one D_β gene segment, seven J_β gene segments, and one C_β gene (Gascoigne *et al.*, 1984; Malissen *et al.*, 1984; Siu *et al.*, 1984a; Kavaler *et al.*, 1984). Nearly 100 rearranged V_β genes have been analyzed to date and these represent 22 unique V_β gene segments (Wilson *et al.*, 1988). Statistical analyses have indicated that there are probably no more than 30 V_β gene segments in the mouse (Patten *et al.*, 1984; Barth *et al.*, 1985; Behlke *et al.*, 1985). The 22 known V_β gene segments may be grouped into 17 different subfamilies, with each subfamily generally

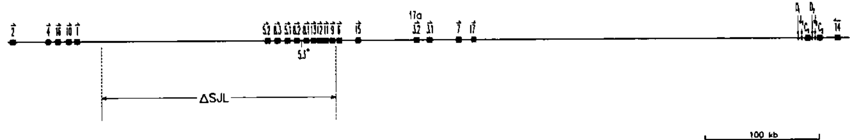


FIG. 9. Physical map of the mouse T cell receptor β-chain locus. The V_β and C_β gene elements are indicated by solid boxes; the D and J gene segment clusters are indicated by lines. Transcriptional orientation is indicated for all V_β gene segments; $V_\beta5.3^*$ is a pseudogene. The region of the β-chain locus that is deleted in SJL mice is indicated by ΔSJL. The $V_\beta3.2$ gene segment is 76% homologous to $V_\beta3.1$ and thus should belong to the $V_\beta3$ subfamily; $V_\beta3.2$ has also been referred to as $V_\beta17a$ (Kappler *et al.*, 1987). A V_β gene segment isolated by Singer *et al.* (1986) has been renamed as $V_\beta17$ in agreement with the existing nomenclature.

defined as a set of V gene segments sharing greater than 75% nucleotide homology (Wilson *et al.*, 1988).

The mouse β-chain family is unique among the immunoglobulin-like loci in that most of the subfamilies contain only a single member. The organization of the β-chain locus has been determined by deletional analysis of T cell lines, pulsed-field gel electrophoresis, and restriction mapping of cosmid clones (Chou *et al.*, 1987a,b; Lai *et al.*, 1987; Linsten *et al.*, 1987; Lee and Davis, 1988). The distance between the 3′-most V_β gene segment and the constant region has been estimated to be from 100 (Lai *et al.*, 1987) to over 250 kb (Chou *et al.*, 1987b; Linsten *et al.*, 1987; Lee and Davis, 1988). It appears that the distance probably is closer to 250 kb, and two comigrating fragments of identical sizes were detected in L cells with $V_\beta3$ and C_β probes (E. Lai, unpublished results). Members of the three multigene subfamilies were found to be located close to each other. The two three-member subfamilies, $V_\beta5$ and $V_\beta8$, have their members interspersed along the chromosome. Thus, the organization of the mouse β chain closely resembles that of the human IgH and TcR β loci, and not the mouse IgH. In SJL, C58, and C57L mice, almost half of the β gene segments have been deleted from the genome and it appears that a single deletion event has occurred in these strains.

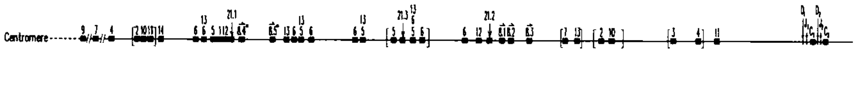
2. *Organization of the Human β-Chain Locus*

The human β-chain gene family is located at chromosomal position 7q32–35 (Table I). The overall organizations of the human and mouse β-chain loci are quite similar, and it appears that the relative order of homologous V_β gene segments is conserved (Lai *et al.*, 1988). DNA sequence analysis has identified 79 unique V_β gene segments that may

be grouped into 21 subfamilies, with 15 subfamilies containing a single member (Ikuta *et al.*, 1985; Leiden and Strominger, 1986; Tillinghast *et al.*, 1986; Concannon *et al.*, 1986; Kimura *et al.*, 1986a; reviewed in Wilson *et al.*, 1988). A physical map of the human β-chain locus is shown in Fig. 10. Using deletional analysis of T cell leukemia DNA, long-range restriction mapping by field-inversion gel electrophoresis, and fine-restriction mapping of cosmid clones, over 40 V_β gene segments were mapped to a 600-kb SalI restriction fragment. In contrast to the mouse β-chain locus, all of the human V_β gene segments mapped to date lie 5′ to the constant region genes.

The V gene segments of the human β-chain locus appear to have evolved by a process of duplication and subsequent divergence since the time of the mammalian radiation event (Lai *et al.*, 1988; Wilson *et al.*, 1988). In some cases, tandem duplications of V_β gene segments have led to the interspersion of small clusters of nonhomologous V_β gene segments. An example of this is the $V_\beta 5$, 6, 13 cluster, of which four are found scattered across the locus. Other tandem duplication events appear to have served only to increase the number of members in an individual V_β gene segment subfamily. An example of this is the closely spaced grouping of $V_\beta 8$ subfamily members.

Comparison of the human and mouse β-chain loci reveals that both species have a similar number of V_β gene segment subfamilies. However, the average number of members per subfamily is about four in humans versus mostly single-member subfamilies in mice. This is reflected in the size of the two loci. All known mouse V_β gene segments and the constant region genes have been mapped to approximately 700 kb of DNA, whereas in humans, a 600-kb region of DNA contains only about half of the known V_β gene segments. Despite the difference in size, the organization of the β-chain loci appears to be well conserved in human and mouse (Lai *et al.*, 1988).



100 kb

FIG. 10. Physical map of the human T cell receptor β-chain locus. The V_β and C_β gene elements are indicated by solid boxes. Unordered V_β gene segments are enclosed by brackets. Two pseudogenes, $V_\beta 8.4$ and 8.5 are indicated with an asterisk. Transcriptional orientation is indicated where known.

B. γ-CHAIN GENE FAMILIES

Functional mouse and human T cell receptor γ-chain variable regions are encoded by V_γ and J_γ gene segments. Like the V_β gene segments, V_γ gene segments are encoded by two exons separated by a small intron. The J_γ gene segments add an additional 15 to 25 amino acids to the γ-chain variable region.

The four mouse C_γ region genes each are encoded by three exons. The first exon is quite large, encoding 110 amino acids (Garman *et al.*, 1986). The second exon encodes 10 to 15 amino acids and the third exon encodes 46 amino acids. The $C_\gamma 2$ gene appears to be a pseudogene and has a defective 5′ splice site bordering the second exon (Garman *et al.*, 1986). The $C_\gamma 1$ and $C_\gamma 3$ genes are over 96% homologous at the level of amino acid sequence. However, the $C_\gamma 1$ gene contains a 15-bp insertion in exon 2 which encodes five additional residues (Garman *et al.*, 1986).

1. *Organization of the Mouse γ-Chain Locus*

The genes for the mouse T cell receptor γ chain are located at chromosomal position 13A2–3 (Table I) and span at least 280 kb. The organization of the mouse γ-chain locus is quite different from the other T cell receptor loci and contains interspersed V and J gene segments as shown in Fig. 11. This organization pattern is similar to that of the

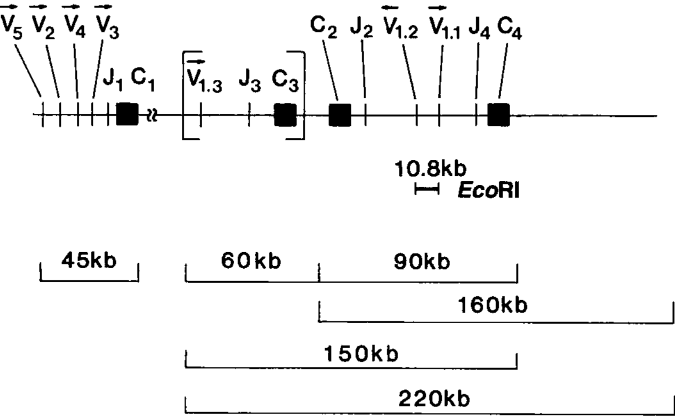


FIG. 11. Physical map of the mouse T cell receptor γ-chain locus. The sizes of various exons and the relative distances between them are not drawn to scale. The transcriptional orientations of the V_γ gene segments are shown as horizontal arrows. For exons included in brackets, the orientation has not been determined relative to the rest of the γ genes.

mouse immunoglobulin λ light chain locus (Eisen and Reilly, 1985) and the shark immunoglobulin heavy chain locus (Hinds and Litman, 1986). A limited number of V_γ gene segments are located 5′ to a single J_γ gene segment and a single C_γ gene in four apparent clusters. At least one of these clusters appears to be in reverse translation orientation relative to the others (Woolf *et al.*, 1988). Rearrangement of V_γ and J_γ gene segments tends to occur within the immediate cluster (Garman *et al.*, 1986; Iwamoto *et al.*, 1986; Kranz *et al.*, 1985; Kronenberg *et al.*, 1989). For example, $V_\gamma5$, $V_\gamma2$, $V_\gamma4$, and $V_\gamma3$ predominantly rearrange to $J_\gamma1$, $V_\gamma1.1$ predominantly rearranges to $J_\gamma4$, and $V_\gamma1.2$ predominantly rearranges to $J_\gamma2$ (Iwamoto *et al.*, 1986; Kronenberg *et al.*, 1989). An exception to this pattern has been reported for one chromosome of the T lymphoma BW5147, which contains a $V_\gamma5$ to $J_\gamma4$ rearrangement that has deleted all of the intervening γ gene segments (Pelkonen *et al.*, 1987). A simplified nomenclature for mouse V_γ gene segments, based on nucleotide sequence homology, is proposed in Table III.

2. *Organization of the Human γ-Chain Locus*

The human γ-chain locus is found at chromosomal position 7p15 as shown in Table I. Compared to the mouse γ-chain locus, the genes encoding the human T cell receptor γ chain are organized in a manner similar to the human and mouse β-chain loci, and are contained in a 160-kb region of DNA (Strauss *et al.*, 1987). The physical map of the human T cell receptor γ-chain locus is shown in Fig. 12. To date, 14 human V_γ gene segments, including four pseudogenes, have been identified and mapped within the locus (LeFranc *et al.*, 1986; Forster *et al.*, 1987; Huck *et al.*, 1988; Kimura *et al.*, 1987b; Strauss *et al.*, 1987). In an effort to simplify the nomenclature of the human V_γ gene segments, we propose the numbering system listed in Table IV. This numbering system groups the V_γ gene segments into subfamilies, based on nucleotide sequence homology. The human γ-chain locus also includes

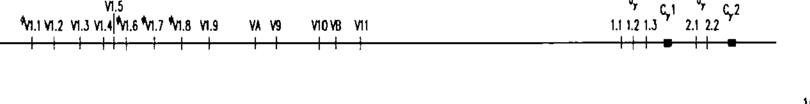


FIG. 12. Physical map of the human T cell receptor γ chain. The whole locus is contained within 150 kb. The exact distances between the V_γ gene segments and from V_γ to J_γ are not known. $V_\gamma1.6$, 1.7, 1.8, VA, and VB are pseudogenes (ψ).

TABLE III
CORRESPONDENCE BETWEEN THE DIFFERENT MOUSE V_γ GENE NOMENCLATURES

Gene	Hayday *et al.* (1985a)	Traunecker *et al.* (1986)	Garman *et al.* (1986)	Heilig and Tonegawa (1986)	Iwamoto *et al.* (1986)	Pelkonen *et al.* (1987)
$V_\gamma1.1$	V10.8B	V2	V1.1	V1	V1	V2
$V_\gamma1.2$	V10.8A	V1	V1.2	V2	V2	V1
$V_\gamma1.3$	V5.7	V3	V1.3	V3	V3	V3
$V_\gamma2$	—	V4	V2	V4	V6	V4.3
$V_\gamma3$	—	—	V3	V5	V4	V4.2
$V_\gamma4$	—	—	V4	—	V5	V4.1
$V_\gamma5$	—	—	—	—	—	V4.4

TABLE IV
CORRESPONDENCE BETWEEN THE DIFFERENT HUMAN V_γ GENE NOMENCLATURES

Gene	LeFranc *et al.* (1986); Forster *et al.* (1987); Huck *et al.* (1988)	Kimura *et al.* (1987b)	Strauss *et al.* (1987)
V1.1	V1	V1	1.1
V1.2	V2	V2	1.2
V1.3	V3	V3	1.3
V1.4	V4	V4	1.4
V1.5	V5	—	—
V1.6	V5	V5	1.5
V1.7	V6	V6	1.6
V1.8	V7	V7	1.7
V1.9	V8	V8	1.8
V2	V9	V9	V2
V3	V10	V10	V3
V4	V11	V11	V4
VA	VA	V9	—
VB	VB	VB	—

five J_γ gene segments and two C_γ genes that are arranged in two clusters approximately 50 kb distal to the 3′-most V_γ gene segment (Strauss *et al.*, 1987). The first cluster contains three and the second cluster contains two J_γ gene segments. The J_γ–C_γ clusters span slightly more than 30 kb of DNA. Like most of the other immunoglobulin-like loci, there are no V_γ gene segments located 3′ of the constant region genes.

C. α- AND δ-CHAIN GENE FAMILIES

Functional mouse and human T cell receptor α-chain variable regions are encoded by V_α and J_α gene segments. Like the V_β and V_γ gene segments described above, V_α gene segments consist of a small leader exon and a large second exon separated by an intron of 100 to 400 bp. An additional 16 to 25 amino acids of the α-chain variable domain are encoded by the J_α gene segments. The single C_α region gene is composed of four exons that, like the C_β, do not reflect separate functional domains. Furthermore, the extracellular domain of the C_α polypeptide is relatively short and the fourth exon encodes only 3′ untranslated sequences. The complete human C_α region contains 112 amino acids. Similar to the β chain, the T cell receptor α-chain polypeptide also contains variable and constant domain-spanning intrachain disulfide linkages.

The most recently discovered T cell receptor gene family encodes the

δ-chain polypeptide and is located completely within the α-chain locus in both mice and humans (Chien *et al.*, 1987b; Hata *et al.*, 1987; D. Loh *et al.*, 1987). Functional mouse and human δ-chain variable regions are encoded by V_δ, D_δ, and J_δ gene segments. Preliminary studies have indicated that the T cell receptor δ chain also can utilize at least one V_α gene segment (Chien *et al.*, 1987b; Gugliemi *et al.*, 1988). Furthermore, the two mouse D_δ gene segments may, like their D_β counterparts, be read in all three reading frames. This may also be true of the human germ line D_δ gene segments. Unlike the other T cell receptor genes, the δ-chain variable region may be assembled from the novel combination of four (V, $D_\delta 1$, $D_\delta 2$, and J_δ) gene segments (Chien *et al.*, 1987a; Boehm *et al.*, 1988). This novel joining results in additional diversity of the final δ-chain variable region.

1. *Organization of the Mouse α- and δ-Chain Loci*

The mouse α- and δ-chain gene families are located at chromosomal position 14C–D as summarized in Table I. The physical map of these loci is shown in Fig. 13. DNA sequence analysis has identified 35 unique V_α gene segments, which may be grouped into 13 subfamilies, all of which have multiple members, ranging from 2 to 10 (Becker *et al.*, 1985; Arden *et al.*, 1985; Wilson *et al.*, 1988). In contrast, only 3 of 17 mouse V_β gene segment subfamilies have multiple members. Preliminary data indicate that V_α gene segment subfamily members may be interspersed as previously observed for the mouse β-chain locus (K. Wang, M. Kronenberg, and E. Lai, unpublished results). However, in contrast to the mouse β-chain locus, it also appears that some V_α gene segments have been duplicated over as much as 100 kb of DNA. This type of long-range duplication also has been observed in the human β-chain locus (Lai *et al.*, 1988). The repertoire of J_α gene segments has been estimated at 50, although only 32 have yet been sequenced (Becker *et al.*, 1985;

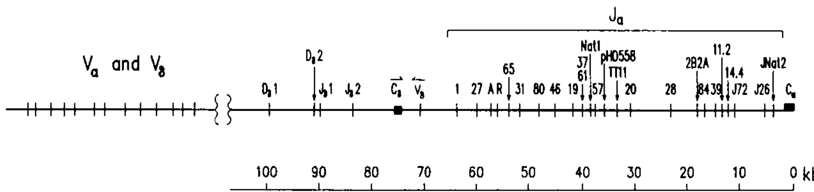


FIG. 13. Physical map of the mouse α/δ locus. [For details on J_α sequences, see Arden *et al.* (1985), Winoto *et al.* (1985) (all numbers), Dembic *et al.* (1986) (JNat2 and Natl), and Hochgeschwender *et al.* (1987) (11.2, 14.4).]

Arden *et al.*, 1985). The structure of the J_α gene segments is different than the J_β and immunoglobulin J gene segments in that the J_α gene segments are substantially longer. However, the J_α gene segments contain many of the conserved residues that are present in the J_β and immunoglobulin J gene segments.

As shown in Fig. 13, all of the identified genes which encode the mouse T cell receptor δ chain are contained within the α-chain locus. This small locus, which spans at least 30 kb, ends approximately 75 kb upstream from the C_α gene and is oriented in the same transcriptional direction as the majority of the α-chain gene segments. Included in the δ locus are a limited number of V gene segments, two D_δ gene segments, two J_δ gene segments, and the δ-chain constant region gene, which is composed of four exons (Chien *et al.*, 1987b). As discussed above, the mouse δ chain has been observed to use at least one V_α gene segment to produce a functional δ-chain polypeptide (Chien *et al.*, 1987a). This would seem to suggest that the δ chain has available a very large repertoire of V gene segments; however, such diversity among δ chain cDNAs has not been observed. Similar to the mouse β-chain locus, at least one V_δ gene segment is located 3′ to the C_δ gene in reverse transcriptional orientation (Iwashima *et al.*, 1988). This indicates that the δ-chain locus employs both looping out and deletion and inversion rearrangement mechanisms (Davis and Bjorkman, 1988). The δ chain genes undergo rearrangement early in development, well before α-chain rearrangement and expression can be detected (Chien *et al.*, 1987a). If the δ-chain genes are unproductively rearranged in a developing thymocyte, then the surrounding α-chain genes may be rearranged, thereby deleting the entire region of DNA containing the δ-chain locus (Chien *et al.*, 1987a). Similarly, the immunoglobulin κ light chain locus is often deleted in cells that have successfully rearranged λ light chain genes, although these loci are not linked on the same chromosome (Hieter *et al.*, 1981a). However, since the expressed T cell receptor δ chain must pair with the product of the δ-chain locus, unsuccessful rearrangement of the γ chain may lead to progressive rearrangement of the α-chain genes and subsequent deletion of the δ-chain locus (Chien *et al.*, 1987b).

2. *Organization of the Human α- and δ-Chain Loci*

The human α- and δ-chain gene families are found at chromosomal position 14q11-12 (Table I). The physical map of these loci is shown in Fig. 14. The α-chain locus contains 58 identified V_α gene segments, 47 identified J_α gene segments, and a single constant region gene (Yoshikai *et al.*, 1985, 1986; Klein *et al.*, 1987; Kimura *et al.*, 1987b; Baer *et al.*, 1987a,b, 1988; Mengle-Gaw *et al.*, 1987; Pircher *et al.*, 1987; Wilson

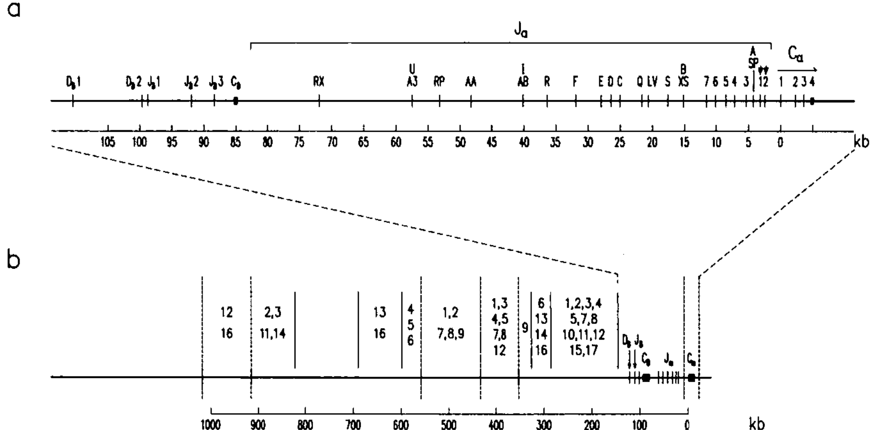


FIG. 14. (a) Physical map of the human α/δ constant region. J_α sequences and locations are summarized from the work of Yoshikai *et al.* (1985), Baer *et al.* (1987a,b, 1988), Mengle-Gaw *et al.* (1987), Finger *et al.* (1986), and Isobe *et al.* (1988). Identical J_α gene segments that have been named by different laboratories are shown by two names above a single J_α gene segment. (b) Organization of the α/δ locus. The numbers above the line represent the subfamilies. The solid lines separating the subfamilies represent pulsed-field gel electrophoresis-separated DNA fragments and the dotted lines represent rearrangement breakpoints.

et al., 1988). The V_α gene segments may be grouped into 22 subfamilies, with 15 subfamilies containing a single member. Preliminary mapping studies with field-inversion gel electrophoresis have indicated that the V_α gene segments are scattered across over 800 kb, with the entire α-chain locus spanning more than 1000 kb (Griesser *et al.*, 1988; Roth *et al.*, 1988; E. Lai, unpublished data). All of the V_α gene segments mapped to date are in the same transcriptional polarity, with their 5′ ends oriented toward the centromere. The J_α gene segments are located throughout an 80-kb region 5′ to the C_α gene. The most distal J_α gene segment is approximately 5 kb from C_α and the distance from C_α to the nearest V_α gene segment is within 200 kb (Griesser *et al.*, 1988; Roth *et al.*, 1988). Ordering of the V_α gene segments by deletional analysis of T cell leukemia DNA indicates a much higher rate of rearrangement in the α-chain locus as compared to the β-chain locus (P. Concannon, R. Wilson, and E. Lai, unpublished data). As discussed below, this may be due to the presence of the δ-chain gene family within the locus.

As in the mouse, the gene family that encodes the human T cell receptor δ chain is located entirely within the α-chain locus (Hata *et al.*, 1987;

Isobe *et al.*, 1988). Isolation of overlapping bacteriophage λ clones containing the 3′-most end of the α-chain locus has shown that the δ-chain constant region is located approximately 81 kb upstream from the C_α gene (Isobe *et al.*, 1988). As shown in Fig. 14a, this 110-kb region contains all of the J gene segments and C genes for both the α and δ chains. The organization of the C_α and C_δ genes is very similar and suggests that they may have arisen by duplication (Takahari *et al.*, 1988). Similar to the mouse δ chain, the human δ chain has been observed to rearrange productively to at least one V_α gene segment, a member of the $V_\alpha 6$ subfamily (Gugliemi *et al.*, 1988). Although this is suggestive of a vast V region repertoire, the three γ-δ-expressing T-cell clones studied to date (Loh *et al.*, 1987; Hata *et al.*, 1987; Gugliemi *et al.*, 1988) have been observed to utilize only two unique V gene segments in productive δ-chain rearrangements. DNA sequence analysis revealed the existence of at least two D_δ gene segments (Loh *et al.*, 1987; Hata *et al.*, 1987; Gugliemi *et al.*, 1988; Boehm *et al.*, 1988). The two J_δ gene segments are located approximately 7 and 13 kb proximal to the δ-chain constant region gene, respectively (Gugliemi *et al.*, 1988; Isobe *et al.*, 1988). The two D_δ gene segments are located approximately 1 and 10 kb 5′ to the $J_\delta 1$ gene segment (Takahari *et al.*, 1988).

The mechanism of rearrangement of the δ-chain locus appears to play a role in the genesis of reciprocal chromosomal translocations involved in human T cell malignancies. Southern blot analysis of T cell DNA from a patient with acute lymphoblastic leukemia (T-ALL) carrying a t(8; 14)(q24; q11) translocation revealed that the 5′-most J_δ gene segment was the site of translocation (Isobe *et al.*, 1988). Similar results were obtained from analysis of DNA from T-ALL cells carrying a t(11; 14)(p13; q11) translocation (Boehm *et al.*, 1988). In this translocation, D_δ–D_δ–J_δ or D_δ–D_δ joins were observed in three out of four breakpoint regions. As mentioned above, the immunoglobulin-like loci appear to be prone to deleterious chromosomal translocation and deletion events because of the frequency of normal gene rearrangement in these loci (Croce *et al.*, 1985; Taylor, 1982; Isobe *et al.*, 1985, 1988; Murre *et al.*, 1985). Since the δ-chain locus is contained within the α-chain locus, this particular region of DNA may be a hot spot for cytogenetic aberrations.

VII. Mechanisms for the Evolution, Recombination, and Diversification of the Immunoglobulin-like Loci

The immunoglobulin-like loci are a model class of gene families for the study of genomic evolution, organization, and regulation. The proteins that they encode serve a vital function in protecting the host

organism from disease. The efficiency with which immunoglobulins and T cell receptor molecules accomplish this task is directly related to the unique ability of these genetic loci to create a remarkable degree of molecular diversity. In a similar manner, the genes of the Major Histocompatibility Complex provide host cells with the ability to present antigen to T cells and consequently serve as markers to permit the immune system to distinguish between self and nonself.

Studies of the genomic organization of the immunoglobulin-like loci, as we have described here, have allowed the characterization of novel mechanisms of gene duplication and rearrangement that are important for both genetic evolution and expression. Physical differences among loci, such as the mouse T cell receptor α-chain and α-chain gene families, as well as differences among analogous loci in different species, such as mice and humans, reveal the development of similar mechanisms with a singular final objective of creating molecular diversity. In all of the immunoglobulin-like loci, a set of discontinuous gene segments is selected from two to three pools containing from 1 to 100 members; these segments are then joined by DNA rearrangement to produce the final coding sequence. This mechanism is known as combinatorial diversity. As we have discussed above, the organization of the members of these gene segment pools may play a major role in the expression of a particular immunoglobulin or T cell receptor molecule.

Specific examples of restricted gene segment rearrangement are evidenced by both the mouse T cell receptor γ-chain locus and the mouse immunoglobulin λ light chain locus. In both of these gene families, the association pattern of V and J gene segments is highly restricted. The organizations of these loci indicate that they most likely arose from duplications of ancestral V-J-C clusters. Furthermore, both of these loci have a very limited repertoire of V gene segments. This limited repertoire is reflected by the relatively minor (5%) contribution of λ-chain polypeptides to the light chain pool in the mouse, as well as the limited diversity of mouse γ-δ T cell receptors. In contrast, the human λ light chain locus, which contains a much larger repertoire of V gene segments, contributes approximately 33% of the total immunoglobulin light chains.

As discussed above, the mouse and human λ light chain loci are also interesting because they provide a good model of the divergence of immunoglobulin-like loci over the course of evolution. As shown in Figs. 1 and 2, mice have two and humans have six C_λ genes, all of which are arranged in clusters together with a 5′ J gene segment. This suggests the occurrence of multiple duplications of J_λ-C_λ clusters since the time of the mammalian radiation event. Similarly, conservation of the relative order of homologous V gene segments in the mouse and human T cell

receptor β-chain gene families suggests that multiple gene duplications occurred across large distances over the course of β-chain evolution.

Simple gene segment duplication, with an end result of increasing the number of members in any given gene segment subfamily, appears to have occurred in virtually every immunoglobulin and T cell receptor locus. Specific examples of this are found in the immunoglobulin heavy chain and κ light chain loci, and possibly the λ light chain locus and the T cell receptor α and β chain loci.

In mammalian immune systems such as those of the human and the mouse, it appears that somatic recombination is a necessary step for the generation of a functional molecule, and is an important mechanism for the generation of diversity. Not all species however, appear to use somatic recombination as a means of generating diversity in their immune systems. Studies of the immune system of a phylogenetically primitive shark revealed that each IgH chain is encoded by single V_H, D_H, J_H, and C_H genes that are linked closely together (Hinds and Litman, 1986). The sequences of C_H genes differ from one another in all exons, and each V_H gene segment may be associated with a single, unique C_H gene (Kokubu *et al.*, 1988). There may be more than 100 individual C_H genes in this species (Kokubu *et al.*, 1987). These data suggest that combinatorial joining does not play a role in the generation of diversity in this lower vertebrate.

A very unusual molecular mechanism for the generation of diversity is found in the chicken λ light chain. The chicken λ-chain locus has been found to contain a single C_λ gene, a single J_λ gene segment located 1.9 kb upstream, and a functional $V_\lambda 1$ gene segment (Reynaud *et al.*, 1985). Twenty-five pseudo-V_λ gene segments lie within 20 kb upstream from the $V_\lambda 1$ gene segment. Despite the fact that the chicken does not have a restricted immune response, it has been found that the V_λ gene segment is joined to the J_λ gene segment in almost all of the B cells in the bursa of fabricius. It appears that the diversity of the chicken λ-chain polypeptide is generated by a gene conversion mechanism between the $V_\lambda 1$ gene segment and the homologous V_λ pseudogene segments located 5′ of $V_\lambda 1$ (Reynaud *et al.*, 1985; Weill and Reynaud, 1987; Thompson and Neiman, 1987).

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Molecular Genetics of Murine Lupus Models

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I. Introduction

Sustained humoral and/or cellular autoimmune responses of sufficient magnitude are currently thought to be the primary cause or secondary contributors to a wide spectrum of human and animal disorders (1). As in any disease entity, inbred animal models with a consistent spontaneous incidence of a given autoimmune disease have been sought to better understand the etiology of these diseases and to devise appropriate new therapeutic measures, and a few have been identified. Of these models, the murine models of lupus/arthritis are among the best suited for study, because their serologic, histopathologic, cellular, and other abnormalities closely match the corresponding human disorders (2).

Below, we will briefly describe these strains and outline the basic conclusions derived from conventional immunopathologic studies. Details in these areas can be found in a previously published review (2). In this article, we will primarily focus on more recent work related to the molecular genetic aspects of these mice, with particular emphasis on B and T cell antigen receptor genes.

II. The Lupus Strains

There are three main types of lupus mice (Table I): New Zealand mice (NZB, NZW, and their hybrids) are of unknown derivation (3,4), whereas MRL mice have a complex genome composed of LG (75%), AKR (12.6%), C3H (12.1%), and C57BL/6 (0.3%), and BXSB mice have been derived from a single cross between C57BL/6 and SB/Le (5-7). In each of these mice, the genetic background predisposes to a late-life disease that becomes clinically manifest and then fatal in the second year of life. In each of these mice, however, there can be a unique accelerating factor that, when acting on the lupus-prone genetic background, causes conversion of the late-life disease to an early-life disease clinically apparent in the first few months of life and fatal within 5-7 months. These

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TABLE I

DERIVATION, H-2, ACCELERATING FACTORS, AND SURVIVAL RATE OF LUPUS MICE

Strains	Derivation	*H-2*	Accelerators	50% survival
NZB	Unknown	*d*	–	15 months
NZW	Unknown	*z*	–	22 months
(NZB × W)F_1		*d/z*	Female hormones	Females, 9 months; males, 13 months
MRL	LG (75%) AKR (12.6%) C3H (12.1%) C57BL/6 (0.3%)	*k*	*lpr* gene	*lpr/lpr*, 4–5 months; +/+, 16–18 months
BXSB	SB/Le (50%) C57BL/6 (50%)	*b*	Y chromosome associated	Males, 5 months; females, 21 months

accelerators are a Y chromosome-associated gene in BXSB contributed from the SB/Le parent; the single-locus autosomal recessive *lymphoproliferation* (*lpr*) gene, which, in a homozygous state, causes a massive lymphoid organ enlargement and expansion of an unusual T cell subset (see further below) that is responsible for severe autoimmunity in MRL-*lpr/lpr* mice, and, apparently, female hormones in (NZB × W)F_1 mice (reviewed in Ref. 2).

Since the time of its original description in MRL mice, the *lpr* gene has been transferred to a number of other standard inbred strains, establishing congenic AKR-, C3H/HeJ-, C57BL/6-, C57BL/10-, BALB/c-, and SJL-*lpr/lpr* mice (8–10). These normal background *lpr* homozygous mice similarly develop lymphadenopathy, but of varying degrees (increasing rank order of lymph node size: AKR-, C57BL/6-, SJL-, MRL-, and C3H-*lpr/lpr*), hypergammaglobulinemia, and diverse autoantibodies. Importantly, it has been documented that the severity and associated pathology of *lpr*-induced disease varies considerably on different strain backgrounds. Thus, in contrast to lupus-background (MRL) *lpr* homozygous mice, the normal-background *lpr* congenics exhibit minimal histopathologic manifestations of autoimmune disease. It has generally been considered that while the expanded *lpr* cells induce the production of diverse autoantibodies, the background genome on which the *lpr* gene acts has profound effects on the quantitative or, more likely, qualitative characteristics of these autoantibodies (e.g., Ig isotype, epitopic specificity, timing of isotype switching from IgM to IgG) and, consequently, on the nature and severity of the induced autoimmune response. Differential effects of genetic backgrounds have been demonstrated with other inducers or accelerators of autoimmune responses, such as bacterial lipopolysaccharide (LPS), whereby autoimmune disease is hastened in lupus-background mice, but fails to appear in normal-background mice despite the induction of autoantibodies in both types of mice (11). The numbers and identity of background genes affecting these processes remain to be determined.

With regard to localization of the *lpr* gene, chromosomal mapping has been unsuccessful thus far despite the fact that approximately 47% of the autosomal genome of the MRL mouse has been tested using 27 linkage markers, including H-2 (5,6). Similarly, the *lpr* gene product is unidentified, and its molecular cloning has therefore remained elusive.

A more recently described autosomal recessive mutation, *generalized lymphoproliferative disease* (*gld*), arose in unmanipulated C3H/HeJ mice (12). C3H-*gld/gld* mice also develop early-onset hypergammaglobulinemia, autoimmune humoral responses, and massive lymphoid hyperplasia; however, as expected from the normal background, few

C3H-*gld/gld* mice develop significant lupuslike nephritis, and none has vascular disease. The *gld* gene has recently been mapped to chromosome 1, in close association with this chromosome's distal At-3 gene (13). Additional mouse strains with the *gld* mutation have also recently been developed, including C57BL/6-, SJL-, AKR.C3H-, CBY.C3H-, and MRL.C3H-*gld/gld*. Although the *lpr* and *gld* mutations were determined to be independent, most, if not all, phenotypic characteristics identified with the atypical *lpr* cells (described in a subsequent section) are applicable to *gld* cells as well, i.e., expanded T cells from these two types of mutants are phenotypically indistinguishable (14).

The natural history and histopathologic, serologic, cellular, virologic, and genetic abnormalities, as well as other aspects, of these mice have been detailed elsewhere (2). Suffice it to say that these conventional analyses have allowed four main conclusions to be reached that, to a large extent, form the foundation upon which current etiologic and molecular studies are based. They are as follows:

1. There is a genetic predisposition toward the disease, and this predisposition, as shown by transplantation experiments (15-17), can be traced to the hematopoietic stem or lymphoid precursor cell. Thus, lethally irradiated lupus mice reconstituted with histocompatible normal stem cells do not develop disease and, conversely, lethally irradiated normal recipients of lupus stem cells do develop lupus disease. The similarity in disease pace in mice transplanted with lupus bone marrow or spleen cells obtained at the prediseased and diseased stages suggests that lupus is not caused by an accumulation of defects at the stem cells, which appear equally abnormal throughout the life of the animals.
2. As a cardinal characteristic, there is a generalized B cell hyperactivity leading to hypergammaglobulinemia and the production of a wide spectrum of autoantibodies. Considerable debate exists as to whether this B cell activation is polyclonal in nature or autoantigen driven, and supportive evidence for each concept and combinations thereof have been provided (18).
3. *In vivo* and *in vitro* studies have clearly shown that T cells are an absolute prerequisite for autoantibody production and disease development. Neonatally thymectomized MRL-*lpr/lpr* mice (19,20) and New Zealand mice rendered homozygous for the *nu* mutation (21) failed to develop lupus. Furthermore, treatment of (NZB $\times$ NZW)F_1 and MRL-*lpr/lpr* lupus mice with anti-L3T4 antibodies abrogated the disease (22,23), and isolated lupus B cells did not produce autoantibodies unless helper T cells or accessory T cell factors were added to the culture (24,25).
4. Finally, a variety of genetic and nongenetic factors (bacterial, viral infections) can independently accelerate or induce the autoimmune

process, but, as stated above, the background genome is the determining factor on the pathogenicity of the autoimmune responses induced, i.e., lupus-background, but not normal-background, mice allow or complement expression of such pathogenicity (nephritis, arteritis, and arthritis) (11).

Following these observations and the recent advances in molecular immunology, it became apparent to us and others that the time was ripe to begin to address lupus at the molecular genetic level. Because of the enormous complexities of these disorders, several avenues of investigation have been pursued, but our main emphasis at present has been placed on the molecular genetics of B and T cell antigen receptor genes. These genes, together with Major Histocompatibility Complex (MHC) genes, constitute the tripartite system that is of paramount importance in antigen recognition and presentation, tolerance induction, idiotypic regulation, and diversification of the immune system. Polymorphic states of these genes as well as their abnormal regulation and expression may affect these processes, thus leading to autoimmune manifestations.

III. Immunoglobulin Germ Line and Autoantibody Genes

Antibodies with specificity for a variety of self-antigens, including nucleic acids, nuclear proteins, and Ig, are one of the hallmarks of systemic lupus erythematosus (SLE) and other generalized autoimmune disorders in man and mouse. These so-called autoantibodies persist at high titers and form circulating immune complexes that deposit in various organs, leading to tissue damage and disease. While most of the disease symptoms can thus be readily explained by immune complex-mediated mechanisms, the causes underlying the high-level autoantibody expression typically associated with these disorders are not understood. As indicated above, cell transfer studies have documented that the murine disease defect resides within the hematopoietic stem cells (15–17). However, in spite of extensive investigations, the exact cellular and molecular abnormalities leading to B cell hyperactivity and autoantibody production remain largely unknown. Several, not necessarily exclusive, possibilities can be entertained: Autoantibody expression in the afflicted individuals or strains of mice may be caused by defects in the complex mechanisms generating the antibody repertoire (reviewed in Ref. 26), including abnormal Ig germ line genes, formation of unusual variable, diversity, and/or joining (V, D, J) gene segment rearrangements, or excessive somatic mutations. Alternatively, the potential to generate lupus-causing autoantibodies may not be unique to lupus-prone individuals; such antibodies may represent normal components of the immune system that, under physiologic conditions, remain unstimulated or suppressed. Their

high-level expression in lupus could result from abnormal response of B cells to regulatory stimuli, defective immunoregulatory capacity of T cells, or stimulation by exogenous or endogenous antigens or polyclonal B cell activators. Other possibilities include the theory that high levels of autoantibodies and pathogenic accumulation of immune complexes might be consequential to the increased biological half-life of such compounds and/or reduced rate of immune complex clearance. Of course, combinations of these concepts are also conceivable.

To further address the above possibilities, we have first attempted to define the genetic origin of, and somatic mechanisms generating, disease-associated autoantibodies from murine models of generalized autoimmune disorders. These and similar studies from other laboratories have been facilitated by advances in cell fusion and molecular cloning techniques, and by unraveling the basic principles of Ig germ line gene organization and generation of antibody diversity in exogenous responses of normal mice. In the first section of this article, we review current studies focusing on an understanding of the genetic origin of autoantibodies at the molecular level with particular emphasis on, but not restricted to, work performed in our laboratory. These studies have addressed the organization of Ig germ line gene heavy and κ light chain gene loci in lupus mice, defined the primary structure and Ig gene segments encoding a variety of lupus-associated autoantibodies, investigated the extent of somatic mutations in autoantibody V genes, and provided estimates for the number of B cell clonotypes participating in autoantibody production.

A. Ig Germ Line Genes in Murine Lupus

1. *Ig Heavy Chain Locus*

Murine Ig heavy chain variable (V_H) genes are organized in at least 10 multigene families with related members that exhibit extensive genetic polymorphism among inbred strains of mice defining several Ig heavy chain variable region gene (*Igh-V*) haplotypes (27–29). The complexity of individual families varies considerably, with a single family (J558) comprising the majority of the entire repertoire (30). To address possible V_H germ line gene defects or *Igh-V* haplotype associations with autoantibody production and lupus disease, we analyzed restriction fragment length polymorphisms (RFLPs) in DNA from all major lupus strains, mice carrying the *lpr* gene, their respective ancestors, and nonautoimmune controls using DNA probes corresponding to all known V_H gene families (31-33). This analysis, examples of which are given in Fig. 1, defined the *Igh-V* haplotypes of all major lupus strains (Table II)

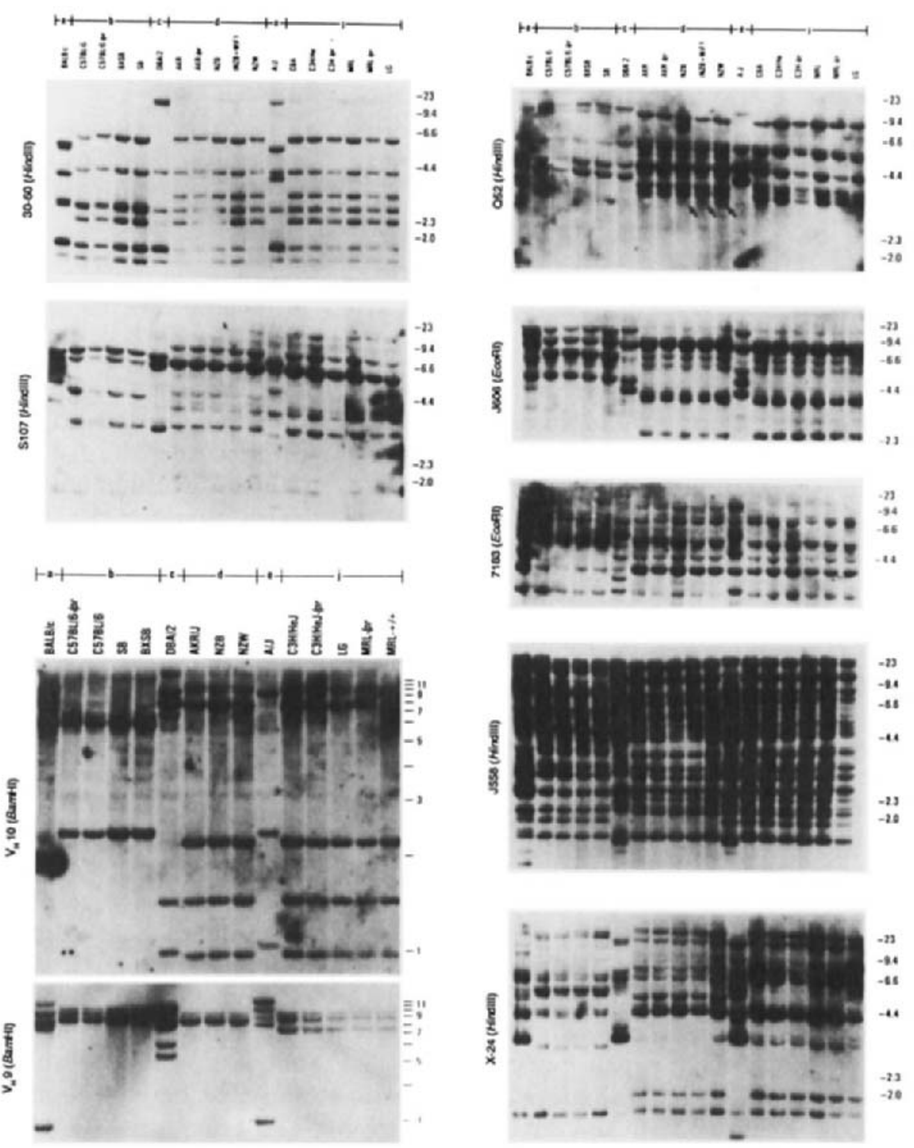
TABLE II
Igh AND *Igκ* HAPLOTYPES OF LUPUS MICE AND THEIR ANCESTORS[a]

Lupus strain	*Igh*	*Igκ*	Ancestors	*Igh*	*Igκ*
MRL	*j*	*a*	LG	*j*	*h*
			AKR	*d*	*a*
			C3H	*j*	*c*
			C57BL/6	*b*	*c*
BXSB	*b*	*c*	C57BL/6	*b*	*c*
			SB/Le	*b*	*c*
NZB	*d*	*b*	Unknown	—	—
NZW	*d*	*c*	Unknown	—	—

[a] *Igh* and *Igκ* haplotypes according to Refs. 27 and 50, respectively.

and revealed that murine lupus is not associated with a particular *Igh-V* haplotype. Furthermore, in lupus mice with known genetic derivation (MRL and BXSB), and in mice congenic for the *lpr* gene, the *Igh-V* loci were transmitted apparently unaltered from their nonautoimmune ancestral strains. NZW and NZB lupus mice, with unknown genetic derivation, have *Igh-V* loci almost indistinguishable from those of nonautoimmune AKR mice. These observations strongly suggested that the V_H germ line gene repertoires of lupus mice may be normal, a conclusion also drawn by Treppicchio and Barrett (34) and subsequently by others (35).

Because murine lupus shows a good correlation between disease onset and switch of polyclonal and autoantibody Ig from predominantly IgM to predominantly IgG, and because IgG autoantibodies have an excellent predictive value for disease expression and are found in eluates from diseased kidneys, we investigated the genomic organization of Ig heavy chain switch regions of the various murine Ig isotypes by RFLP analysis with appropriate probes. No evidence for gross abnormalities was detected (36), suggesting that enhanced switching may occur under the influence of abnormal levels of, or in response to, T cell-derived factors, as we have already suggested (24,37,38). To investigate the possibility of subtle, but functionally significant, abnormalities in Ig regulatory sequences that may have escaped detection by RFLP analyses, we cloned and sequenced the Igh enhancer region in two strains that develop lupus early in life (BXSB and MRL-*lpr/lpr*) (36). The reference BALB/c sequence was identical to that of BXSB, and the MRL-*lpr/lpr* Igh enhancer differed by a single nucleotide. This possibly allelic difference resides outside of the enhancer core regions (39,40) and is, therefore, probably functionally irrelevant.

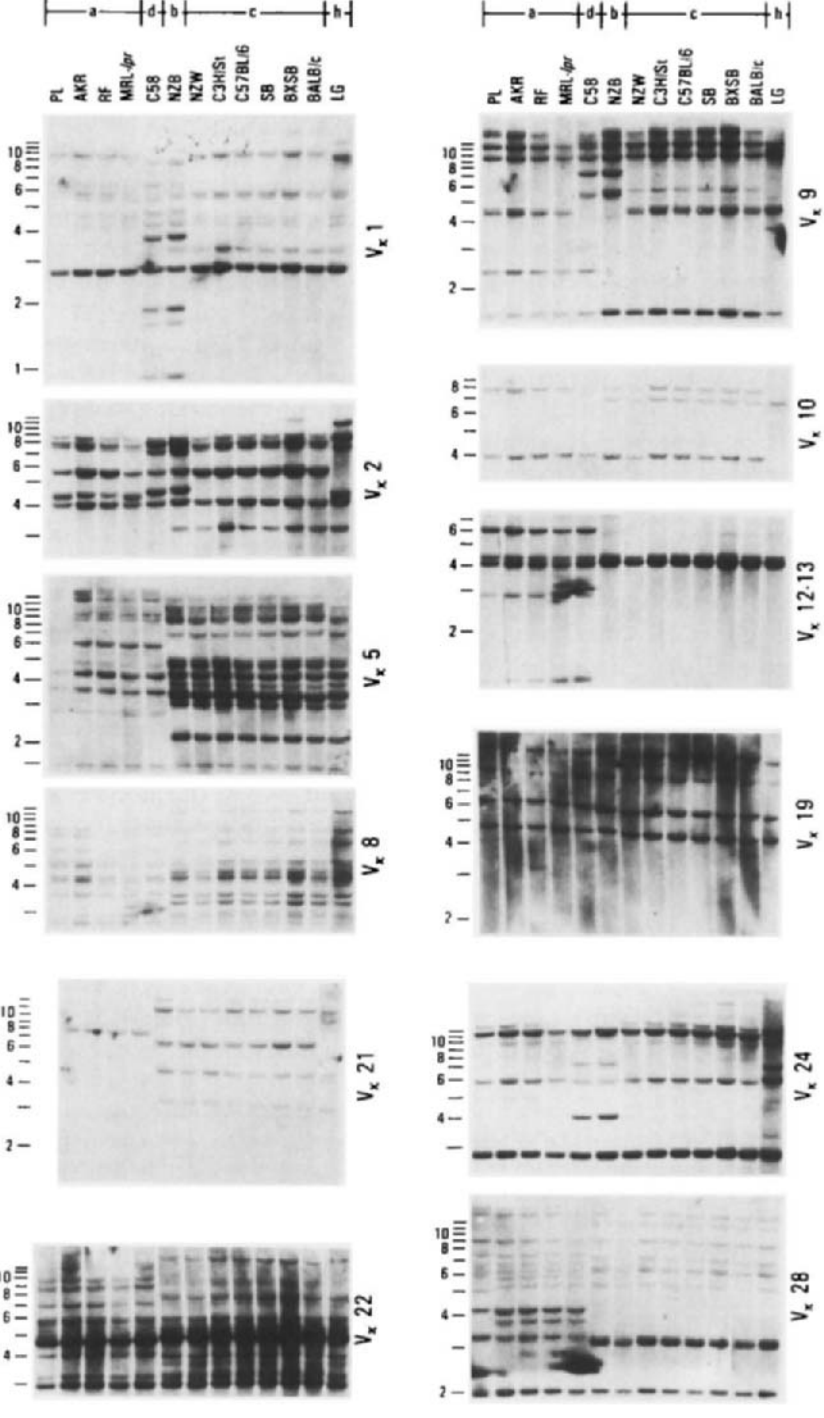


2. *Ig κ Light Chain Locus*

The organization and complexity of the murine *Igκ* gene complex is less well defined than that of the *Igh* locus. Based on amino acid similarity, 24 $V_κ$ groups have been distinguished (41) but additional $V_κ$ groups exist (42–44). The number and complexity of corresponding $V_κ$ gene families are not known and estimates vary considerably (44–46). The murine *Igκ* locus is polymorphic (44,47–49), albeit possibly to a lesser extent than the *Igh* locus. We recently initiated extensive RFLP analyses of the *Igκ* locus in a large number of mouse strains using DNA probes corresponding to 16 $V_κ$ protein groups ($V_κ$1, 2, 5, 8, 9, 10, 11, 12-13, 14, 19, 21, 22, 23, 24, and 28 and a novel $V_κ$ group termed $V_κ$RF) (50). This study, which extends previous analysis of the $V_κ$ locus by others (44,45,51), estimates the complexity of the investigated $V_κ$ families (from 1 to over 30 members) and reveals the presence of eight *Igκ* haplotypes in inbred mice.

In this analysis (presented in part in Ref. 32), we included all major lupus strains and mice congenic for the *lpr, gld, motheaten* (*me*), and *tight skin* (*tsk*) genes, all of which induce autoimmune disease and/or autoantibody expression on various genetic backgrounds. Congenics for other genes of immunologic interest and various mutant strains were also tested. As exemplified in Fig. 2 for $V_κ$1, 2, 5, 8, 9, 10, 12-13, 19, 21, 22, 24, and 28 gene families, the $V_κ$ loci of autoimmune mice with known genetic derivations appear essentially identical to their nonautoimmune ancestral strains, i.e., MRL and BXSB lupus mice derived their $V_κ$ locus essentially unaltered from their AKR (haplotype *a*) and C57BL/6 or SB (both haplotype *c*) progenitors, respectively. NZW mice with unknown genetic derivation share their $V_κ$ locus with normal mice of the Igk^c haplotype (such as BALB/c and C57BL/6). Among lupus mice, only NZB revealed an unusual, possibly recombinant, *Igκ* haplotype, termed $Igκ^b$: 10 of their analyzed $V_κ$ families ($V_κ$5, 8, 10, 12-13, 14, 19, 21, 22, 23, and 28), as well as the *Jk/Ck* complex, belong to the *c* haplotype, whereas the remaining loci ($V_κ$1, 2, 9, 11, 24, and $V_κ$RF) are very similar to, but not always identical with, the

FIG. 1. Autoradiographs of restriction enzyme-digested, size-separated liver DNA from several lupus and other inbred strains of mice probed with [^{32}P]DNA corresponding to nine murine V_H gene families. The strains have been organized according to their *Igh-V* haplotypes (indicated by lowercase letters between bars above strain designations). The restriction enzyme used for digestion and the V_H gene family probed are indicated on the left, and size markers are on the right side of each blot. Arrows in the autoradiography corresponding to the Q52 family highlight an additional band in all three NZ strains that is not present in the Igh^d prototype strain, AKR.


a
d
b
c
h
PL
AKR
RF
MRL-lpr
C58
NZB
NZW
C3H/St
C57BL/6
SB
BXSB
BALB/c
LG
V κ 1
V κ 2
V κ 5
V κ 8
V κ 21
V κ 22
V κ 9
V κ 10
V κ 12-13
V κ 19
V κ 24
V κ 28

corresponding loci in C58 mice (haplotype *d*). The extent, if any, to which the NZB *Igκ* haplotype contributes to disease etiology in this strain remains to be determined, since nonautoimmune strains such as LG and C58 also exhibited unique *Igκ* haplotypes.

Our study contrasts with a similar analysis performed by Bona (52), who found RFLPs between MRL and AKR, and between C57BL/6 and the congenic C57BL/6-*tsk* strains with $V_κ1$, $V_κ10$, and $V_κ19$ DNA probes. Bona concluded that the $V_κ$ genes, which are preferentially used by autoantibodies (see below), might derive from a set of germ line genes present only in autoimmune-prone mouse strains. The reasons for these significant differences are presently unexplained; however, close scrutiny of fragment patterns obtained in the two laboratories shows one major additional fragment in the MRL and C57BL/6-*tsk* (as well as in nonautoimmune C3H and LG) lanes that is present at approximately the same molecular weight in three of their blots ($V_κ1$, $V_κ10$, and $V_κ19$), but that is absent from corresponding lanes on our blots. Although other explanations might exist, this suggests that the unique fragments might be artifacts resulting from, for example, plasmid contaminations in the respective DNA preparations.

The above studies (except Ref. 52), in concert with previous classical genetic analyses (53,54) and the observation that lupuslike symptoms and/or autoantibodies can be induced in normal-background mice by a variety of means (8-11,55-57), strongly suggest that (1) defects within the Ig heavy and κ light chain loci are unlikely primary causes for murine SLE, and (2) that there are no simple associations of *Igh* and *Igκ* haplotypes with murine lupus.

B. Ig GENE SEGMENTS ENCODING AUTOANTIBODIES

1. *Autoantibody-Specific Genetic Elements*

Although the above evidence suggests normal Ig germ line genes in lupus mice, autoantibodies may still be encoded by unique V (or D) gene segments that are not rearranged or used in response to exogenous antigens. To investigate such a possibility, which might suggest a defect in the control of V-(D)-J rearrangement in lupus disease, we cloned and

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FIG. 2. Autoradiographs of restriction enzyme-digested, size-separated liver DNA from several lupus and other inbred strains of mice probed with [^{32}P]DNA probes corresponding to several $V_κ$ gene families. Lowercase letters above strain designations denote their *Igk-V* haplotypes as defined by RFLPs. The 1-kb pair size markers are given on the left and the $V_κ$ gene probe is given on the right of each blot. Shown are *Hin*dIII digests except for $V_κ10$ and $V_κ28$ (both Pst I), and $V_κ19$ (*Bam*HI). Corresponding results were obtained with additional restriction enzymes and probes for $V_κ11$, $V_κ14$, $V_κ23$, and $V_κ$RF gene families.

sequenced mRNAs encoding heavy and light chains from 12 monoclonal autoantibodies. Table III summarizes the characteristics of these monoclonals and the structural genetic elements (V, D, and J gene segments) encoding them. The respective sequences for most of them have been reported (32,43,50,58,59). While J and constant (C) gene segments ($C\mu$ and $C\kappa$ were sequenced from a representative autoantibody) (58) were identical or almost identical to those of BALB/c mice, some D segments could not be classified. However, novel D segments are frequently seen in antibodies to exogenous antigens and can be ascribed to alterations occurring during V/D/J joining, additional uncharacterized D families in the murine genome, allotypic differences, and a novel mechanism for D segment inversion. Of interest, all unassigned D segments were clearly different, and the three FL16.1 D segments shared by $(B \times W)F_1$ anti-DNAs were used in two reading frames and in context with different N-region sequences, resulting in quite different primary structures of the third complementarity-determining regions (CDR-3). Furthermore, the CDR-3 length, which is considered important for antigen and antiidiotype recognition, was not conserved among these autoantibodies with different specificities. Almost all V genes investigated could be unambiguously assigned to existing V gene families. The only exceptions were the MRL-DNA4 V_H gene (32) and the MRL-RF24 V_κ gene (43), which define new V_H and V_κ families, termed V_H10 (29) and V_κ-RF (50), respectively. However, the organization of corresponding germ line genes in lupus and normal mice of the same haplotype are indistinguishable by RFLP analysis (32,33), and V_H10 genes are also expressed in hybridomas from LPS-stimulated splenocytes of nonautoimmune mice (R. Dildrop, personal communication). Many of the autoantibody V genes were very similar, and in one instance even identical, to those encoding antibodies to exogenous antigens in normal mice (60). Other investigators have reported additional nucleic acid sequences of anti-DNA (61–66), anti-bromelaine-treated red blood cells (BrRBCs) (67), and rheumatoid factor (RF) antibodies (68–70) from autoimmune and normal mice. With the exception of the V_H gene encoding anti-BrRBC antibodies, all V_H and V_κ sequences were closely related to V genes used in exogenous responses and/or belonged to known V gene families. The evidence available at present, therefore, strongly suggests that autoantibodies do not employ unique Ig gene segments but are encoded by the same (or at least an overlapping) germ line gene repertoire used by antibodies to exogenous antigens. This conclusion, which has recently been reached for human RF autoantibodies as well (71), is in full agreement with a major postulate for generation of antibody diversity, i.e., that an individual V gene participates in the generation of different specificities.

TABLE III

GENETIC ELEMENTS ENCODING MURINE LUPUS-ASSOCIATED AUTOANTIBODIES

Monoclonal Ab code	Strain	Specificity	Isotype	Heavy chain			Light chain	
				V_H[a]	D	J_H	V_κ[b]	J_κ
BXW-DNA7	(NZB × W)F_1	ssDNA	IgM	J558	DFL16.1	J_H1	n.d.[c]	n.d.
BXW-DNA14	(NZB × W)F_1	ssDNA	IgM	J558	DFL16.1	J_H2	$V_\kappa2$	$J_\kappa1$
BXW-DNA16	(NZB × W)F_1	ssDNA	IgM	J558	DFL16.1	J_H2	$V_\kappa9$	$J_\kappa2$
MRL-DNA10	MRL-*lpr*	ssDNA	IgM	J558	n.a.[d]	J_H3	$V_\kappa1$	$J_\kappa4$
MRL-DNA4	MRL-*lpr*	ssDNA	IgG_{2a}	V_H10	n.a.	J_H3	$V_\kappa1$	$J_\kappa2$
MRL-DNA22	MRL-*lpr*	ssDNA,dsDNA	IgM	J558	n.a.	J_H4	$V_\kappa5$	$J_\kappa4$
MRL-RF7	MRL-*lpr*	IgG_{2b},ssDNA	IgM	J558	n.a.	J_H1	$V_\kappa22$	$J_\kappa4$
MRL-RF14/9	MRL-*lpr*	IgG_{2a}	IgM	J558	n.a.	J_H4	$V_\kappa28$	$J_\kappa1$
MRL-RF24	MRL-*lpr*	$IgG_{1,2a,3}$	IgM	J558	Q52	J_H4	$V_\kappa RF$	$J_\kappa1$
MRL-RF28	MRL-*lpr*	$IgG_{2a,b}$	IgM	n.d.[c]	n.d.	n.d.	$V_\kappa28$	$J_\kappa1$
MRL-RF33	MRL-*lpr*	IgG_1	IgM	n.d.	n.d.	n.d.	$V_\kappa23$	$J_\kappa5$
MRL-Histone7	MRL-*lpr*	Histones	IgG_3	Sm7	SP2	J_H4	$V_\kappa5$	$J_\kappa5$

[a] V_H gene family designations according to Refs. 27–29. Sm7 is a potential new V_H gene family formerly included in the large J558 family.

[b] V_κ groups according to Ref. 41. The MRL-RF24 V_κ sequence could not be assigned to any known V_κ group and represents the prototype of a new, single-member V_κ gene family, provisionally termed $V_\kappa RF$ (43,50).

[c] n.d., Not determined.

[d] n.a., Not assigned.

2. *Genetic Restriction among Autoantibodies*

Common structural genetic elements among autoantibodies with different specificities might point toward common inducing agents, provide a structural explanation for how these molecules cicumvent antiidiotypic or other forms of immunologic regulation, and possibly establish a basis for specific therapeutic interventions using antiidiotypes. Preferential usage of V_H genes by autoantibodies with different specificities has indeed been suggested, particularly of those of the 7183 V_H gene family (72), and it was concluded that autoimmune disease may be related to a defective control of V_H gene expression (73). However, as reviewed recently (74), numerous studies have clearly shown that autoantibodies can be encoded by all known V_H gene families, and the proposed 7183 V_H gene overrepresentation, particularly with respect to autoantibodies from spontaneously autoimmune mice, can be questioned (Table IV). Moreover, 7183 V_H genes can be found in many exogenous responses, clearly showing that this family is in no way specifically or exclusively expressed in self-reactive antibodies. More recently, preferential use of V_κ gene families ($V_\kappa 1$, $V_\kappa 10$, and $V_\kappa 19$) by autoantibodies has been suggested (52). As in the case of heavy chain genes, the respective gene families are also encoding exogenous responses, one of them ($V_\kappa 1$) even at an unusually high frequency (75), and many other V_κ gene families have been found to encode autoantibodies (Table V). In this context, it is worth mentioning that an apparent nonrandom representation of V_H and V_κ gene families in collections of hybridoma autoantibodies should be interpreted with caution, because neither the precise V germ line gene number nor the expression frequency is known. Moreover, skewed representation might be due to small sample size, nonrandom representation of autoantibody specificities, and, if RNA hybridization analyses (RNA dot blotting; Northerns) are being used, technical reasons, as discussed in detail elsewhere (74). The majority of current data suggests that the overall murine lupus-associated anti-self response shows little, if any, genetic restriction.

Of course, this does not exclude genetic restriction among autoantibodies of a given specificity, although this kind of restriction is more likely related to the structural requirements for generation of a particular binding site than to possible defects in V gene usage. Genetic restriction is common among antibodies against defined haptens and epitopes on complex exogenous antigens (76,77), and has also been suggested for several autoantibody specificities. The strongest example of restrictive use of structural genetic elements by autoantibodies of a given specificity was observed in anti-BrRBC antibodies; all six monoclonals analyzed

TABLE IV
V_H GENES ENCODING 174 MURINE MONOCLONAL AUTOANTIBODIES FROM AUTOIMMUNE AND NONAUTOIMMUNE MICE[a]

V_H gene families[b]	Strains of MAb origin			Estimated complexity
	All (174)	Autoimmune (113)	Nonautoimmune (61)	
V_H1 (J558)	52.3	56.6	44.3	60-1000
V_H2 (Q52)	5.2	2.7	9.8	15
V_H3 (36-60)	5.2	6.2	3.3	5
V_H4 (X-24)	1.2	1.8	0	2
V_H5 (7183)	19.5	11.5	34.4	12
V_H6 (J606)	2.3	2.7	1.6	10
V_H7 (S107)	1.7	2.7	0	4
V_H8 (3609)	1.2	0.9	1.6	9
V_H9 (VGAM3-8)[c]	0	0	0	5
V_H10 (DNA4)	1.2	1.8	0	3
V_HBrRBC	3.4	2.7	4.9	?
a.a.[d]	5.7	8.8	0	—
n.a.[e]	1.2	1.8	0	—

[a] V_H gene use is expressed as a percentage of the total number of monoclonal autoantibodies in a given collection (indicated in parentheses). For comparison, the column at the right shows the estimated genomic complexity in absolute numbers of the various V_H gene families (27-30).

[b] V_H gene family designations according to Refs. 27-29. V_HBrRBC is a possibly new V_H gene family expressed in autoantibodies to BrRBCs (67).

[c] After completion of the study from which this table is taken (74), several IgG MRL-*lpr/lpr* RF autoantibodies encoded by V_H9 genes were observed (69).

[d] a.a., Ambiguous assignment.

[e] n.a., Not assigned.

(three each from NZB and CBA mice) apparently used the same V_H, D, J_H, V_κ, and J_κ segments (67). Restricted V_κ gene usage ($V_\kappa1$, $V_\kappa8$, $V_\kappa19$, and $V_\kappa24$) was reported for induced monoclonal RFs from nonautoimmune mice (78), however, this form of genetic restriction may not apply to spontaneous RFs from MRL-*lpr/lpr* mice, which serve as a model for rheumatoid arthritis (43,59,69). Arant *et al.* suggested that MRL-*lpr/lpr* and MRL-+/+ anti-Igs are preferentially encoded by 36-60 V_H genes (79), a conclusion that is in contrast to reports from our (59,80) and another laboratory (69) showing only 1 of over 50 RFs being encoded by a 36-60 V_H gene, with the majority deriving from J558 and the remainder from S107, V_H9, and Sm7 (a possible new V_H gene family formerly included in J558). The reasons for these strikingly different results are unclear, but may reflect differences in fine specificity,

TABLE V
V_κ GENE UTILIZATION BY 51 MURINE MONOCLONAL AUTOANTIBODIES[a]

V_κ family[b]	Specificity			Percentage
	RF	DNA	Others	
$V_\kappa 1$	3	3	0	11.8
$V_\kappa 2$	0	1	0	2.0
$V_\kappa 4$	2	0	0	3.9
$V_\kappa 5$	0	1	1	3.9
$V_\kappa 8$	8	1	1	19.6
$V_\kappa 9$	0	1	6	13.7
$V_\kappa 19$	8	8	0	31.4
$V_\kappa 21$	0	1	0	2.0
$V_\kappa 23$	2	0	0	3.9
$V_\kappa 24$	2	0	0	3.9
$V_\kappa 28$	1	0	0	2.0
V_κRF	1	0	0	2.0

[a] Shown is the number of autoantibodies, grouped by specificity [RF, DNA, others (RNA, histone, BrRBC, multispecific)], encoded by gene(s) of the indicated V_κ families. The column at the right indicates relative frequency at which the respective gene family was observed.

[b] V_κ families according to Ref. 41. V_κRF is a tentatively new V_κ family defined by the MRL-RF24 V_κ gene (43,50) that could not be assigned to known V_κ families.

because the anti-Igs of Arant *et al.* reacted with Fab or intact Ig, but not with Fc. The third group of self-specificities, anti-DNA antibodies, analyzed in greater detail, can be encoded by a large number of different V_H and V_κ gene families and, hence, this group shows little, if any, genetic restriction (32,58,61–66,81). Human anti-DNAs can also be encoded by quite diverse V_H genes (82), however, this response may be more restricted in man than in mice (83). The relatively large number of different genetic elements that fulfill the criteria for generation of Ig and nucleic acid binding sites is consistent with multiple and diverse epitopes recognized on these antigens.

C. Somatic Mutation

The question of whether autoantibodies are germ line encoded or result from somatic mutations in antibodies originally directed against exogenous antigens is important because it defines the developmental stage at which a given B cell clone acquires self-specificity and escapes

tolerance induction. As will be discussed below, the presence and distribution of somatic mutations in various portions of the V region sequence may further provide useful hints regarding autoantibody-inducing agents. Finally, autoimmunity might result from disturbed regulation of the mechanisms responsible for somatic mutation in the maturation of normal immune responses. Based on suggestive evidence in the literature, two entirely different models can been proposed: (1) autoantibodies may be somatic mutants of "normal" antibodies (84) that, in lupus disease, escape regulation and/or are generated at an abnormal rate due to pathologically increased somatic mutations; (2) unmutated germ line genes may encode anti-self specificity that is lost during somatic mutations physiologically associated with maturation of antibody responses (85). In the latter scenario, the inability to mutate Ig sequences might contribute to the high levels of autoantibodies typical of such disorders.

In lupus mice, only a single germ line gene (the V_H11 germ line gene of the S107 family; Ref. 65) has been isolated thus far, limiting direct evaluation of somatic mutations considerably. However, additional indirect evidence has been obtained by analysis of clonally related autoantibodies (based on identical V/D/J and V_κ/J_κ junction sequences and Ig gene rearrangements), independent isolation of identical V genes from different sources, and identity of autoantibody V genes with germ line genes in the literature. For example, one of our IgM anti-DNAs expresses a V_κ gene identical to the BALB/c V_κ1-A germ line gene (86), and another IgM anti-DNA V_κ sequence is identical to that encoding an antidextran antibody (87). Moreover, two clonally unrelated IgM anti-DNAs (32) and two clonally unrelated IgM RFs (unpublished data) share identical V_H gene-encoded regions, respectively, strongly suggesting that unmutated V germ line genes can be expressed in murine lupus-associated autoantibodies.

Although spontaneous lupus autoantibodies may be encoded by V genes in germ line configuration, such antibodies do not have to be devoid of somatic mutations, particularly after class switch. Thus, several mutations have been identified in IgG_{2a} anti-DNAs from a phosphocholine-immunized (NZB × W)F_1 mouse (65). Moreover, clonally related IgG and IgA MRL-*lpr/lpr* RFs and IgG anti-DNAs (63,69), and IgG (NZB × W)F_1 anti-DNAs (62), show clear evidence for somatic mutations and, hence, resemble antibodies to exogenous antigens in which the frequency of somatic mutations is also associated with class switch (88). Therefore, gross abnormalities (complete lack of or abnormally increased somatic mutation in Ig genes) appear unlikely causes for the high-level autoantibody expression observed in these autoimmune disease models. A similar conclusion has also been reached for the human system (71).

D. Inducing Agents and Clonotypic Restriction

Whether lupus-associated anti-self responses are triggered by the corresponding autoantigens, result from polyclonal B cell activation, or are induced by cross-reactive exogenous antigens ("antigenic mimickry") has yet to be resolved. Certain molecular characteristics of the anti-self response may be useful to distinguish between the above possibilities. Polyclonal responses are genetically and clonotypically unrestricted, may or may not undergo class switch, and any somatic mutations that might be present can be expected to be random. In contrast, (auto)antigen-driven responses may be genetically restricted, may show clonotypic convergence (i.e., reduced number of clonotypes after class switch), and exhibit selection for productive mutations in complementarity-determining regions of their V genes.

Although the large number of V, D, and J gene segments engaged in autoantibody formation suggests polyclonal activation, this cannot be taken as proof because complex (auto)antigens may elicit a similarly diverse response. Moreover, there is increasing evidence in the literature to support an (auto)antigen-driven origin of autoantibodies. While the majority of monoclonal IgM RFs and all IgM anti-DNAs studied in our laboratory were clonally unrelated (32,59), corresponding specificities of the IgG and IgA isotypes analyzed by others showed clonotypic relatedness (62,63,69), suggesting that these responses may exhibit clonotypic convergence. Further support for an antigen-driven model is provided by the increased frequency of productive mutations in complementarity-determining regions as opposed to mutations in framework regions that were observed among clonally related hybridoma IgG and IgA RFs and IgG anti-DNAs from MRL-*lpr/lpr* and related mice (63,69).

IV. Genomic and Expressed T Cell Antigen Receptor Repertoire

Several studies in animal models have clearly documented the primary role of T cells in the pathogenesis of systemic and organ-specific autoimmune diseases, either in the form of helper activities required for autoantibody production or self-reactive cytotoxic T cells. Thus, as stated above, neonatally thymectomized MRL-*lpr/lpr* mice do not exhibit the lymphoid hyperplasia and early-life severe lupus seen in control animals (19,20), and (NZB × NZW)F_1 *nu/nu* mice do not develop autoimmune disease unless a syngeneic thymus is engrafted (21). The involvement of T cells in the induction of autoimmune syndromes has also been supported by cell transfer experiments (89–92) and by the inhibitory or

ameliorating effects of administered anti-L3T4 (CD4) antibodies in spontaneous models of autoimmunity, such as murine strains with lupus (22,23), and in the NOD mouse with insulin-dependent diabetes (93,94), as well as models of experimentally induced autoimmune syndromes such as collagen arthritis (95), allergic thyroiditis (90), experimental allergic encephalomyelitis (EAE) (96), and myasthenia gravis (97). In fact, preliminary evidence has suggested that an allelic variant of the T cell antigen receptor (TcR) $V\beta6$ gene is necessary for the development of experimental collagen arthritis in mice (98). Moreover, a limited set of TcR $V\alpha$-$J\alpha$ and $V\beta$-$J\beta$ genes has been identified in T helper clones specific for the encephalitogenic portion of mouse myelin basic protein (MBP), and a monoclonal antibody recognizing the product of one of these genes was found to block T helper clone recognition of the specific encephalitogenic MBP peptide *in vitro* and to significantly reduce the susceptibility of appropriate mice to peptide-induced EAE (99-101).

For the reasons cited above, we and others believe that a detailed investigation into the genomic composition and expression of TcR genes in mice predisposed to autoimmunity will be paramount to our efforts to determine why tolerance to self is circumvented. Significant TcR genomic polymorphisms may be identified that could correlate with the autoimmune predisposition. Furthermore, aberrant TcR expression patterns might emerge that will indicate either selective activation of certain clones or abnormalities in thymic selection and maturation processes in these diseases. Below, we summarize the current understanding of TcR gene biology and describe our recent studies on the genomic composition and expressed repertoire of these genes in several autoimmune and normal mouse strains.

A. TcR Gene Diversity and T Cell Maturation

The two types of murine TcR heterodimeric structures (α:β and γ:δ) and their corresponding genes have been well defined (reviewed in 102-104), with the α:β structure found on the majority (~98%) of mature peripheral helper ($CD4^{+}8^{-}$) and cytotoxic ($CD8^{+}4^{-}$) MHC class II- or class I-restricted T cells, respectively. The γ:δ is found on a small (~2%) but distinct population of peripheral T cells, on the majority of dendritic T cells in the skin, and on intraepithelial gut lymphocytes; the function of the γ:δ-bearing cells remains unknown. Both heterodimers are independently expressed on the cell surface of these T cell populations in a noncovalent association with the multichain (five chains; γ, δ, ϵ, ζ, η) invariant CD3 complex.

The mouse β-chain locus (chromosome 6) consists of an estimated 20-30 V genes, arranged in a minimum of 17 one- to three-member

subfamilies, and two C-region genes, each with a single upstream D element and six functional J elements (Fig. 3). Strains of mice (SJL, SWR, C57L, and C57BR) have been identified that have deleted a large fraction (~50%) of these Vβ segments, yet appear immunologically normal (105). The murine α-chain locus (chromosome 14) consists of perhaps 100–150 V genes arranged in 15–20 subfamilies, each consisting of 1–10 members and a single C-region gene with a large number (~50%) of upstream J elements (no Dα gene element has been identified). The δ locus (chromosome 14) is unique in that it is located between the V and J segments of the α gene and is deleted in α:β-bearing T cells. Although there is some overlap in V region usage, the V gene segments used for α and δ chains are largely distinct, with only approximately seven V segments identified for the δ chain. Finally, the TcR γ locus (chromosome 13) is less diverse, with three different functional Jγ–Cγ loci and approximately seven Vγ gene segments. TcRs appear to diversify primarily by recombination of their various genetic elements, but not by somatic hypermutation, which occurs for Ig genes.

Although there is no complete consensus among the prevailing views, it is reasonably established that the normal pathway of T cell differentiation involves the following steps (reviewed in Refs. 106–109; Fig. 4): (1) emigration of precursor T cells ($CD4^-8^-$, TcR^-) from the bone marrow to the thymic cortex; (2) transcription of the genes encoding the multichain CD3 complex; (3) rearrangements of the γ and δ loci, initial rearrangements of the β locus, and acquisition of surface γ:δ dimeric structures together with CD3 in a few such cells; (4) in the

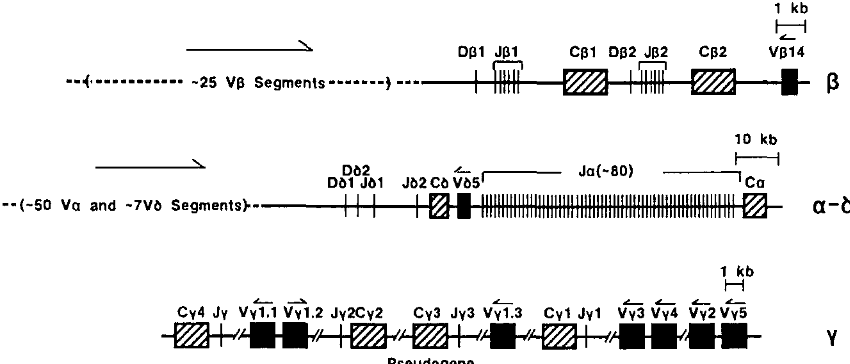

FIG. 3. Genomic organization of the T cell receptor loci in the mouse. Transcriptional orientations are indicated with arrows.

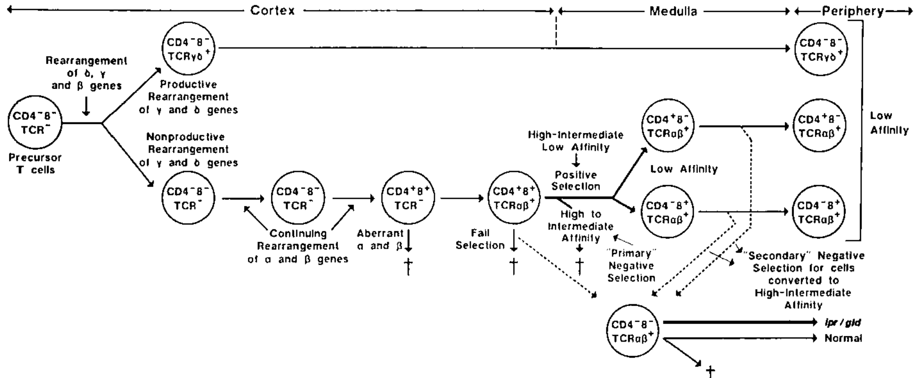

FIG. 4. Developmental pathways for murine T cells (see text).

majority of CD4$^-$8$^-$ cells where γ,δ rearrangements are nonproductive, continued maturation, with further β-chain gene rearrangements and mRNA expression, followed by acquisition of CD4 and CD8 accessory molecules (CD4$^+$8$^+$ or double-positive cells), with CD8 appearing prior to CD4; (5) the subsequent rearrangement of α-chain genes and the appearance of surface CD3 together with α:β dimeric structures on ~50% of these cells; (6) the so-called positive (110–112) and negative (113–117) selection processes, i.e., positive selection for cells having TcRs that will recognize antigen fragments only if presented by self-MHC, and negative selection against cells having TcRs that would confer autoreactivity in the mature T cells; and, finally (7) the development of some of these cells into mature CD4$^+$8$^-$ (helper) or CD8$^+$4$^-$ (cytotoxic) thymocytes and their eventual exit to the periphery. It is generally thought that positive selection precedes negative selection, primarily because positive selection seems to involve an interaction between the TcR and MHC molecules expressed on thymic cortical epithelial cells (109,118), the thymic cortex being the site at which double-positive thymocytes are found. Similarly, negative selection requires the presence of cells derived from bone marrow (macrophages, dentritic cells) (109,119), which are believed to be found primarily in the thymic medulla, a site occupied by more mature thymocytes. It is far from clear, however, what the actual sequence of events is, because several studies suggest, somewhat paradoxically, that both positive and negative selections begin at the double-positive stage (107,120,121). Janeway (107) has offered several plausible scenarios for these events; Sprent and Webb (109) have suggested that, to the contrary, negative selection of cells displaying TcRs

with intermediate to high anti-self-MHC affinity precedes positive selection of cells displaying TcRs with low anti-self-MHC affinity; Marrack and Kappler (108) suggested that these events are related to the different spectrum of self-peptides bound to thymic epithelial cells (positive selection) and self-peptides bound to bone marrow-derived cells (negative selection). Whichever model of thymic selection is correct, it is now clear that positive selection determines the CD4/CD8 phenotype of the developing thymocyte (110–112), this determination being based on whether the TcR has reactivity for self class I (CD8 selected) or class II (CD4 selected). Although the exact mechanism of this selection process has not been delineated, it clearly leads to a down-regulation of the unselected CD4 or CD8 accessory molecule, presumably the result of either a preprogrammed event or, alternatively, through delivery of an active negative signal (107), giving rise to single-positive $CD8^+4^-$ or $CD4^+8^-$ cells, with the $CD4^+8^-$ subset appearing first. A portion of these cells, selected for low anti-self-MHC reactivity, are then exported to the periphery where they form the pool of self-MHC-restricted helper ($CD4^+8^-$) and cytotoxic ($CD8^+4^-$) T cells. Out of the $>50 \times 10^6$ T cells produced in the thymus per day, only $1–2 \times 10^6$ per day are exported as mature functional T cells (108,109). Thus, the majority of T cells die intrathymically, presumably because (1) their TcR gene rearrangements are not productive, (2) their TcRs do not exhibit reactivity for self-MHC so as to be positively selected, or (3) they are eliminated by negative selection.

In addition to the $CD4^+8^-$ and $CD8^+4^-$ TcR $\alpha{:}\beta^+$ cells, a minor subset of $CD4^-8^-$ TcR $\alpha{:}\beta^+$ cells consisting ~2–30% of $CD4^-8^-$ thymocytes (themselves representing ~5% of the total thymocytes) has been identified (106,122–125), but its origin remains enigmatic. This subset appears relatively late in ontogeny (between birth and day 5 of life, increasing to ~20–30% of double-negative thymocytes in 2- to 4-week-old mice) and thus, unlike immature double-negative cells, cannot be the precursor to the TcR $\alpha{:}\beta^+$ single-positive cells already present at birth. Furthermore, this double-negative TcR $\alpha{:}\beta^+$ subset, in contrast to immature $CD4^-8^-$ cells, is unable to recolonize thymuses, nor can it give rise to medullary T cell types, further substantiating its late developmental stage (124). As is postulated later herein, these cells, exhibiting many phenotypic characteristics similar to the abnormally expanded T cells of autoimmune mice homozygous for the *lpr* or *gld* mutations, may represent a heterogeneous population derived from (1) cells that never express CD4 and CD8, (2) cells that have failed to be selected, and (3) moderately self-reactive cells that have escaped clonal deletion but have down-regulated their accessory molecules.

From the foregoing discussion, it becomes apparent that TcR genomic modifications (allelic variants, genomic deletions), alterations imposed during thymic positive and negative selection, and oligoclonal restrictions in autoaggressive T cells might be important in the pathogenesis of autoimmune disorders. Aspects of our investigations in these areas are outlined below.

B. Germ Line TcR α and β Genes in Autoimmune Strains

At least two situations can be envisioned in which the germ line TcR genes could contribute to autoimmunity: (1) autoimmune strains could exhibit alterations in TcR genomic organization (deletions or duplications of certain VDJC elements) which would alter the repertoire and may lead to autoreactivity through immunoregulatory imbalances, and (2) autoimmune strains could have unique allelic variants of TcR V genes, including one or more V genes predisposing to the development of autoreactive T cells. Mutations in promoter regions or in the heptamer-nonamer rearrangement signals might also affect the ability to transcribe or rearrange, respectively, certain gene segments.

1. *Cα and Cβ Genes*

In an intial structural analysis of the TcR locus (126), we screened genomic DNAs of classical autoimmune strains along with additional ancestral strains for gross alterations in TcR Cα and Cβ genes. Hybridization of the α-chain C-region probe to *Eco*RI-digested DNA identified genomic DNA fragments (4.4 and 6.8 kb) encoding the Cα gene, but revealed no polymorphisms among the strains tested. Hybridization of *Bam*HI-digested DNA with a TcR β-chain (CDJ) probe also disclosed nonpolymorphic DNA fragments (6 and 10 kb) in 16 of the 18 strains tested. The two exceptions were NZW mice, in which only one hybridization band (9 kb) was detected, and (NZB $\times$ NZW)F_1, which had the expected composite NZB and NZW profiles (6, 9, and 10 kb). Considering the published restriction map for the TcR β gene locus, additional digests, and actual cDNA cloning and sequencing of the NZW DJC elements (126), we concluded that this profile was caused by an ~8.8-kb deletion beginning at the first exon of Cβ1 and extending to the beginning of the first exon of Cβ2, thereby creating a Cβ locus lacking the Cβ1, Dβ2, and Jβ2 segments (Fig. 5).

A meiotic recombination event can be evoked to explain this deletion, with an unequal crossing over between the precisely aligned first exon of the Cβ1 gene on one chromatid and the Cβ2 gene on its homologue. Based on Southern blot analysis only, Kotzin *et al.* (127) independently derived similar conclusions. Due to this deletion, only

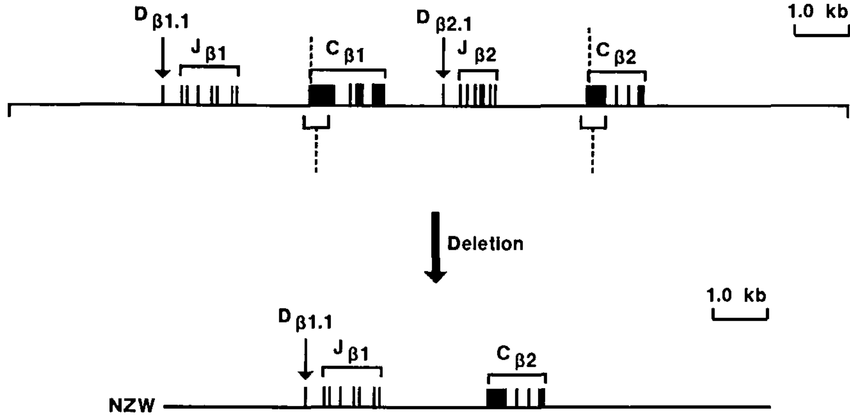

FIG. 5. A schematic representation of the TcR β-chain deletion identified in NZW mice. Dashed lines denote breakpoint regions as determined by sequence analysis.

Dβ1-Jβ1, and not Dβ2-Jβ2 or Dβ1-Jβ2, joinings are possible in NZW mice; thus, these mice may be able to generate considerably less TcR β-chain diversity than other strains. In spite of this seemingly dramatic impairment, the effects on T cell responses of these mice are not readily apparent. Furthermore, despite the contrary claims (128), our studies with (NZB × NZW)F_2 mice (129) and those of Kotzin and Palmer (130) with NZB × NZW backcrosses to NZB have not disclosed any primary role for this deletion in the development of autoimmune manifestations in these mice.

2. *Vβ Haplotypes*

We first used a complete set of 17 Vβ subfamily probes (i.e., Vβ1-17, according to nomenclature in Ref. 131) to analyze genomic blots containing DNA from a number of normal, autoimmune-susceptible or autoimmune-contributing murine strains not previously characterized in the literature, including autoimmune BXSB, MRL, NZB, NZW, and NOD (non-obese diabetic) mice and their ancestors (when known) (132). Using *Eco*RI, *Hin*dIII, and *Bam*HI, we detected no new RFLPs beyond those previously described (105) defining the two Vβ haplotypes, i.e., the $V\beta^a$ haplotype that includes the above-described Vβ-deleted strains (SWR, SJL, C57L, and C57BR with deletions in Vβ5, 8, 9, 11, 12, and 13), and the $V\beta^b$ haplotype that includes all other non-Vβ deleted strains.

3. Vα Haplotypes

When genomic blots of various strains were hybridized with nine Vα subfamily probes (Vα1, 2, 4, 5, 6, 7, 8, 9, and 11), numerous RFLPs were detected (Fig. 6), identifying four distinct haplotypes, which we have designated $V\alpha^a$, $V\alpha^b$, $V\alpha^c$, and $V\alpha^d$ (132). Specific RFLPs were detected for each haplotype and for all of the Vα subfamilies analyzed, in most cases regardless of which restriction enzyme was used. In contrast to the situation in the murine Vβ locus, no Vα haplotypes were found in which one or more entire subfamily had been deleted; however, deletions of some specific genes or gene sets within some subfamilies were detected (data not shown).

Table VI lists the strain distribution among the four Vα haplotypes. The larger group carries the $V\alpha^a$ haplotype, including a number of normal laboratory strains (BALB/c, CBA, and A/J), the autoimmune MRL mouse and its major progenitor strains (LG, AKR, and C3H), and the autoimmunity-contributing strain (SB/Le). The C57 strains carry the $V\alpha^b$ haplotype (or a subtype thereof), as does the autoimmune-prone BXSB mouse, which is a cross between the C57BL/6 and SB/Le parental strains. SJL and SWR, on the other hand, are $V\alpha^c$, as are NZB, PL/J, and NOD mice. Finally, $V\alpha^d$ is carried only by NZW mice of all the strains tested. No apparent relationship of MHC with Vα haplotype emerged from these studies.

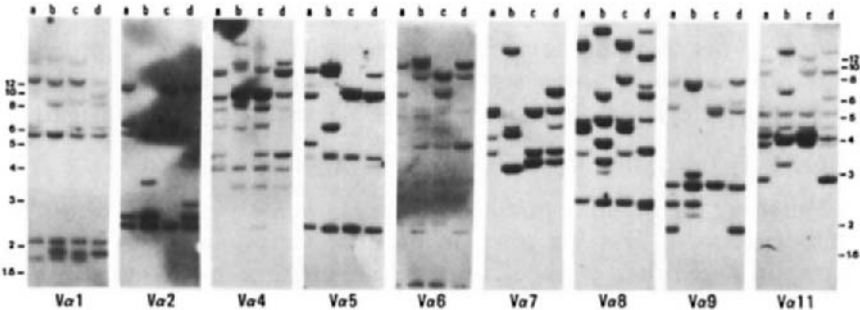

FIG. 6. RFLPs distinguishing murine TcR Vα haplotypes. Lanes a–d correspond to Vα haplotypes $V\alpha^a$, $V\alpha^b$, $V\alpha^c$, and $V\alpha^d$. Genomic DNAs were obtained from strains BALB/c (lane a), BXSB (lane b), NZB (lane c), and NZW (lane d). Vα4 and Vα5 panels are *Bam*HI digests; all others are *Hin*dIII digests. Size markers (in kilobases, kb) are indicated (for blots Vα1, Vα2, Vα7, Vα8, and Vα9 at the left, and for blots Vα4, Vα5, Vα6, and Vα11 at the right).

TABLE VI
STRAIN DISTRIBUTION AMONG THE FOUR TcR Vα HAPLOTYPES

Vα haplotype	Strains
a	BALB/c, AKR, C3H, CBA, A/J, LG, SB/Le, MRL
b	C57BL/6, C57BL/10, BXSB, C57L, C57BR (subtypes)
c	SJL, SWR, NZB, PL/J, NOD
d	NZW

Based on these findings, it can be stated that in murine models of systemic autoimmunity, correlations with Vα haplotype were not clearly apparent. Thus, the prototypic lupus models were distributed in all four Vα haplotypes. In addition, on the basis of the nine Vα subfamilies analyzed in this study, autoimmune MRL and BXSB mice are shown to have Vα and MHC haplotypes (i.e., $V\alpha^{a}/H\text{-}2^{k}$ and $V\alpha^{b}/H\text{-}2^{b}$, respectively) identical to at least one of their normal progenitor strains. It therefore seems unlikely that disease expression in these lupus models is caused by abnormalities encoded in a particular Vα (or Vβ) haplotype per se or by novel combinations of Vα haplotypes with MHC alleles. However, these examples do not preclude the possibility that, in particular cases, an otherwise normal Vα haplotype may nonetheless contribute to the unique constellation of factors allowing the autoimmune response to occur. Of interest in this regard, the $V\alpha^{c}$ haplotype was found to be common to a diverse group of autoimmune-prone or autoimmune-contributing strains, but whether this association is fortuitous or relevant to the disease process remains to be investigated.

C. EXPRESSED TcR Vβ REPERTOIRE IN AUTOIMMUNE MODELS

Following the genomic studies outlined above, we examined the expression patterns of TcR Vβ genes in primary lymphoid organs of autoimmune and other types of mice in an attempt to identify unique expression patterns or abnormalities in T cell maturation and selection phenomena, particularly in tolerance-related clonal deletions.

1. Multiprobe TcR Vβ RNase Protection Assay

To define TcR gene expression characteristics of autoimmune and normal mice, we adapted and modified an RNase protection assay (132,133) whereby the quantity and quality of a mRNA species in the total cellular RNA from any tissue source can be analyzed.

In this assay, diagrammatically depicted in Fig. 7, a single-stranded antisense radioactive RNA probe of defined length, complementary to the mRNA sequence to be quantitated, is hybridized in solution to the target mRNA under conditions of probe excess and high Rot value, such that essentially all target mRNA present becomes hybridized to the probe. After hybridization, the excess probe is removed by RNase digestion, leaving the hybridized probe:mRNA duplex intact (i.e., protected). The protected probe is electrophoresed on polyacrylamide sequencing gels and identified as a specific band by autoradiography or other means (e.g., densitometry). The strength of the radioactive signal or densitometric peak in a given band is directly proportional to the quantity of protected probe corresponding to that band, and hence to the quantity of target mRNA present in the original RNA sample.

With a goal of having an accurate and rapid assay for determining the entire TcR V gene usage at the RNA level, we modified this basic assay into what can be described as a fingerprinting method, or multiprobe RNase protection assay. Briefly, cDNA probes corresponding to all known Vβ genes (and several Vα genes) have been made through recombinant DNA techniques into defined-length fragments (from ~40 to ~360 bp long) and then subcloned into pGEM, a

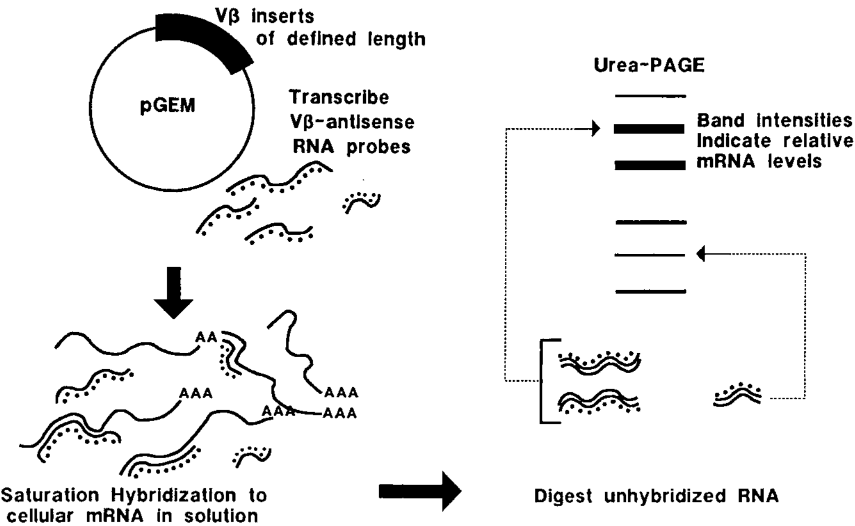

FIG. 7. A schematic representation of the multiprobe RNase protection assay for the analysis of expressed TcR repertoires in lymphoid cells (see text).

riboprobe vector. Importantly, the probe design has been made in such a way that the size of a given protected band uniquely identifies a single Vβ (or Vα) gene. The fingerprinting or ladder RNase protection assay for analyzing multiple genes simultaneously works as follows: Several radiolabeled individual antisense riboprobes (a probe set), each of a defined length recognizing different Vβ (or Vα) mRNAs, together with an aliquot of the RNA preparation to be analyzed, are hybridized in a single tube, are digested with RNase A + T1, and are then run on a single lane of a polyaccrylamide sequencing gel. High and low molecular weight probes for Cβ are also included in each Vβ probe set to normalize for total TcR mRNA from lane to lane. In the final autoradiography (or densitometric profile), intensities of different bands correspond to the level of the TcR V gene mRNAs present, thus creating a V gene fingerprint for the relevant T cell population. The use of probe sets over individual probes increases the information that can be obtained from a given amount of RNA, and increases the accuracy of the results, because the relative levels of the individual probes within a probe set are internally controlled. Good band definition of Vβs can currently be performed with six to eight probes/probe set. Vβ expression patterns, using the above-outlined approach on total mouse thymocytes and double-positive thymocyte RNAs, are shown in Fig. 8, where the reference prototype template with four probe sets is also shown. As expected, the profiles of total thymocytes and double-positive cells (consisting of ~80% of the total thymocytes that, in their majority, have not yet undergone thymic selection, but highly transcribe Vβ genes) are identical.

2. *Genomic Impositions on TcR Vβ Gene Expression*

When unselected thymocytes (sorted $CD3^-$ thymocytes) of the two Vβ haplotypes (Vβ^a deleted and Vβ^b nondeleted) were compared with regard to their Vβ gene expression, it became apparent that the genomic deletions in the former haplotype had affected TcR repertoire expression among the various Vβ genes. Thus, for compensatory reasons, and not unexpectedly, most of the residual Vβ genes of a Vβ-deleted mouse exhibited a higher expression in this population than did the corresponding genes of Vβ-nondeleted mice. Exceptions to this general rule were, however, noted. For example, the Vβ15 gene was, to the contrary, underexpressed in the Vβ-deleted haplotype compared to the Vβ-nondeleted haplotype, and Vβ16 was overexpressed to a greater extent than can be explained by compensatory expression. Differences in Vβ gene expression within a Vβ haplotype were also observed; for example, in the Vβ-nondeleted haplotype, there was a generally lower expression of the Vβ9 and Vβ17 genes. Moreover, the Vβ17a (Vβ3.2) gene was expressed only in the Vβ-deleted haplotype, confirming previous

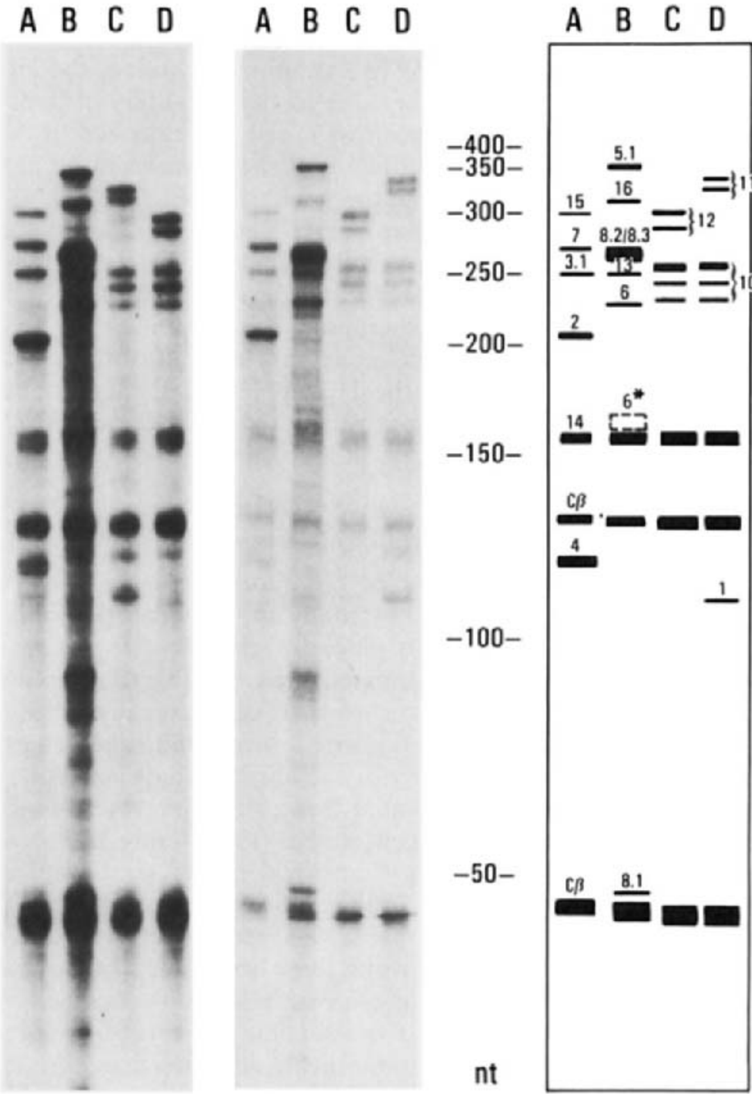

FIG. 8. Results obtained by the RNase protection assay with unseparated total thymocytes (left) and double-positive ($CD4^+8^+$) thymocytes (center), and the corresponding, diagrammatically depicted, prototypic template (right) with four probe sets (A, B, C, and D) encompassing most of the known murine Vβ genes; nt, nucleotide.

immunofluorescence studies with an anti-Vβ17a antibody (113). Recent studies (134) have indicated that this gene is present in the genome of both Vβ haplotypes, but in the Vβ nondeleted haplotype, because of a single base substitution resulting in a termination codon, the level of mRNA from this gene is low either due to the instability of nonfunctional mRNA or to the lack of positive T cell selection with these rearrangements in the thymus. In addition, allelic polymorphisms of Vβ1, 3, 6, and 10, previously noted at the genomic level (105), were also detected in RNAs of the two Vβ haplotypes. Recent preliminary studies (98), cited above, suggested that the Vβ6 allele of the $V\beta^b$ haplotype is necessary for responses to collagen and development of arthritis in mice of the appropriate $H\text{-}2^q$ background. Further studies on the relationship of these Vβ (and Vα) allelic variants to responsiveness against foreign and self-molecules and to the development of diseases, including autoimmune diseases, are therefore warranted, as is the definition of their primary structure.

3. Tolerance- or Negative Selection-Related TcR Vβ Repertoire Modifications

Because the germ line T (and B) cell repertoire is immense and, therefore, capable of recognizing essentially any antigenic determinant, negating mechanisms must be in place by which highly self-reactive lymphocyte clones are eliminated, inactivated, or otherwise suppressed to create a tolerant state. With regard to B cells, recent studies (135) with double transgenic mice expressing a neo-self-antigen (hen egg lysozyme) and a high-affinity antilysozyme antibody have suggested that B cell tolerance is primarily mediated by an antigen receptor inactivation process. In contrast, more recent studies (135a), using $H\text{-}2^k \times H\text{-}2^d$ transgenic mice expressing an IgM anti-$H\text{-}2^k$ MHC class I antibody, have suggested that autospecific B cells can be controlled by clonal deletion. With regard to T cells, in spite of the extensive literature on suppressor cells and immunologic networks, there has been persistent support for the notion that there is a major censorship function exercised in the thymus. This censorship or negative selection prevents autoaggressive (high-affinity antiself) T cells from maturing and migrating, but allows cells with the capacity to recognize a foreign antigen in association with an MHC restriction element to pass this barrier.

Definitive evidence for the clonal deletion of T cells expressing products of certain Vβ genes exhibiting high reactivity to self-I-E and/or *Mls* determinants has recently been obtained by utilizing monoclonal anti-V specific antibodies to demonstrate that T cells expressing self-reacting receptors are absent from the pool of medullary thymocytes and peripheral T cells, but are present among cortical, as yet unselected,

thymocytes. Thus, I-E-restricted (probably associated with an unknown B cell-specific self-peptide) thymic clonal elimination of mature T cells expressing the Vβ17a (Vβ3.2) gene product was initially documented by Kappler *et al.* (113) in certain types (Vβ^a haplotype, I-E$^+$) of mice (C57BR) that encompass this gene in their germ line repertoire. Further tolerance-related clonal eliminations of Vβ6- (115) and Vβ8.1-expressing (114) mature T cells were subsequently identified in I-E$^+$ mice bearing *Mls*^{a} determinants.

The Mls antigens (reviewed in Refs. 136,137), primarily expressed on B cells, are defined by their ability to stimulate unidirectional strong primary T cell proliferative responses in mixed lymphocyte reactions involving MHC-identical strains. They appear to be encoded by at least two loci, *Mls*-1 and *Mls*-2. The *Mls*-1 locus is on chromosome 1 and has two alleles, the *a* (active) and the *b* (null) allele. The chromosomal location of *Mls*-2 locus is unknown, but it also contains two alleles, the *a* (active, formerly designated *Mls*^{c}) and the *b* (null) allele. Strains expressing both *Mls*^{a} and *Mls*^{c} have previously been referred to as *Mls*^{d}. In general, *Mls*^{b} T cells respond to each of the three other *Mls* types, but do not stimulate any of these cells. In contrast, *Mls*^{d} cells stimulate T cells of each of the other *Mls* types, but do not respond to any *Mls* type. Finally, only one combination of mutual stimulation is seen between *Mls*^{a} and *Mls*^{c}, although in all cases, *Mls*^{c} reactivity is weaker. The *Mls* genes and their products are unknown.

Utilizing the above-described multiprobe RNase protection assay, we verified the above findings at the RNA level, thus further documenting clonal deletion as a means of tolerance induction. Thus, as depicted in Fig. 9A, Vβ17a-specific mRNA is present in spleens of the I-E$^-$ SJL, C57L, and SWR strains, but is absent in spleen RNAs of the I-E$^+$ C57BR strain. Furthermore (Fig. 9B), no protected Vβ6 bands are seen in spleen mRNAs of strains such as AKR (*Mls*^{a}) or NZB and DBA/2 (*Mls*^{d}), and this phenomenon is dominantly expressed in crosses of *Mls*^{d} mice (NZB or DBA/2) with an *Mls*^{c} (NZW) mouse. Moreover, Vβ8.1 mRNA is lacking in spleens of the *Mls*^{a} AKR mouse, but is present in spleens of *Mls*^{b} (MRL and C57BL/6) or *Mls*^{c} (C3H) mice (Fig. 9C).

Following the above confirmation, we then proceeded to utilize our four-probe set RNase protection assay and RNAs of thymocytes and spleen cells of various murine strains; we hoped to identify abnormalities or additional tolerance-related phenomena that might be relevant to the expression of autoimmune syndromes. The results with probe set A (Vβ15, 7, 3.1, 2, and 14) indicated the absence or significant reduction of Vβ3.1 mRNAs in spleens of NZB, NZW, and C3H mice, and of Vβ7 mRNAs in NZB and AKR mice (Fig. 10A). With probe set B (Vβ5.1, 16, and 6), the expression of Vβ5.1 and Vβ16 was found to be very low or absent in spleen RNAs of MRL, NZB, and C3H mice as

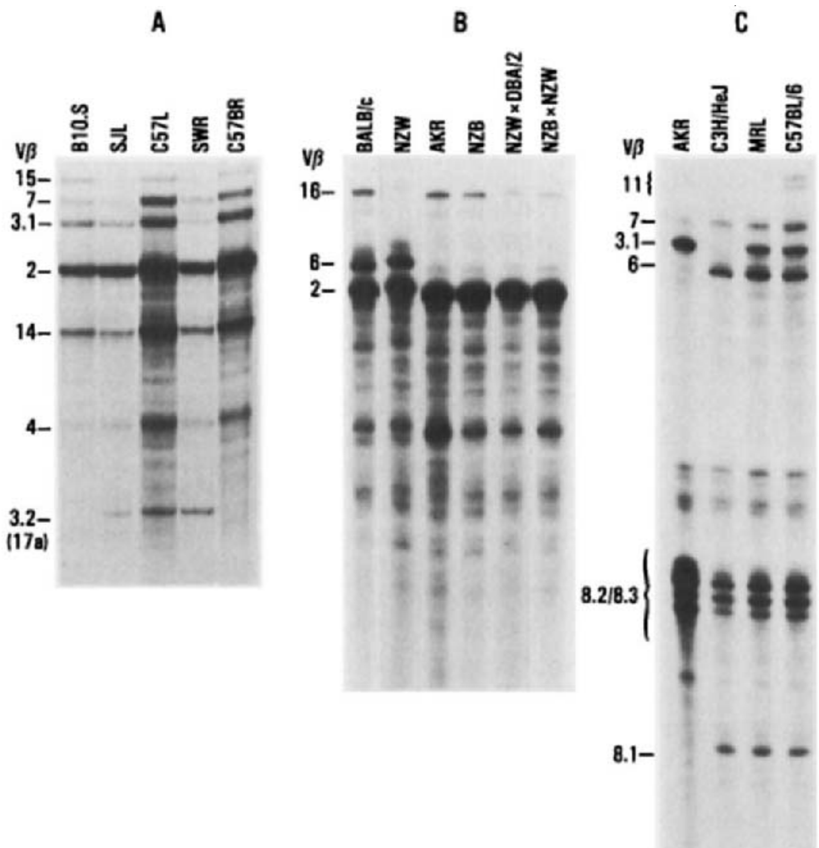

FIG. 9. Confirmation by the RNase protection assay of the tolerance-related clonal deletions of splenocytes expressing Vβ17a (Vβ3.2) (I-E related) (A), Vβ6 (I-E/*Mls*ᵃ related) (B), and Vβ8.1 (I-E/*Mls*ᵃ related) (C) (see text).

well as in the (NZB × NZW)F_1 and (NZW × DBA/2)F_1 crosses, but was present in spleen RNAs of a number of other strains tested (Fig. 10B). Finally, the results with probe set C + D (Vβ10, 11, and 12; data not shown), indicated that in addition to the genomically imposed complete deletions of Vβ11 and Vβ12 in SJL and SWR mice, partial reduction of Vβ11 and Vβ12 expression was detected in NZW and two of its crosses, as well as in a number of other laboratory strains. Additional studies indicated deletion of Vβ9-expressing cells in *Mls*ᵃ (AKR) or *Mls*ᵈ (DBA/2) mice (data not shown in Fig. 10).

As discussed above, the studies of Kappler *et al.* (113) demonstrated that the Vβ17a (Vβ3.2) clonal deletion is I-E mediated, and the studies

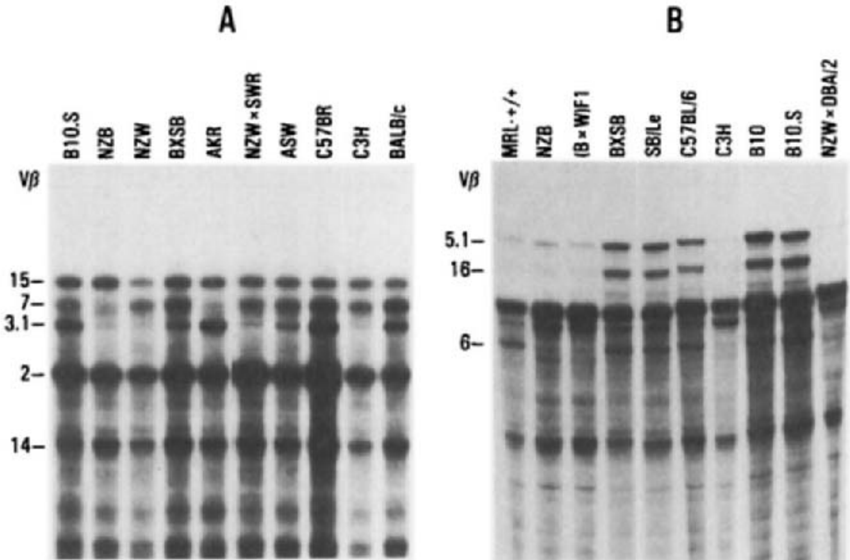

FIG. 10. Clonal deletion of Vβ7 and Vβ3.1 (A) and Vβ5.1, Vβ16, and Vβ6 (B) identified by the RNase protection assay (see text).

of McDonald *et al.* (115) and Kappler *et al.* (114) demonstrated that the Vβ6 and Vβ8.1 deletions, respectively, are I-E/*Mls*^{a} related. The correlations with I-E and *Mls* of the new deletions (Vβ3.1, 5.1, 7, 9, 11, 12, and 16) identified in our studies were therefore investigated.

As depicted in Table VII, based on published *Mls* typings and inferred *Mls* typings derived from this and the above-cited tolerance-related clonal deletion studies, it appears that the Vβ3.1 deletion is most likely mediated by recognition of self-I-E in conjunction with *Mls*^{c}. Thus, all I-E$^+$/*Mls*^{c} (or I-E$^+$/*Mls*^{d}) mice analyzed failed to express Vβ3.1 in their peripheral (spleen) T cells, whereas I-E$^-$/*Mls*^{c} mice, such as A.SW, did not exhibit this clonal deletion. However, a group of I-E$^-$/*Mls*^{c} strains that have in common an *H-2*^{b} allele at the I-E locus (NOD, SB/Le, BXSB, C3H.SW, BALB.B, D2.GD) can exhibit partial deletion of Vβ3.1. On the other hand, Vβ7 and Vβ9 deletions appear to be I-E/*Mls*^{a} related (like Vβ6 and 8.1). Moreover, deletions of Vβ5.1, 11, 12, and 16 occur in a large number of, but not all, I-E$^+$ strains (data not shown). These latter observations indicate that clonal modifications imposed by self-I-E recognition extend beyond the originally described Vβ17a deletion in the C57BR mouse, and are therefore of broader significance.

TABLE VII
CORRELATION OF Vβ3.1 DELETION WITH *Mls*[c] AND OF Vβ7 AND Vβ9 DELETIONS WITH *Mls*[a]

Strain	*H-2*	I-E	*Mls**			Expression of	
			a	*b*	*c*	Vβ3.1	Vβ7 and Vβ9
DBA/2	*d*	+	+		+	−	−
NZB	*d*	+	+		+	−	−
BALB/c	*d*	+			+	−	++
C3H/HeJ	*k*	+			+	−	++
NZW	*z*	+			+	−	++
PL/J	*u*	+			+	−	++
C57BR	*k*	+		+		+++	++
C57L	*b*	−		+		+++	++
SJL	*s*	−		+		++	++
SWR	*q*	−	+			++	++
AKR	*k*	+	+			++	−
MRL-+/+	*k*	+		+		++	++
C57BL/6	*b*	−		+		++	++
B10.S	*s*	−		+		++	++
A.SW	*s*	−			+	+	++

**Mls* typings derived from the literature or inferred from Vβ clonal deletions.

The deletions of anti-self-TcR-expressing cells have been considered to occur at the double-positive stage of thymic differentiation. This conclusion is based on the fact that cells bearing TcRs that confer autoreactivity to class II MHC ligands (such as Vβ17a for self-I-E and Vβ6 or Vβ8.1 for self-I-E/*Mls*[a]) are deleted not only from the class II MHC-specific $CD4^+8^-$ subset, but also from the class I MHC-specific $CD8^+4^-$ subset (115,120,121). However, we detected Vβ11 and Vβ12 deletions in the $CD4^+8^-$, but not in the $CD8^+4^-$, thymocyte subset, suggesting that negative selection might continue after conversion of the double-positive cells to the single-positive type, presumably commensurate to increased avidity of TcR–MHC interactions associated with acquisition of higher TcR density.

4. *Origin of lpr and gld Cells*

The *lpr* and *gld* autosomal recessive mutations are prime examples of single-gene defects associated with a massive expansion of a unique, phenotypically and functionally similar (Table VIII) lymphocyte subset that appears to be responsible for the heightened polyclonal and autoimmune responses observed in mice of varying genetic backgrounds homozygous for these mutations (reviewed in Ref. 2). The appearance

TABLE VIII
PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF *lpr/gld* CELLS

Surface phenotype
Thy-1$^+$, CD3^{low}, TcR α:β^{low}, CD4$^-$8$^-$, CD5^{low}, Ly-5$^+$ (B220), Ly-6c$^+$, Ly-22$^+$, Ly-24$^+$ (Pgp-1), PC.1$^+$, Jlld$^+$ (30% of cells), IL2-R$^-$, ℓ K$^+$ channels^{high}
Functional phenotype
Low proliferative response to mitogens, alloantigens, and anti-CD3, anti-TcR or anti-Ly-6c antibodies; low cytotoxic responses; deficient agonist-induced phosphoinositide hydrolysis and constitutive phosphorylation of CD3-ζ chain; high spontaneous production of B cell differentiation factors

of this expanded population has been shown to be thymus dependent (19,20). Initial immunofluorescent and cytotoxicity studies demonstrated that >90% of the cells in the enlarged lymph nodes of *lpr* or *gld* homozygous mice are positive for Thy-1 antigen and express low levels of the Ly-1 (CD5) antigen. Additional studies (reviewed in Ref. 2) demonstrated that the expanded *lpr* and *gld* Thy-1$^+$, dull CD5$^+$ lymph node cells were negative for both T cell subset markers CD4 and CD8, but expressed other cell surface antigens, including Ly-5 (B220), Ly-6c, Ly-22, Ly-24 (Pgp-1), PC.1, and an abundance of the so-called ℓ K$^+$ channels. Some of the proliferating lymph node cells (30–40%) also express one of the heat-stable antigens (HSAs) detected by the monoclonal antibody Jlld, but they are IL-2-R$^-$. These double-negative (CD4$^-$8$^-$) cells also show rearrangement and significant expression of full-length TcR α- and β-chain transcripts and bear surface TcR α:β and CD3, but at reduced density compared to normal T cells. Finally, Southern blotting (138–140) and analysis of cDNA clones (141) have indicated the polyclonal nature of the *lpr* and *gld* cells, with a predominant expression (65% of cDNA clones) of Vβ8.2 and Vβ8.3 gene products (141). A striking feature that has emerged from these combined findings is that the *lpr/gld* cells express both mature (TcR α:β) and immature (CD4$^-$8$^-$, CD5^{low}) T cell markers. Because of this, the relationship of these cells to the known normal T cell subsets has been difficult to establish.

Recognition of the thymic differentiation pathways and of the I-E/*Mls*-related clonal deletions outlined above has provided a valuable new population-level marker for monitoring the critical selection-related events in thymocyte maturation and, moreover, offers a unique opportunity to further address the issue of *lpr* and *gld* cell origin. The concept we tested was whether these abnormally expanded double-negative TcR α:β-bearing cells had been subjected to the I-E/*Mls*-imposed T cell repertoire modifications, as outlined above. RNA, extracted from sorted

populations of $CD4^-8^-$ T cells taken from the enlarged lymph nodes of the AKR-, MRL-, C3H-, and C57BL/6-*lpr/lpr* mice and C3H-*gld/gld* mice, was characterized for Vβ mRNA expression by protection analysis. The results of this analysis (Table IX) were unambiguous, revealing essentially the identical set of tolerance-related clonal deletions found in mature peripheral (spleen) T cells of the respective non-*lpr/gld* congenic strains (deletions of Vβ6, 7, 8.1, and 9 in $I\text{-}E^+/Mls^a$ mice; Vβ3.1 in $I\text{-}E^+/Mls^c$ mice; and low expression of Vβ5.1, 11, 12, and 16 in all $I\text{-}E^+$ mice).

These findings, taken together with evidence indicating the requirement for CD4 participation in I-E- (120) and I-E/Mls^a-induced (121) clonal deletions as well as for CD8 participation in class I-restricted clonal deletions (142), point strongly to the existence of a previously unrecognized pathway of late thymic differentiation. Simply stated, thymocytes destined for the lineage giving rise to *lpr/gld* (and probably to a subset of the heterogeneous normal double-negative TcR $\alpha{:}\beta^+$ cells) have at least passed through the double-positive stage in which tolerance-related clonal deletions occur and, subsequently, are in some way induced to down-regulate their accessory molecules, thereby effectively becoming "secondary" double-negative, but TcR $\alpha{:}\beta^+$, cells. The derivation of these cells through a CD4/CD8 loss pathway is further supported by a recent report (Ref. 23, cited above) in which *in vivo* treatment of MRL-*lpr/lpr* mice with anti-CD4 antibody inhibited accumulation of the abnormal $CD4^-8^-$, TcR $\alpha{:}\beta^+$ cells.

We can envisage two possible mechanisms by which down-regulation of accessory molecules can occur in these cells, as was depicted diagrammatically in Fig. 4: (1) if programmed down-regulation of CD4/CD8

TABLE IX
I-E/*Mls* TOLERANCE-RELATED CLONAL DELETIONS IN SPLEEN/LYMPH NODE CELLS OF *lpr/gld* CONGENIC STRAINS

Strain	*H-2*	I-E	*Mls*	I-E/*Mls* Vβ deletions	I-E Vβ deletions
AKR	*k*	+	*a*	6, 7, 8.1, 9	5.1, 11, 12, 16
AKR-*lpr/lpr*				6, 7, 8.1, 9	5.1, 11, 12, 16
C3H	*k*	+	*c*	3.1	5.1, 11, 12, 16
C3H-*lpr/lpr*				3.1	5.1, 11, 12, 16
C3H-*gld/gld*				3.1	5.1, 11, 12, 16
MRL-+/+	*k*	+	*b*	—	5.1, 11, 12, 16
MRL-*lpr/lpr*					5.1, 11, 12, 16
C57BL/6	*b*	−	*b*	—	—
C57BL/6-*lpr/lpr*					

is the rule for nonpositively selected $CD4^+8^+$ cells (followed by cell death), then double-negative TcR $\alpha{:}\beta^+$ cells could represent a short-lived intermediate cell type in this process; (2) a second possiblity, which we currently favor, and which is supported by some highly suggestive, although indirect, evidence, is the involvement of a novel back-up form of tolerance induction, perhaps directed at some thymocytes expressing moderately autoreactive TcRs. Such moderately autoaggressive cells might be present at the double-positive stage and converted to double-negative cells via the so-called "primary" negative selection process, wherein the highly autoaggressive clones are deleted. Alternatively, such cells might be generated during a postulated "secondary" negative-selection process exerted on single-positive cells that changed via maturation and higher receptor level expression from low to moderate/high-affinity autoreactive cells; the highly reactive cells will be eliminated, while some of the moderately reactive cells may escape deletion but will still down-regulate their CD4/CD8 molecules. The recently reported findings of Kisielow *et al.* (142) using transgenic mice expressing a self-reactive TcR specific for the male HY antigen suggest that CD4/CD8 loss may indeed be such a tolerance-related event. In this investigation, the peripheral transgene-expressing T cell population in male (i.e., autoreactive) mice, but not in female (nonautoreactive) mice, was found to be composed almost entirely of cells that had lost the CD8 accessory molecule required for efficient HY antigen recognition; most of these cells (58%) were of the double-negative phenotype, with the remainder being $CD4^+8^{low}$ (35%) or $CD4^+8^-$ (7%). We interpret such cells to represent probable escapees from clonal elimination that were secondarily "tolerized" by accessory molecule down-regulation before being allowed to exit to the periphery. The presence of such high numbers of these cells is presumably an artifact of the transgene system, in which the capacity of the clonal elimination process is likely to be overwhelmed. An alternative explanation, i.e., that these cells represent expansion of a TcR $\alpha{:}\beta^+$ subset that never expressed CD4/CD8, must also be considered, but is generally not supported by current evidence, indicating that such a subset probably does not exist (106). Moreover, even if such cells existed, it is not clear why they would be positively selected and expanded in the absence of CD8 accessory molecules.

Based on the above scheme, the *lpr* and *gld* cells would represent a magnified and otherwise modified version of a distinct, posttolerization subset of the heterogeneous normal double-negative TcR $\alpha{:}\beta^+$ thymocytes. In the normal setting, the moderately self-reactive cells from this pathway that escape clonal deletion will be subjected to tolerance-induced accessory molecule down-regulation and slowly accumulate in the thymus to constitute a portion of the minor late-appearing

double-negative TcR $\alpha{:}\beta^+$ population. Some of these cells might migrate to the periphery (see below), but the majority would be expected to eventually die intrathymically due to their lack of accessory molecules and resultant inability to efficiently fulfill their function. In contrast, *lpr* and *gld* mice, due to undefined modifications introduced by these mutations, produce a high number of such cells in the thymus, a large proportion of which leak into the periphery, populate lymph nodes and spleen, and continue to proliferate.

In advancing this concept, it might therefore be suggested that double-negative TcR $\alpha{:}\beta^+$ *lpr/gld* cells (and a subset of normal double-negative TcR $\alpha{:}\beta^+$ cells) have essentially autoreactive TcR specificities. If such cells are exported in large numbers into the periphery, as in *lpr* and *gld* homozygous mice, autoimmune manifestations can clearly ensue. For example, such double-negative cells might retain sufficient autoreactivity in spite of their lack of CD4/CD8, and induce or accelerate autoimmunity by secretion of appropriate B cell differentiation/promoting lymphokines (37). Alternatively, a portion of these cells may reacquire accessory molecules and become fully functional and autoreactive. Indeed, acquisiton of accessory molecules by *lpr* and normal double-negative T cells cultured *in vitro* has been demonstrated (143–146).

The above-postulated autoreactive potential for double-negative TcR $\alpha{:}\beta^+$ cells is further supported by several other studies. Thus, double-negative splenic T cells isolated from normal (BALB/c, DBA/2) mice upon concanavalin A (Con A) activation acquired CD4 and, in contrast to other Con A-stimulated T cell subsets, induced syngeneic B cells to proliferate and differentiate into Ig-secreting cells at high levels (146), a situation not unlike that seen spontaneously with the constitutively activated *lpr* cells (37). Such activated peripheral double-negative TcR $\alpha{:}\beta^+$ normal lymphocytes also proliferated upon incubation with syngeneic dentritic cells and were inhibited by anti-class-II antibodies, further documenting their autoreactive potential (145,146). Induction of autoimmune disease by transfers of double-negative, Thy-1$^+$-Ly-1^{low} spleen cells from normal BALB/c mice (147), or thymic cells from cyclosporin-treated mice (148,149) severely depleted of CD4$^+$8$^-$ and CD8$^+$4$^-$ cells, into syngeneic nude mice has also been documented. Furthermore, in (NZB $\times$ W)F_1 lupus mice (25), and in humans with lupus (S.K. Datta, personal communication), helper *in vitro* activity for anti-DNA production was found not only in CD4$^+$8$^-$ but also, surprisingly, at an equal if not higher degree in CD4$^-$8$^-$ spleen and peripheral T cells, respectively. Interestingly, studies in mice suggest that the frequency of double-negative TcR $\alpha{:}\beta^+$ cells in thymuses and spleens differ in various genetic backgrounds (106,146,150) and thus may

be genetically controlled. Such genetically imposed expansions of these bona fide autoreactive double-negative TcR $\alpha{:}\beta^+$ cells may contribute in an important way to the induction of murine and even human systemic autoimmune diseases.

V. Conclusions

To summarize, the above studies addressing the Ig germ line gene organization in lupus-prone strains of mice suggested that the disease can develop in different Ig heavy and light chain haplotypes, and that the Ig germ line genes in lupus mice are probably normal. Analyses of the Ig gene segments expressed in monoclonal autoantibodies from autoimmune mice revealed that similar, and in some instances even identical, gene segments are expressed in autoantibodies and in antibodies to exogenous antigens, and that antiself and antiforeign responses are encoded by the same, or at least an overlapping, germ line gene repertoire. A large variety of Ig V, D, and J gene segments can encode autoantibodies with different specificities, and both germ line genes and somatically mutated genes can be expressed in such antibodies. As in responses to certain exogenous antigens, autoantibodies with a given specificity may exhibit varying degrees of genetic restriction. Evidence has been obtained that, in an individual lupus mouse, the number of autoantibody-secreting clonotypes decreases after class switch, while that of productive mutations increases, suggesting that antiself responses might be (auto)antigen-driven responses, but this conclusion should be considered tentative.

In addition, studies addressing the TcR germ line and expressed repertoire of autoimmune and other laboratory mice indicated, as with Ig genes, that there is no unique autoimmunity-associated TcR α or β gene haplotype, and the germ line TcR repertoire in autoimmune mice is similar to that in normal mice. The inherent program of Vβ gene expression in unselected thymocytes is related to Vβ genomic organization and imparts a significant skewing effect on the Vβ repertoire that, in general, persists in the mature T cell population. Furthermore, all readily detectable tolerance-related Vβ clonal elimination phenomena can apparently be attributed to either I-E or I-E/*Mls* reactivity, including deletion of clones expressing Vβ3.2 (17a) in Vβ^a haplotype I-E$^+$ mice, deletion of clones expressing Vβ6, 7, 8.1, and 9 in I-E$^+$/*Mls*^{a} mice, deletion of cells expressing Vβ3.1 in I-E$^+$/*Mls*^{c} mice, and deletions of Vβ5.1, 11, 12, and 16 in I-E$^+$ strains. Lupus-prone mice show no generalized defect in the expression of these major tolerance-related clonal eliminations.

Finally, based on our investigations of I-E and *Mls* tolerance-related

Vβ clonal deletions, we propose that the abnormally proliferating, autoimmunity-inducing/enhancing double-negative TcR $\alpha{:}\beta^+$ *lpr* and *gld* cells are not related to immature $CD4^-8^-$ thymocytes, but instead are derived from $CD4^+8^+$ precursors through a process resulting in down-regulation of both accessory molecules. Suggestive evidence supports the concept that such CD4/CD8 down-regulation may be the result of a novel negative selection mechanism acting on thymocytes with moderately autoreactive TCRs that escape conventional clonal elimination. These cells are similar to a subset of the heterogeneous double-negative TcR $\alpha{:}\beta$ minor normal T cell population. Exportation and expansion of these cells in the periphery may be important in the development of systemic autoimmunity.

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Heterogeneity of Cytokine Secretion Patterns and Functions of Helper T Cells

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I. Introduction

Since the discovery that cell-surface antigen differences among T cells (24,44) allowed the separation of cytotoxic T lymphocytes (CTLs) from T helper (Th) cells, several lines of evidence have suggested that further subdivisions exist within the Th cell population. Although functional evidence using mixed cell populations suggested different types of Th cells, the lack of discriminatory cell-surface markers has hampered efforts to clearly define the different subtypes. The development of *in vitro* T cell clones led in 1985 to the description of four types of helper T cell clones (94), and in 1986 two types of Th clones (Th1 and Th2) were defined on the basis of different patterns of cytokine secretion (125). These patterns have been confirmed in several panels of Th clones, and this is currently the most clear-cut criterion for separation of mouse Th subtypes. Because the two types differ in the synthesis of many cytokines, and the cytokines have a major role in the regulation of immune responses, the two types of Th cells have markedly different functions. Although the Th1 and Th2 cytokine patterns are the major recognized phenotypes of long-term mouse T cell clones, there is mounting evidence that other cytokine secretion patterns exist and that the Th1 and Th2 phenotypes may represent mature stages of Th cells. In this review, we describe the properties of Th1 and Th2 clones, the functions of the cells and their cytokines, their contributions to immune regulation, and the existence and properties of precursor Th cells.

II. T Cell Cytokine Secretion Patterns

Because cytokine functions are complex and overlapping, the development of precise, monospecific bioassays and enzyme-linked immunosorbent assays (ELISAs) was essential before a clear picture of T cell cytokine synthesis could be obtained (reviewed in Refs. 126 and 127). The first description of Th1 and Th2 cytokine differences (125) showed

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that mouse Th cells synthesizing and secreting interleukin 2 (IL-2) and interferon γ (IFN-γ) could be distinguished from clones secreting IL-4 [identified by its activities as T cell growth factor (TCGF), mast cell growth factor, and enhancement of synthesis of IgE and Major Histocompatibility Complex (MHC) class II antigens by B cells]. Lymphotoxin (LT) was synthesized coordinately with IFN-γ (197), and these results were confirmed and extended by mRNA analysis, additional bioassays, and monoclonal antibodies (27). IL-5 and a cDNA clone of unknown function, P600, were expressed only by Th2 clones (27,173), and preliminary results indicate that IL-6 is also expressed by Th2 but not by Thl clones [T. R. Mosmann and F. Lee, unpublished (66)]. Several other cytokines or cytokine-like molecules were expressed by both Thl and Th2 clones, but in significantly greater amounts by Thl clones, including granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF), and three more cDNA clones of unknown function, TY5, P500, and H400 (18,27). In contrast, Th2 clones expressed relatively larger amounts of preproenkephalin (ppENK), the precursor of several enkephalin hexapeptides (221). IL-3 was expressed by all clones at widely differing levels. The current information on the mouse Thl and Th2 cytokine synthesis patterns is shown in Table I. Although human Thl-like and Th2-like clones have been described, most human *in vitro* T cell clones express mixed patterns of cytokine secretion (110,139,201). This apparent discrepancy between mouse and human Th clones is discussed in more detail in Section IX, and most of the following discussion refers mainly to mouse T cells.

CTLs also produce cytokines when stimulated by antigen-presenting cells (APCs), particularly some of the Thl cytokines, such as IL-2, GM-CSF, and IFN-γ (91,148). We recently examined a panel of alloreactive $CD8^+$ T cell clones and found that they secrete the same pattern of cytokines as do Thl clones (Table I; T. A. T. Fong and T. R. Mosmann, unpublished). The only exception was the synthesis of IL-2, which occurred in some clones but not in others, as reported previously (91). This could represent a real division among CD8 clones, or it could be an extreme form of the instability of IL-2 expression in Thl clones. *In situ* mRNA hybridization studies of normal CD8 cells (116) showed that a majority of these cells could express IL-2 mRNA when stimulated with calcium ionophore and phorbol ester, although only transient expression was obtained using milder stimulation conditions. Most of our CD8 clones were cytotoxic, and we found no positive or negative correlation between cytotoxicity and IL-2 secretion in our panel of long-term CD8 clones.

Recently, another group of T cells has been described; these express a different T cell antigen/MHC receptor consisting of a $\gamma\delta$ dimer (14)

TABLE I
PROPERTIES OF MOUSE T CELL CLONES

Feature	CTL	Thl	Th2
Surface markers			
L3T4	−	+	+
LY1	−	+	+
LYT2	+	−	−
Cytokines[a]			
Interferon-γ	++	++	−
Interleukin 2	+/−[b]	++	−
Lymphotoxin	+	++	−
GM-CSF	++	++	+
Tumor necrosis factor	+	++	+
TY5	++	++	+
P500	++	++	+
H400		++	+
Interleukin 3	+	++	++
Met-enkephalin	+	+	++
Interleukin 4	−	−	++
Interleukin 5	−	−	++
Interleukin 6	−	−	++
P600	−	−	++
B cell help			
IgM, IgG_1, IgA		+	++
IgG_{2a}		++	+
IgE		−	++
Delayed-type hypersensitivity		++	−
Macrophage activation	++	++	+

[a] Cytokine expression was evaluated by bioassays, ELISAs, and RNA hybridization.
[b] Some but not all CTL clones produce IL-2.

instead of the $\alpha\beta$ dimer expressed on CTLs and Th cells. The $\gamma\delta$ cells do not express either the CD8 antigen (found on CTLs) or the CD4 antigen (found on Th cells) (101). One subtype of $\gamma\delta$ cells, the dendritic T cells, expresses a limited diversity of γ and δ chains (14) and is found in the epidermis, whereas T cells of another subtype express different V_γ and V_δ genes and are associated with the gut (13). One type of dendritic $\gamma\delta$ T cell expressed the Thl pattern of cytokines (R. Tigelaar and T. R. Mosmann, unpublished), but it is not yet known if other cytokine patterns exist within the $\gamma\delta$ population.

In vitro, some mast cell lines synthesize IL-4 (19) and other cytokines of the Th2 pattern in response to cross-linking of surface IgE (M. Plaut and W. E. Paul, personal communication). So far, this is the only non-T cell type that has been reported to synthesize IL-4 or IL-5. This is

particularly interesting because Th2 cells and their cytokines are implicated in strong allergic responses. If normal mast cells synthesize Th2 cytokines, this may represent an amplification mechanism for allergic responses.

III. Functions of Th1 and Th2 Cytokines

A. Th1-Specific Cytokines

IL-2 was initially discovered by its ability to stimulate proliferation of T cells *in vitro*. Although for several years IL-2 and TCGF were considered to be synonymous, we now know that IL-4 (125) and P40 (203,206) are also TCGFs, although the details of their action on different cell types may vary (see Section VI). IL-2 also has additional functions, including the induction of differentiation of CTL precursors and proliferation and differentiation of B cells (108,132,161). IL-2 is produced by Th1 cells and some CTLs.

Because the antiviral activity of IFN-γ was discovered at an early stage, this cytokine was termed an interferon, although some of its other activities may be more significant than is its antiviral activity. Activities on macrophages include activation of increased expression of Ia antigens and Fc receptors for IgG (212), increased killing of intracellular parasites, and increased antibody-dependent cell-mediated cytotoxicity (ADCC) (129,130,133,138,153). IFN-γ also has a variety of effects on B cells, including induction of proliferation (41), inhibition of proliferation (121), preferential induction of IgG_{2a} synthesis by B cells (179), and antagonism of all of the activities of IL-4 on B cells (33,120,121,151). Th2 but not Th1 proliferation is inhibited by IFN-γ (47,53) and IFN-γ synergizes with LT/TNF to inhibit proliferation (117) and cause differentiation of myeloid cell lines (200). In addition to Th1 and CTLs, IFN-γ is synthesized by natural killer (NK) cells (87).

LT and TNF bind to the same receptor with similar affinities and thus probably have similar biological activities. Synthesis of the two cytokines differs, however—LT is produced by Th1 clones and CTLs, whereas TNF is produced by Th1 clones, CTLs, Th2 clones, and especially macrophages (9). TNF and LT have a very wide spectrum of activities, including cytotoxicity, induction of differentiation of granulocytes, fever induction, and inhibition of lipid uptake, leading to cachexia (reviewed in Refs. 9 and 114). Lymphotoxin can kill B cells during interaction of Th1 cells with B cells presenting antigen (197), although under other circumstances LT and TNF can act as B cell growth factors (90).

B. Th1-Preferential Cytokines

GM-CSF stimulates growth and differentiation of several cell types in the myeloid lineage, particularly granulocytes and macrophages and their precursors (reviewed in Ref. 31). In addition to its production by Th1, Th2, and CTLs in response to antigen, GM-CSF may be produced by other cells, such as keratinocytes (98) and bone marrow stromal cells (157). The activities of GM-CSF in hemopoiesis may thus be regulated by normal bone marrow elements during steady-state hemopoiesis, and T cells may provide an additional source during strong immune responses in which it is likely that hemopoietic cells will need to be replenished, as well as provide a local source to enhance myeloid cell differentiation and function in the vicinity of the infection.

TNF, although produced mainly by macrophages and Th1 cells, is also expressed by Th2 cells, at least at the mRNA level (27). In other respects, TNF has very similar properties to LT.

Several cDNA clones have been isolated from a Th2 cDNA library (218) on the basis of their expression in abundant amounts only after stimulation of the T cells (18,221). Three of these cDNAs, TY5, P500, and H400, are expressed by all T cell clones, but are expressed more abundantly in Th1 than in Th2 clones (18,27). P500 has also been isolated independently and was named TCA3 (22). The function of the proteins corresponding to these cDNAs is not yet known, but their sequences are consistent with secreted proteins, and TY5 has been identified as a major secreted protein of activated T cells (18). These three cDNAs have moderate homology with one another and with other genes in mouse and human (18). They comprise a family of at least five members, and one of the members, macrophage inflammatory protein, is a mediator of inflammatory responses (214). Because this family of genes is expressed at higher levels in the Th1 cells that mediate inflammation (Section IV), it will be important to test the T cell members of this gene family for inflammatory functions. This family of five "inflammatory" proteins is also distantly related to another family of proteins, including platelet basic protein and neutrophil-activating factor (18).

C. Th2-Specific Cytokines

IL-4 has a large number of biological activities on various cell types (reviewed in Ref. 128) and as a result was originally described by several names, including B cell differentiation factor γ, B cell growth factor, B cell stimulatory factor-1, and macrophage fusion factor (54). In addition to being a cofactor for proliferation of B cells (69), IL-4 induces activation (152) and increased Ia (134,160) and Fcϵ receptor (71)

expression on resting B cells, and increased Ia expression on macrophages (219). B cells are also induced to undergo immunoglobulin class switching to IgG_1 (75,209) and IgE (8,33,35,103). IL-4 is also a cofactor for proliferation of mast cells (57,124,125,178), T cell clones (46,57,124,125,178), CTL precursors (144), mature T cells (70), and thymocytes (220). Hemopoietic cells respond in a complex manner to IL-4, such that IL-4 enhances the effects of some colony-stimulating factors and inhibits colony growth in other cases (142,156). Many cell types bear receptors for IL-4 (109,137), and so there may be more effects of IL-4 still undiscovered. IL-4 is synthesized by the Th2 subset of T cells (27,125) and also by some mast cell lines *in vitro* (19).

IL-5 was originally identified as a B cell growth factor that stimulated activated B cells to proliferate (183). When the recombinant cDNA clone for IL-5 was isolated (136,217), two other activities of IL-5 were confirmed, i.e., eosinophil growth and differentiation factor (172) and killer helper factor (188). IL-5 also enhances the secretion of IgA by B cells stimulated with lipopolysaccharide (LPS) (12,37,131). IL-5 is synthesized by the Th2 subset of T cells (27) and also by some mast cell lines (M. Plaut and W. E. Paul, personal communication).

IL-6 has a large number of activities and was originally identified by a number of bioassays and given several names. Only when cDNA clones encoding IL-6 were isolated did it become clear that all of these effects were mediated by a single molecule (30,59,64). IL-6 stimulates proliferation of plasmacytomas and hybridomas (204,207), thymocytes (66), and hemopoietic progenitor cells (30). IL-6 also induces mature CTLs from thymocytes (117,187), secretion of acute-phase proteins by hepatocytes (55), and stimulates B cells to produce antibody (64).

D. Th2-Preferential Cytokines

One of the major induction-specific proteins synthesized by Th2 cells is the precursor of the met-enkephalin neuropeptides (ppENK) (221). This was initially discovered by isolating and sequencing one of the abundant clones in a Th2 cDNA library; the functions of ppENK synthesized by T cells are not yet known. It is possible that ENK is used to communicate between the immune and nervous systems, e.g., by reducing pain during inflammatory reactions. Alternatively, ENK may have quite separate functions in each system, because the nervous and immune systems are generally separate. In this regard, ENK has been reported to stimulate natural killer cells (216). Finally, the extent of cleavage of the precursor by T cells is not known, and so it is conceivable that the ppENK precursor has functions that are not shared with the enkephalin hexapeptides.

IL-3 is synthesized by all T cells that we have tested, including Thl,

Th2, and CTL clones. The amounts made by different clones vary widely, and in a panel of Th1 and Th2 clones both types synthesized similar amounts (125), although results from a larger panel of clones suggest that Th2 cells may synthesize more than Th1 cells. IL-3 stimulates proliferation and differentiation of a wide variety of hemopoietic progenitors and induces the formation of *in vitro* colonies containing cells of the erythroid, myeloid (macrophages and granulocytes), megakaryocyte, and mast cell lineages (reviewed in Ref. 123). IL-3 appears to be produced by T cells but not by bone marrow stromal cells, and may thus represent an "emergency" regulator of hemopoiesis that is used to enhance the generation of more leukocytes during strong immune responses, when the hemopoietic system is likely to be depleted.

IV. Functions of Th1 and Th2 Cells

A. Delayed-Type Hypersensitivity (DTH)

When injected simultaneously with antigen into the footpads of naive mice, Th1 clones cause an antigen-specific and MHC-restricted inflammatory reaction that peaks at about 24 hours (26). Th2 clones do not produce a swelling reaction under these conditions (26). One of the essential mediators of this DTH response is IFN-γ, which is responsible for some but not all of the antigen-specific swelling reaction induced by most Th1 clones (51a). IFN-γ has also been implicated in the migration of lymphocytes into inflammatory sites (76), and other Th1 cytokines that may be involved in DTH reactions are LT and GM-CSF, which also have effects on macrophages and granulocytes.

The DTH responses in several species can be separated into two types. Tuberculin-type DTH is characterized by a mononuclear cell infiltrate, slow kinetics (peaking at about 48 hours), and substantial induration (45,210). In contrast, Jones–Mote DTH involves more granulocytes in the infiltrate, more rapid kinetics (peaking at about 24 hours), and causes softer swelling, with more edema than cellular infiltration (85,158). By these critiera, DTH induced by Th1 clones resembles the Jones–Mote form of DTH (51a). If further experiments confirm this similarity, it will be important to determine the cell type(s) that are responsible for tuberculin-type DTH. Possibilities include Th1 cells in conjunction with Th2 cells, Th1 and other accessory cells, or a hitherto-undiscovered type of T cell.

B. Helper Functions of Th1 and Th2 Cells

One of the major functions of helper T cells (the one for which they are named) is to provide signals for activation, proliferation, and

differentiation to B cells that have encountered an antigen. Considering the differences in cytokines produced by the murine Th1 and Th2 subsets, it is not surprising that there are significant differences in this complex and multistep function. Indeed, each of the four Th products that play an important role in B cell help (IL-2, IL-4, IL-5 and IFN-γ) are subset specific. The mechanisms of T cell help and the roles of cytokines and Th subsets in that process have been the subject of several extensive recent reviews (1,36,78,126,208), and the reader is referred to these for a more thorough treatment of the subject and for a more complete bibliography.

Several strategies have been employed for studying the functions of mouse Th clones *in vitro* and for analyzing the roles of specific Th products in these functions. One approach is to use Th clones as the carrier-specific population providing antigen-specific, Ia-restricted help for B cell antihapten responses. However, the frequency of antigen-specific B cells is quite low, even in primed B cell populations, and much of the immunoglobulin produced often comes from antigen-nonspecific bystander responses (R. L. Coffman, unpublished). For this reason, it is necessary to use detection methods that are antigen specific, such as hemolytic plaque assays or antigen-specific ELISA or radioimmunoassays. The low number of responding cells also means that the response of minor isotypes is quite small and difficult to quantitate accurately, especially in primary responses. These limitations can be overcome by using B cell populations that are highly enriched for hapten-specific B cells (181).

A second strategy is to use Th clones specific for molecules on the surface of all B cells, such as Ia, Mls, or H-Y antigens, thus rendering the response effectively polyclonal yet still antigen specific and MHC restricted. A significant refinement of the polyclonal approach is the use of clones specific for rabbit Ig and of rabbit antimouse Ig antibodies as the antigen. The antibodies bind to all B cells with high affinity, and processing and presentation of the rabbit antibody by the B cell are required for T cell activation (28,198). This system, therefore, appears to involve the same sequence of events as any other antigen-specific response, but it results in the stimulation of a majority of B cells (103).

The initial step in T cell-mediated differentiation of resting B cells is their activation to enter cell cycle. In most but not all cases (104,105), this function requires direct T-B cell contact and cannot be mediated by secreted products of Th cells, either singly or in combination (95,102,135,180,213). Both subsets appear, in general, to be capable of delivering this cell-mediated activation signal to unfractionated B cell populations (36,94) and to purified small B cells (93,195,199). Boom

et al., however, have isolated a small, dense population of splenic B cells which can be activated by a Th2 clone but not by a Th1 clone (15).

1. *Helper Functions of Th2 Cells*

The ability of many Th2 clones to provide efficient help for B cell growth and differentiation has been demonstrated in a number of laboratories (15,36,42,93,94,181,196,198,199). Th2 clones can stimulate significant clonal expansion and Ig secretion by 50–80% of splenic B cells in limiting dilution cultures (103) and can stimulate purified populations of small, resting B cells. IL-4 and IL-5 appear to be the principal soluble mediators involved in the helper function of Th2 clones (15,36) (R. L. Coffman, unpublished). The addition of neutralizing monoclonal antibodies to IL-4 causes relatively little inhibition of total Ig production in Th2-stimulated B cell cultures (15,36), whereas the addition of anti-IL-5 antibody causes quite substantial inhibition of Ig production (15,155,173). The combination of anti-IL-4 and anti-IL-5 leads to even greater inhibition than anti-IL-5 alone. Several recent experiments demonstrate that Th and B cells form stable conjugates as a consequence of specific antigen recognition by the Th cell (96,97,170,171) and that cytokines may be secreted preferentially near the portion of the Th cell that receives activation signals (145). It seems possible (but difficult to prove) that a fraction of the cytokines produced by the Th cell is delivered to the B cell across this area of close contact and that this fraction is protected from inhibition by anticytokine antibodies. Thus, the use of anticytokine antibodies to study helper functions may result in an underestimation of the importance of individual cytokines. The importance of IL-4 and IL-5 has also been shown in experiments in which Th2 products are used to promote the proliferation and differentiation of B cells that have been activated by direct contact with a Th1 clone (36). In this system, both IL-4 and IL-5 act as growth and differentiation factors and the combination of both cytokines stimulates growth and differentiation as effectively as does a Th2 supernatant. The exception to this general conclusion is the IgE response, which requires IL-4 and appears unaffected by IL-5 (see below).

Large B cells can proliferate and secrete Ig directly in response to IL-5, whereas purified small B cells require the contact-mediated Th signal to become responsive to IL-5 (63,155). IL-5 thus appears to be the factor responsible for the factor-mediated bystander responses induced by Th2 clones (155). This stimulation of large B cells is relatively weak compared to stimulation by a polyclonally activating Th clone or by LPS (R. L. Coffman, unpublished). This could mean either that IL-5 is an inefficient stimulus for large B cells or that only a subpopulation of large

B cells is responsive. Young NZB/NZW F_1 mice, which later in life develop a lupuslike autoimmune disease, have an increased proportion of IL-5-responsive B cells, and *in vitro* culture of these cells with IL-5 induces production of substantial amounts of autoantibody (63).

2. *Helper Functions of Thl Cells*

The ability of the Thl subset to function as helpers for B cells is considerably more controversial. Thl clones have been reported to provide help for antigen-specific responses from primed B cells (36,42,56) and for primary responses from hapten-purified unprimed B cells (181). In other laboratories, Thl clones fail to provide help for primary responses either to specific antigens (93,94) or in the rabbit Ig polyclonal system (15). We have tested a panel of Thl clones for their ability to stimulate T cell and antigen-dependent polyclonal responses (36) (R. L. Coffman, unpublished). These clones were nearly as efficient at promoting proliferation of both small and large B cells, but were far less efficient at stimulating Ig production than were Th2 clones with similar specificities. Additional proliferation and substantial Ig production could be obtained from these cultures by adding IL-2 or, in many cases, a mixture of IL-4 and IL-5. Further enhancement of Ig production could often be achieved by inhibiting much of the IFN-γ activity with an anti-IFN-γ antibody. Thus, in this culture system, IL-2 levels appear to be too low, and IFN-γ levels too high, for optimum helper function. These results do show, however, that a substantial level of B cell help can be mediated entirely by Thl cells and their products. These observations may explain some of the apparent differences in Thl function observed in different laboratories. For example, some Th clones produce little or no IL-2, especially after long periods of time in culture (27,215).

Although Thl clones can provide helper activity under certain circumstances, they also have the potential to inhibit B cell responses that have been stimulated by either subset of Th cell. IFN-γ at low concentrations acts as a specific antagonist of IL-4-mediated effects on B cells (33,35,120,179) and, at higher concentrations, acts as an inhibitor of B cell growth and differentiation (33,126). At higher T to B ratios, Thl clones can even be directly cytotoxic for activated B cells. Thl clones have, in fact, been shown to be inhibitory for responses stimulated by Th2 clones (6,17,52), and much of this inhibition can be blocked with anti-IFN-γ antibodies (R. L. Coffman, unpublished).

C. Isotype Regulation by Th Clones

Thl and Th2 clones differ consistently in their ability to stimulate IgG_{2a} and IgE responses. Hapten-purified unprimed B cells make a significant IgG_{2a} response to haptenated carrier when help is provided

by a Th1 clone, but not by a Th2 clone (181). Similarly, polyclonal stimulation with Th1 clones, but not with Th2 clones, leads to substantial IgG_{2a} responses if exogenous IL-2 is added to the cultures (36) (R. L. Coffman, unpublished). Several lines of evidence suggest that IFN-γ is required for this IgG_{2a} response, such as the enhancement of IgG_{2a} in LPS-stimulated B cell cultures (179) and the enhancement or inhibition of IgG_{2a} responses *in vivo* with IFN-γ or anti-IFN-γ, respectively (48). This has not been confirmed directly in Th-stimulated cultures; neither the addition of anti-IFN-γ to Th1-stimulated cultures nor the addition of IFN-γ to Th2-stimulated cultures significantly affects IgG_{2a} responses (36,181).

Most Th2 clones provide help for quite respectable IgE responses, whereas Th1 clones stimulate no detectable IgE under normal conditions (36) (R. L. Coffman, unpublished). The Th2-mediated IgE response is quite dependent upon IL-4 and can be totally inhibited by anti-IL-4 or IFN-γ. This is consistent with earlier studies that demonstrated that IL-4 is required for IgE production in LPS-stimulated B cell cultures and that this was inhibitable by IFN-γ (33–35,106,179). The differences in the expression of IL-4 and IFN-γ seen in Th1 and Th2 clones fully explain their different abilities to stimulate IgE. Th1 clones can stimulate IgE production if IL-4 and anti-IFN-γ antibodies are added to the cultures (R. L. Coffman, unpublished). Stimulation of hapten-purified B cells with a Th2 clone also stimulates a much larger IgG_1 response than does a Th1 clone, and this response is largely sensitive to anti-IL-4 and IFN-γ (181). *In vivo*, anti-IL-4 can almost totally inhibit both polyclonal (49) and antigen-specific primary and secondary (50) IgE responses, and IFN-γ can also inhibit polyclonal IgE responses (48).

D. Human Th Clones

The cytokine expression patterns of human $CD4^+$ Th clones do not fall clearly into different subsets. Large panels of clones have only been tested for IL-2, IL-4, and IFN-γ expression, and clones expressing all of the possible combinations of these cytokines have been observed (43,110,139,141,164,201). Similar diversity also exists among $CD8^+$ clones (110,139). A majority of the $CD4^+$ clones of each type can stimulate IgM and/or IgG production (110,141,164,201). In contrast, all clones that stimulate IgE production belong to subsets that express IL-4 (43,110,141,201). Some clones produce both IL-4 and IFN-γ and among these clones there is an inverse correlation of IFN-γ production and IgE helper function (43,141). As in the mouse, this is consistent with observations that IgE production by human B cells requires IL-4 and can be inhibited by IFN-γ (140). The roles played by IL-2, IL-4, and IFN-γ in the other helper functions of these clones have not been clearly

elucidated as yet; however, both IL-2 and IFN-γ have potent B cell growth and differentiation activities in a variety of mitogen-stimulated B cell culture systems (84). Maggi *et al.* report IgG helper function in a majority of the $CD4^+$ clones that do not secrete detectable levels of IL-2, IL-4, or IFN-γ (110).

E. Macrophage Activation

Both Thl and Th2 clones produce cytokines that activate macrophages, although Thl clones are much more effective. IFN-γ activates macrophages to kill intracellular parasites more effectively and to increase synthesis of Ia antigens and Fcγ receptors, and synergizes with LT to activate ADCC. GM-CSF also enhances the production of macrophages and their migration to the site of an immune reaction, so that it may act in concert with the macrophage-activating factors to increase macrophage function at a local site. Activation of macrophages by IL-4 has also been reported for Ia expression and intracellular killing (40,219), although in both cases these functions may be induced by IL-4 much less effectively than by IFN-γ (40,153). In addition to the cytokine effects, Th cells also deliver cell-mediated activation signals to macrophages, and, as in the case of T–B interactions, both Thl and Th2 are able to deliver the cell-mediated signals (182a).

V. Precursors of Th1 and Th2 Differentiation States

A. Evidence from Bulk Cultures

When $CD4^+$ spleen T cells from normal mice are stimulated with various polyclonal or antigen-specific reagents, the cytokine pattern that is produced does not match either the Thl or Th2 pattern, nor any mixture of these types (N.E. Street, J. H. Schumacher, and T. R. Mosmann, unpublished). In unseparated spleen cell populations, significant levels of IFN-γ may be contributed by $CD8^+$ lymphocytes, while $CD4^+$ cells produce only small amounts of IL-4, IL-5, or IFN-γ, and express IL-2 at high levels. In other studies, only IL-2 and IFN-γ could be detected in supernatants from mitogen-stimulated normal spleen cells (184). It is not clear if these discrepancies are due to differences in the balance of Thl and Th2 activation in the mouse colonies, or in the sensitivities of the assays used. The cytokine pattern could still be explained by Thl and Th2 cells if it were postulated that IL-4, IL-5, and IFN-γ, but not IL-2, were subject to considerable negative regulation. In contrast, experiments designed to test the existence of suppression of cytokine synthesis have shown that IL-2

synthesis is subject to considerable suppression in normal spleen cell populations, whereas suppression of IFN-γ, IL-4, and IL-5 synthesis was only seen rarely (N. E. Street, J. H. Schumacher, and T. R. Mosmann, unpublished). This means that the balance between the cytokines is even more difficult to reconcile with the Thl and Th2 patterns, and so it is very unlikely that normal spleen cells consist merely of a mixture of Thl and Th2 cells, and much more likely that at least one precursor stage exists, synthesizing IL-2.

B. Evidence from Clones

The Thl and Th2 cytokine synthesis patterns have been found in panels of long-term mouse T cell clones in a variety of laboratories. However, other cytokine secretion patterns exist, especially among short-term clones in tissue culture (92) (A. Glasebrook, personal communition; N. E. Street, H. Bass, D. F. Fiorentino, T. A. T. Fong, J. Leverah, and T. R. Mosmann, unpublished). In particular, clones exist which synthesize both Thl and Th2 cytokines. We recently followed the cytokine secretion patterns of a large number of T cell clones at early times after establishment in tissue culture (e.g., 2–8 weeks) and found that the distinct Thl and Th2 phenotypes were seen most readily with cells from mice that had been immunized with antigens that stimulate a strong Thl or Th2 response *in vivo*. T cell clones from mice that had not been deliberately immunized tended to make lower quantities of cytokines and showed mixed cytokine patterns, although clear Thl or Th2 clones could develop in these populations at later times. These experiments suggest the existence of precursors of Thl and Th2 phenotypes, but do not yet prove whether the precursors are committed to a particular phenotype before expressing the mature cytokine secretion phenotype, or whether a common precursor can be induced to differentiate into either Thl or Th2 cells. Since both responder and stimulator cells were obtained from strongly immunized mice, the commitment to Thl or Th2 phenotypes could have occurred *in vivo* or *in vitro*.

C. Cell-Surface Antigen Expression

Since cell-surface markers have been so useful in separating functionally distinct lymphocyte populations, it is important to consider the available surface markers on Th cells. Several monoclonal antibodies (MAbs) have been described that subset mouse, rat, and especially human $CD4^+$ T cells (5,10,11,16,122,191). Some of these MAbs recognize determinants on various isoforms of the CD45 protein (leukocyte common antigen, T200/B220). The expression of this molecule varies among lymphocytes, and three introns near the N-terminus are variably

expressed by alternate splicing (192). Of the eight isoforms theoretically possible, at least three are expressed by different lymphocytes. Some MAbs recognize determinants on the nonvariant part of the CD45 polypeptide, whereas others recognize determinants on one of the three variably expressed exons. The determinants recognized by the latter group are collectively referred to as CD45R (i.e., CD45 restricted) determinants, although this nomenclature does not distinguish between determinants located on different exons. MAbs 2H4 and HB11 (122,191) recognize determinants encoded by exon A of human CD45, while OX22 and 16A recognize exon B of rat and mouse CD45, respectively (5) (K. Bottomly, personal communication), and UCHL1 (10) is expressed reciprocally with HB11/2H4.

The expression of various CD45R determinants distinguishes functionally different subsets of $CD4^+$ T cells in mouse, man, and rat, but it is not yet determined if these markers distinguish different stages of cells in the same lineage, different lineages, or, perhaps most likely, a combination of both possibilities. The human $HB11^+/2H4^+$ cells lose expression of the 2H4 determinant after activation (2,32,175,191), and activated rat and mouse T cells similarly lose expression of the OX22 antigen (147) and the 23G2 epitope (11), respectively. This evidence all suggests that some CD45R epitopes are lost after activation, and so a particular CD45 molecule is not expressed uniformly on a particular lineage of cells.

There are also data that could be interpreted as evidence for the expression of different epitopes by different lineages of CD4 T cells. Arthur and Mason (5) showed that IL-2 is produced mainly by the $OX22^+$ subset, while B cell help was mediated by the $OX22^-$ cells. Mouse $CD4^+$ $16A^{hi}$ cells produced IL-2, whereas $16A^{lo}$ cells produced IL-4 (16). However, this observation is complicated by the fact that *in vitro* T cell clones showed reversed patterns, i.e., IL-4-producing Th2 clones express higher 16A epitope levels. It is also possible that a particular population of cells, e.g., recently stimulated T cells, may contain a disproportionate amount of either Th1 or Th2 cells, depending on the type of stimulation administered to the animal. In such cases, the subpopulation in question might appear to define either Th1 or Th2 cells, although in reality the subpopulation would be defined by its activation state.

Taken together, the evidence suggests that CD45 epitopes may be both activation and lineage markers, and that these determinants may only be useful for defining functional lineages within a carefully defined subset of $CD4^+$ T cells in the same activation state, but not in normal mixed populations. For example, $OX22^-$ cells may comprise activated Th1 cells as well as nonactivated Th2 cells or their precursors. This raises the possibility that double-antibody studies using CD45R reagents in

conjunction with activation markers (e.g., Pgpl) may be able to clearly define functional subsets. The results of Hayakawa and Hardy (60) are interesting in this regard, since they have used two MAbs (recognizing determinants that have not yet been defined) to separate out four subpopulations of CD4$^+$ T cells. Populations I and III expressed cytokines and functions similar to Thl and Th2 cells, respectively, whereas population II had intermediate properties and may be related to the precursors identified in cloning experiments (see above). The extension of this approach to additional pairs of MAbs may help to resolve the current rather confusing picture. Several additional MAbs that do not recognize CD45 determinants (10) may also be useful, although it is possible that Thl and Th2 cells do not express simple lineage markers that are uniquely associated with one lineage, such as the CD4 and CD8 markers, which are such useful lineage markers on helper and cytotoxic T cells.

The Pgpl cell surface marker is expressed on subsets of CD4$^+$ and CD8$^+$ T cells. In both cases, the Pgpl$^-$ cells appear to correspond to virgin, unstimulated T cells, and the memory cells that have been previously exposed to antigen are found in the Pgpl$^+$ population (20). The Pgpl$^+$ and Pgpl$^-$ populations both express similar levels of IL-2 after stimulation, but the Pgpl$^+$ cells express much higher levels of IFN-γ (21). This suggests that the precursors of both CTLs and Thl cells produce IL-2 but not IFN-γ, in accordance with the evidence discussed above. In preliminary experiments, the Pgpl$^+$ population is also responsible for the majority of the IL-4 and IL-5 production (R. C. Budd, J. H. Schumacher, and T. R. Mosmann, unpublished).

D. Other Types of Th Heterogeneity

Several studies of normal T cell function have suggested that heterogeneity exists in the helper T cell compartment. The Tl and T2 subpopulations (3,4,86,177) probably correspond to precursors and mature T cells rather than to Thl and Th2 subtypes. Tl cells are short-lived, as evidenced by rapid disappearance after thymectomy, resistant to antithymocyte serum administered *in vivo*, and include precursors but not effectors of DTH and B cell help. These may correspond to the IL-2-producing precursors discussed above and to the Pgpl$^-$ T cells described in the preceding paragraph. In contrast, T2 cells are relatively long-lived, sensitive to antithymocyte antiserum, and include the effector cells for B cell help. These cells may correspond to a mature effector population that has previously been exposed to antigen, including Thl and Th2 cells and also Pgpl$^+$ cells.

Limiting dilution experiments showed that two different types of T helper cell could be defined—those that helped a single cell, presumably involving cell contact, and those that could help bystander

cells (72,112,211). These populations are unlikely to be equivalent to Th1 and Th2 cells, since Th1 and Th2 can provide both cognate and noncognate help to B cells (see above). One or both of the helper types defined in limiting dilution cultures may be included in the IL-2-synthesizing precursor cell population, especially since the majority of T cells that help B cells in limiting dilution cultures synthesize IL-2 (146). Since we know neither the number of differentiation states that precede the Th1 and Th2 phenotypes nor their ability to provide B cell help, we cannot yet decide the relationship of the Th1, Th2, and precursor cell types to the functional types of helper T cells defined in limiting dilution cultures.

T helper cells have also been subdivided on the basis of the expression of cell-surface determinants recognized by alloantisera raised against MHC *I* region differences (185,186). These two subsets were effective at different stages of a B cell response, and only the Ia^+ cells bound to a nylon-wool column (186). The determinant recognized by the anti-*I* region antisera has not been fully characterized, and these populations are difficult to reconcile with any of the other types of T cell heterogeneity described above.

E. Hypothetical Model of T Cell Phenotypes

Attempting to bring together some of the evidence in this section, we propose the model of T cell heterogeneity shown in Fig. 1. Th precursors (Thp) are $Pgp1^-$, are present in the T1 population, and synthesize IL-2 but few or no other cytokines. After activation by antigen, Thp secrete IL-2 and start to differentiate into more mature cells secreting additional cytokines. Before differentiation into Th1 and Th2 cells, the Th cells progress through a stage (Th0) in which they secrete cytokines characteristic of both Th1 and Th2 cells. There may be other intermediate stages (indicated as "?") between the Th precursors and the Th1 and Th2 mature phenotypes. If the antigen stimulation is strong and sustained, the cells are driven to differentiation into the final Th1 and Th2 phenotypes. These mature cells may be analogous to the memory cells of the B lineage, which are committed to a particular Ig isotype. We emphasize that the intermediate stages, between Thp and Th1/2 cells, may have functional attributes that are different from either Th1 or Th2 cells, and these possible intermediates may play an important role in normal immune responses. After an immune response has successfully eliminated the foreign organism, the T cells remain $Pgp1^+$ and enter a long-term resting, memory pool. These T cells may or may not retain their Th1 and Th2 phenotypes on subsequent stimulation.

Although this model is able to account for much of the data described

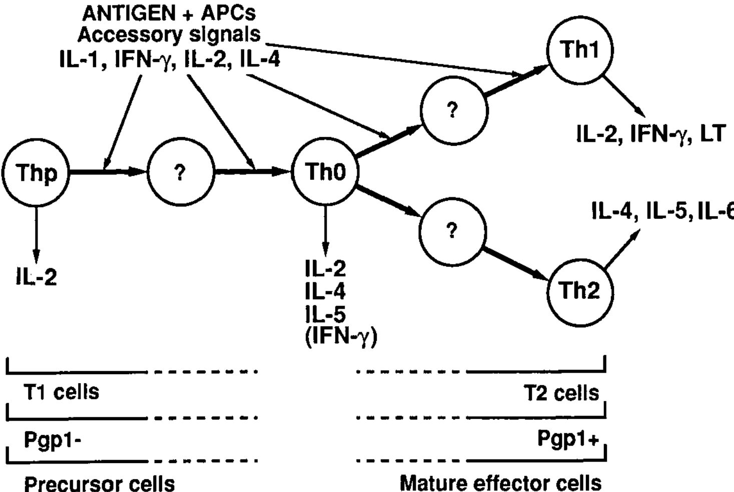

FIG. 1. Possible relationships of Th subpopulations. The branch point into the Thl and Th2 lineages could also occur at an earlier stage than the Th0 cell, e.g., the Thp or earlier. Cells marked "?" indicate that additional differentiation states may exist. The Thl and Th2 states may represent activated cells, and these phenotypes may or may not be retained in the memory cell population.

in previous sections, there are obviously several other models that are still tenable, and we have proposed the model in Fig. 1 as a working hypothesis to provide a framework for future experiments. In particular, it is still possible that the Thl and Th2 precursors are precommitted before exposure to antigen, or that several complex cytokine secretion patterns exist, controlled by environmental factors as well as the differentiation state of the Th cell.

VI. Differential Induction of Thl and Th2

A number of immunogens provoke responses which appear to involve predominantly one of the two Th subsets. Because it is not yet possible to clearly assay these subsets *in vivo*, virtually all of the evidence for the involvement of specific subsets is inferred from the nature of the response. Thus, responses composed largely of DTH, either with very low antibody production or antibodies mostly of the IgG_{2a} isotype, may be regarded as Thl-mediated responses. Examples of antigens that appear to stimulate predominantly Thl responses include fixed *Brucella abortus* (48) and many viruses (39). Responses consisting of large antibody responses,

including significant production of IgE, and weak or absent DTH are regarded as being mediated by Th2 cells with minimal involvement of Thl cells. Examples of antigens which can provoke these responses include goat antimouse IgD and many helminth parasites (49–51). There are several well-documented examples of antigens or pathogens that induce either a predominant Thl or a predominant Th2 response, depending upon the strain of inbred mouse used, including human type IV collagen (194), human fibrinopeptide B (194), and the protozoan parasite *Leishmania major* (61).

At least two distinct classes of explanation can be envisioned for the preferential response to certain antigens. One is that Thl and Th2 cells have at least partially different specificity repertoires. Peterson *et al.* (143) have shown in B10.Br mice that the fine specificity of Th cells which proliferate *in vitro* in response to human fibrinopeptide B [presumably Thl cells (78)] is different than the fine specificity of functional helper cells responding to this peptide (presumably mostly Th2 cells). These fine specificity differences may be related to inherently different variable (V) region repertoire expression in Thl and Th2 cells, or to different modes of presentation of different antigens, such that the final, selected repertoires are different. At present, there are few additional data pertinent to the expression of different repertoires and it will not be discussed further. The second class of explanation is that the stimuli required for proliferation and effector function are qualitatively and/or quantitatively different for the two subsets.

There are at least three different possibilities that need to be considered in the establishment of a Thl- or Th2-biased response. First, the bias may be achieved either by selective activation/growth of Thl or Th2 cells, or by driving the differentiation of uncommitted precursors to Thl or Th2 phenotypes. Second, the selective activation or regulation may occur at the levels of precursor Th stages rather than at the "mature" Thl and Th2 cell stages. Third, cross-regulation of Th subsets (Section VII) may modify the Th populations subsequent to the differential activation step.

Considering the possibility that Thl and Th2 cells are induced by different antigens, one basis for this differential activation could be differences in the type of antigen-presenting cell required by each Th subset. A variety of quite different cell types can process and present antigen, including B cells (29), macrophages (202), dendritic cells (74), Langerhans cells (65,73,176), Kupffer cells (165), astrocytes (189), and endothelial cells (65). With this diversity of APC types, it seems plausible that some of these cells can preferentially activate one Th subset. Splenic dendritic cells, for example, have been shown to present antigen to T cells

that can proliferate *in vitro* in response to antigen (Th1), but not to functional B cell helpers (Th2), whereas splenic and peritoneal macrophages can present antigen to both subsets (154). In general, however, attempts to discern the pattern of such preferences by reinterpreting the existing literature have given complex answers:

1. Antigen-specific B cells appear to be the principal APCs in murine lymph nodes (80,100,111,163). Depletion of B cells *in vivo* by anti-μ treatment leads to defects in the priming of T cells that can transfer DTH (62) and proliferate *in vitro* (62,80,162,163). Both types of assay suggest a defect in the generation of Th1 cells in B-depleted lymph nodes, and have been used to support the idea that B cells present preferentially to Th1 cells (78). However, T cells that transfer B cell help are also primed 10- to 40-fold less efficiently in B-depleted lymph nodes (163) and this population presumably includes many Th2 cells. In contrast, B cell depletion does not seriously affect antigen presentation in the spleen, and both helper (79,80,162) and DTH (62) T cells can be primed in B-depleted spleens.
2. Mice expressing a transgene-encoded Eα on most APCs except for B cells produce normal antibody levels to an E-restricted antigen, but cannot be primed for *in vitro* T cell proliferation to the same antigen (205). However, transgenic mice expressing the Eα on B cells, but not on some other types of APCs, are defective in the *in vivo* priming of both T cell functions. This suggests that certain types of APCs may preferentially stimulate Th2 cells, but the patterns of expression of these transgenes are too complex to draw more specific conclusions.
3. Young SJL mice are defective in the priming of DTH to hepatitis virus, although they produce normal antibody responses (113,182). This defect can be corrected by injection of macrophages from older SJL mice, which have normal DTH responses. The simplest interpretation of these experiments is that Th1 cells are more dependent upon macrophages than are Th2 cells.
4. The type of Th response of BALB/c mice to infection with *L. major* can be radically altered by varying the number of $CD4^+$ T cells present at the time of infection. The response of BALB/c mice to *L. major* is almost entirely a Th2 response, but it can be shifted to a Th1 response by depleting most Th cells with anti-L3T4 antibody (61,168). Similarly, BALB/c nude mice develop resistance (requiring a Th1 response) to *L. major* if reconstituted with small numbers of T cells prior to infection, but develop a strong Th2 response to the same infection if reconstituted with larger numbers of Th cells (119). Thus, the type of response produced by infection depends in some way on the number

of Th present, rather than on any obvious difference in antigen presentation. These results may depend more on cross-regulation between Th subsets (Section VII) than on preferential activation/differentiation. These apparently contradictory observations suggest that the rules for preferential stimulation of different Th subsets, or for specific differentiation of these subsets from a common precursor, may be quite complex.

Janeway has proposed that the density of presented antigen is a critical factor, with low-density presentation leading to stimulation of the Th2 subset and high-density presentation being required for stimulation of Th1 cells (78). This model is consistent with the observations that many intracellular pathogens, the antigens of which should be presented at high density by infected cells, elicit strong Th1 responses, including DTH, IgG_{2a} antibody, *in vitro*-proliferating T cells, and IFN-γ production. The model also predicts that, for soluble antigens, antigen-binding B cells will present the appropriate antigen at high density and preferentially stimulate Th1 cells, whereas phagocytic cells will present much less antigen and preferentially stimulate Th2 cells. Another factor which may be important in preferential stimulation is the requirement of Th subsets for factors elaborated by the APCs. Th2 cells require IL-1 as a cofactor for growth and express IL-1 receptors, whereas Th1 cells do not require IL-1 and do not express detectable IL-1 receptors (58,99,107,190). Thus IL-1-producing APCs should stimulate Th2 cells better than APCs that do not produce IL-1 (e.g., B cells), although B cells can stimulate Th2 clones effectively if exogenous IL-1 or macrophages are added to the culture (159). Thus the distinction between IL-1-producing and -nonproducing APCs may be important *in vivo* only when systemic or local IL-1 concentrations are limiting.

VII. Cross-Regulation of Th1 and Th2 Differentiation States

Although IL-2 is the major growth factor for most or all T cell clones, there are several complexities in the proliferative responses of Th1 and Th2 clones to different cytokines (47,58,99). IL-2 is a strong growth factor for both types, and IL-4 is able to stimulate growth, to a lesser extent, at short times after antigen stimulation. At later times, Th2 clones still respond to both cytokines, while Th1 clones respond to IL-2 only, although IL-2 and IL-4 can still synergize to stimulate Th1 growth. IL-1 is required by Th2 but not Th1 clones as a costimulant for the induction of growth factor responsiveness by antigen and APCs.

Several features of immune responses suggest that Th1 and Th2 cells are mutually inhibitory and that at least some of these effects are

mediated by soluble cytokines. This inhibition may operate at the levels of effector function, cytokine synthesis, and proliferation. Th1 effector function and IL-2 and IFN-γ synthesis are inhibited during a strong Th2 response (T. A. T. Fong, N. E. Street, and T. R. Mosmann, unpublished). In addition to the different requirements for stimulation of proliferation, Th1 and Th2 clones exhibit different responses to inhibitory cytokines. IFN-γ inhibits the growth of Th2 but not Th1 clones in response to either IL-2 or IL-4. A Th2-derived inhibitor of Th1 proliferation can be inferred from the experiments described in Ref. 67, using recent information to reinterpret some of the results.

Since there were several lines of evidence suggesting Th2 inhibition of Th1 function, we have recently examined Th2 supernatants for inhibitory cytokines effective on Th1 cells, and we have partially purified a cytokine that inhibits the synthesis of cytokines by Th1 clones (D. F. Fiorentino, M. W. Bond, and T. R. Mosmann, unpublished). This cytokine synthesis inhibitory factor (CSIF) is distinct from interleukins 1 through 7, IFN-γ, GM-CSF, and TGFβ, and may represent a novel cytokine that is involved in the mutual inhibition of Th1 and Th2 cells. This cytokine may also be important for the B cell helper function of Th1 clones. As described above, Th1 clones are often poor B cell helpers unless the quantities of various cytokines are adjusted to optimal levels. It is possible that the Th2-derived CSIF may effect this change in mixed populations of cells, so that most Th1 cells would be excellent helpers in the presence of even a small Th2 response but would not help effectively in the absence of Th2 activation. This might explain the puzzling discrepancy between the effective help provided by some Th1 clones *in vitro*, contrasted with the inhibition of antibody production seen in the presence of a strong DTH (presumably Th1) response (89). The various cytokine contributions to Th1 and Th2 growth and function are diagrammed in Fig. 2.

Although the cytokine cross-inhibitory effects may account for the reciprocal regulation of Th1 and Th2 cells in strong immune responses, this does not explain how the immune system is returned to a state of balance after the infection is resolved. The regulatory properties of certain isotypes of antibody may be related to the restoration of balance (38,149,150,128a). IgG_{2a} antibodies, expected to be produced during a preferentially Th1 response, lead to inhibition of DTH reactions and enhancement of antibody production, whereas IgG_1 antibodies, expected to be more abundant during a Th2 response, have the opposite effects. Thus the cytokine effects, which should occur within hours of the infection and persist only until antigen is removed, result in enhancement of like responses and inhibition of unlike responses. In contrast,

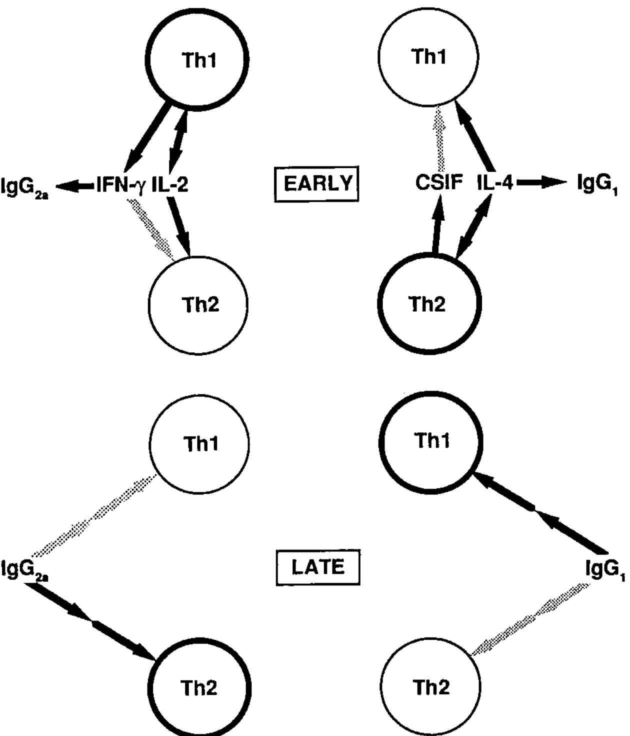

FIG. 2. Interregulation of Thl and Th2 cells. Solid arrows indicate positive effects (e.g., enhancement of proliferation), and stippled arrows indicate negative effects. Left, Thl response; right, Th2 response.

antibody effects would be expected to occur later, when antibody levels rise a few days after the response is initiated, and would tend to counteract the bias imposed by the cytokines.

It is also intriguing to speculate on the role of CTLs in this process. Since CTLs produce the Thl cytokine pattern, activation of CTLs would be expected to influence the overall balance of a response in favor of Thl cells, with corresponding effects on antibody synthesis. We have proposed (126) that early activation of CTLs during viral infection may result in a preferential Thl helper cell response, which could explain the tendency of viral immune responses to produce more IgG_{2a} than IgG_1 when compared to protein antigen responses (39).

An extra layer of complexity is added by the realization that there are probably other types of Th cells in addition to Thl and Th2, and

that these precursor cell stages may have different responses to the regulatory agents described here. Regulation may influence both the function and proliferation of the precursors, as well as affecting their differentiation into Thl and Th2 cells.

VIII. Contributions of Th1 and Th2 to Immune Responses

During an immune response that involves Thl but not Th2 cells (Fig. 3A), several predictions can be made about effector functions. DTH should be strongly induced, but B cell activation would probably not occur due to the inhibitory effects of an excess of IFN-γ and LT produced in an overwhelming Thl response without Th2 involvement. Macrophage functions would be strongly induced by IFN-γ, LT, and GM-CSF, and so intracellular parasites would be effectively killed. If a strong Thl response was accompanied by a moderate Th2 response (Fig. 3B), several features might change. The dilution of the Thl cytokines, the positive effects of IL-4, IL-5, and IL-6, and the modifying effects of Th2-derived CSIF (see above) may all combine to render the Thl cells effective B cell helpers, leading to good antibody production with a bias toward IgG_{2a} (because of IFN-γ production). DTH may be inhibited by the Th2 cells, by CSIF, or by other mechanisms. In general, the activation of Thl cells leads to several effector functions causing cytotoxicity (Fig. 4), and so Thl activation may be most appropriate for dealing with intracellular parasites such as viruses and intracellular bacteria and protozoans.

A strong Th2 response accompanied by a moderate Thl response (Fig. 3C) would be expected to lead to strong antibody production, because of IL-4, IL-5, and IL-6 production, with a possible bias away from IgG_{2a} toward IgG_1, induced by IL-4. IgE should not be prominent, since IFN-γ would still be present. DTH should be weak or absent, since Th2 cells appear to inhibit this Thl function. Finally, in a strong Th2 response without Thl activation (Fig. 3D), high antibody levels would be expected, with no detectable DTH. In particular, IgE would be expected in the presence of high levels of IL-4 and the absence of IFN-γ. High IL-5 levels may result in eosinophilia and macrophage function may be partially activated by IL-4, but not to the same extent as during a Thl response. Mast cells may also be expanded by the actions of IL-3 and IL-4, and so the Th2 response would induce several features of an allergic response, including IgE and the cells that use IgE as a surface receptor to recognize antigens (Fig. 5).

Because CTLs produce the Thl set of cytokines, any response that involves strong CTL activation should give a strong selective advantage

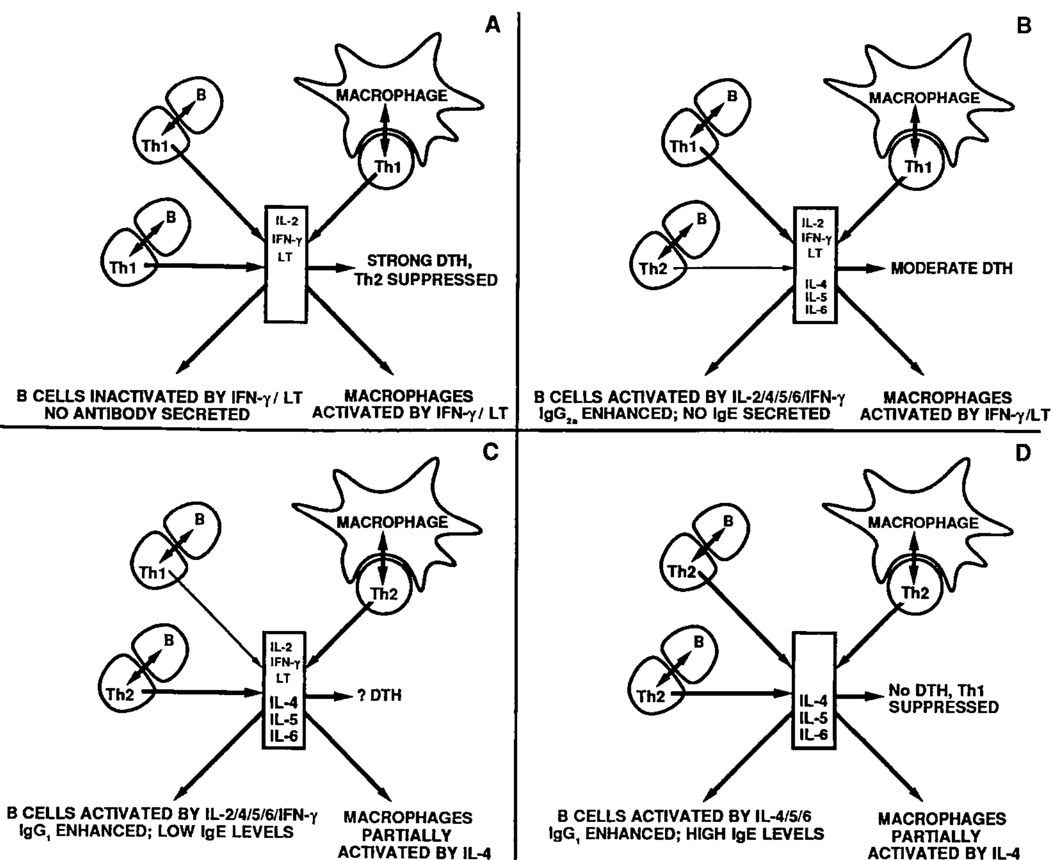

FIG. 3. Predicted consequences of selective Th1 and Th2 activation. Heavy and light arrows indicate high and low cytokine secretion levels, respectively. A, high Th1 response; B, high Th1, low Th2 response; C, low Th1, high Th2 response; D, high Th2 response.

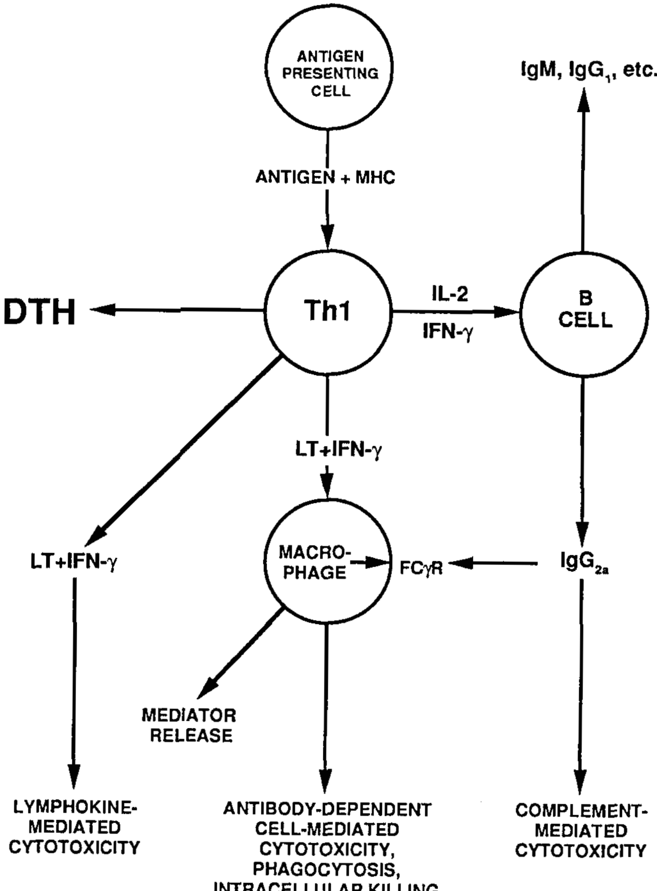

FIG. 4. Thl-mediated effector functions.

to the activation and growth of Thl helpers. As a result, we would expect that CTL responses would be preferentially associated with Thl responses and that a pure Th2 response, with high IgE levels, would not involve CTL activation. This model also leads to the interesting prediction that Thl and CTLs may have similar regulatory properties, especially with regard to their susceptibility to Th2-derived inhibitors such as CSIF.

In addition to the involvement of Thl and Th2 cells in normal responses, it is very likely that the precursors described in Fig. 1 are also functional. The very early precursor, Thp, may correspond to the Tl cell that is not immediately functional (3), but intermediate stages, such

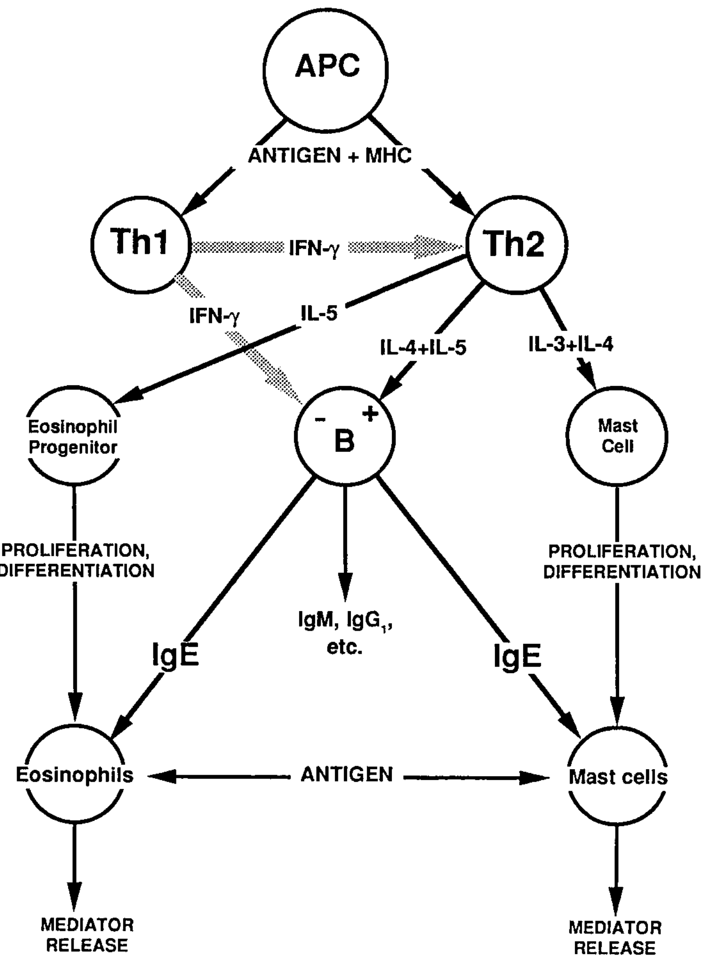

FIG. 5. Thl and Th2 regulation of IgE responses. Solid and stippled arrows indicate positive and negative effects, respectively.

as the proposed Th0 cell, may produce high cytokine levels and have good functional capabilities. Some suggestion of this comes from limiting dilution studies of B cell helpers, in which most of the early helpers produced IL-2 (146). These may correspond to an IL-2-, IL-4-, and IL-5-secreting cell that is quite common early in T cell cloning experiments. In addition to strong helper function, this cell might also strongly enhance T cell growth, because of the production of two T cell growth factors but no inhibitor. This cell would be useful early in an

immune response, when vigorous clonal expansion is necessary, whereas more tightly regulated functions are required later in the response.

Normal, short-term responses may be very complex, since the final Th effector function will be a composite of the functions of Thl, Th2, and various precursors. In general, they may fall into categories B and C (Fig. 3). With our present level of understanding, the extreme responses (A and D, Fig. 3) often seen against various chronic parasite infections or infestations may be more easily interpreted. Similarly, certain adjuvants may have chronic effects that eventually result in simple, extreme responses. It is possible that only a long-term response will drive differentiation far enough so that the complete cross-regulation of Thl and Th2 can occur, and severely biased Thl and Th2 responses can be obtained. In addition to the parasite responses described below, adjuvants such as Freund's complete adjuvant (FCA) and *Brucella abortus* (BA) tend to result in antibody responses biased toward IgG_{2a}, and sometimes DTH, while IgE is not induced. In contrast, alum/*Bordetella pertussis* (ABP) immunizations tend to produce high IgE levels. Interactions between adjuvants are complex, depending on the timing of administration of the two agents (77), which could be explained by the opposing effects of cytokine and antibody regulatory effects (Section VI).

The infestation of rodents with the intestinal helminth *Nippostrongylus brasiliensis* (Nb) provokes the three responses that are characteristic of the mammalian response to many metazoan parasites: eosinophilia, mast cell hyperplasia in mucosal tissues, and substantially increased IgE levels (7,23,82,83). All three responses are T cell dependent and are largely absent in nude or T-depleted mice (81,88,115,118,166). This pattern of responses suggests a strong preferential Th2 response with minimum involvement of Thl cytokines, especially IFN-γ. The findings that the IgE response to Nb can be completely inhibited by anti-IL-4 (49) and that blood and tissue eosinophilia can be totally inhibited by anti-IL-5 (37a) provide strong support for this interpretation. Each cytokine *in vivo* mediates the specific functions suggested by its *in vitro* activities, and anti-IL-4 causes no inhibition of eosinophilia, nor does anti-IL-5 inhibit IgE production. Thus, these two responses appear to be coordinately regulated by the same Th subset but by two different subset-specific cytokines. Further support for the role of Thl and Th2 cytokine patterns in this response comes from studies in which the ability of T cells to synthesize IL-4 and IL-5 was greatly enhanced in spleen and mesenteric lymph nodes from Nb-infested mice, while IL-2 and IFN-γ syntheses were suppressed (N. E. Street and T. R. Mosmann, unpublished).

The infection of mice with the intracellular protozoan parasite *L. major* provides the clearest example of the different consequences of a Thl or a Th2 response to a pathogen. Infection of a susceptible

mouse strain such as BALB/c with *L. major* induces a strong antibody response with little or no DTH to *L. major* antigens (68). This response exerts little effective control of parasite growth and the infection soon spreads beyond the initial lesion with fatal consequences. Several types of evidence support the interpretation that Th2 cells are preferentially activated in BALB/c mice. Lymphoid organs from infected mice have significant levels of IL-4 mRNA but no detectable IFN-γ mRNA, and these mice have marked elevations in serum IgE, which can be abrogated by treatment of the mice with anti-IL-4 antibodies (61). In addition, enhanced susceptibility can be transferred to these mice by an *L. major*-specific Th clone (174). The response of resistant strains, such as C57BL/6 and C3H, presents a sharp contrast. This response is characterized by strong DTH, little or no antibody production and strong IFN-γ production (68,168,169). These mice develop a localized lesion which heals rapidly and they are immune to reinfection. Such mice have low serum IgE levels (61) and substantial amounts of IFN-γ mRNA but undetectable IL-4 mRNA in their spleens and lymph nodes (61,168), and their T cells generate large amounts of IFN-γ when challenged with antigen *in vitro* (169). IFN-γ appears to be the most significant component of this response and has been shown to activate macrophage killing of *Leishmania in vitro* (129,133). Transfer of *L. major*-specific Th1 clones into infected BALB/c mice confers complete resistance to the infection (174).

These two very different responses closely resemble two forms of leishmaniasis in man. Visceral leishmaniasis (kala-azar) is characterized by rapidly disseminating and ultimately fatal disease, high antibody levels, little or no DTH, and defective IFN-γ and IL-2 production in response to antigen *in vitro* (25,167). In contrast, patients with cutaneous leishmaniasis or even normal individuals in endemic areas have strong DTH but minimal antibody to *Leishmania* antigens, and produce significant IL-2 and IFN-γ levels *in vitro* (25,167). Thus, the type of Th response which predominates in response to *Leishmania* infection can have profound consequences for the outcome of the disease.

IX. Human Helper T Cell Subsets

In spite of the clear dichotomy of mouse Th1 and Th2 clones, and the accumulating evidence for their involvement in normal immune responses, human T cells do not appear to segregate into two clear subsets. Many human T cell clones secrete both Th1 and Th2 cytokines (110,139), although Th1 and Th2 clones can be found (110,201). Recent evidence (described above) that precursor stages exist for mouse T cells, secreting both Th1 and Th2 cytokines, suggests that the two species may

be more similar than was first thought, if it is suggested that mouse clones *in vitro* tend to differentiate into either Th1 or Th2 clones, whereas human T cell clones tend to be more stable as the earlier, mixed cytokine secretion phenotype. Another reason for proposing that the two species are quite similar is that the cytokine functions are very similar in mouse and human immune systems. Further evidence for Th1-like and Th2-like functions in humans is obtained from immune responses to parasites, which often exhibit properties similar to those seen in the mouse responses in which Th1 and Th2 cells have been implicated, and from general features of the immune system, such as the tendency for DTH and IgE responses to be mutually exclusive. In the future, improvements in subsetting techniques for mouse and human Th cells, and the ability to assess cytokine production by single cells, may help to resolve the apparent species differences.

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ADVANCES IN IMMUNOLOGY, VOL. 46

The Leukocyte Integrins

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I. Introduction

Cellular adhesion and recognition mechanisms are among the most basic requirements for the evolution of multicellular organisms. During the development of an embryo, cellular adhesion proteins can impart position-specific information which guides cell migration, localization, and the transfer of information between cells. As cells are triggered to differentiate to form tissues or organs, adhesion proteins help to maintain the organization and integrity of the body. The immune system is composed of a network of cells in which cellular recognition mechanisms have been highly specialized. The function of the immune system is to distinguish self from nonself and to eliminate the latter. Two major protein families, the integrin family and the immunoglobulin superfamily, have evolved to guide cell–extracellular matrix and cell–cell interactions for both developmental processes and immune function. The immunoglobulin superfamily, which includes the polymorphic antigen-specific receptors of lymphocytes, has recently been reviewed (Williams and Barclay, 1988). This review will focus on the molecular biology of the leukocyte integrins, LFA-1, Mac-1, and p150,95, and on their role in mediating inflammation.

Three recent developments have underscored the importance of the leukocyte integrins as adhesion receptors of the immune system: (1) The recognition that the leukocyte integrins are evolutionarily related to other integrins, such as the fibronectin receptor and platelet glycoprotein IIb/IIIa, which guide cell localization during embryogenesis and wound healing; the leukocyte integrins provide a similar mechanism in the immune system for guiding leukocyte localization during inflammation. (2) Identification of intercellular adhesion molecule-1 (ICAM-1), a ligand for LFA-1, which is induced during inflammation and may regulate

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leukocyte migration and localization; this receptor–ligand pair demonstrates the first known interaction between a member of the integrin family (LFA-1) and a member of the immunoglobulin superfamily (ICAM-1). (3) Discovery and characterization of immunodeficiency patients who are genetically deficient in their expression of the leukocyte integrins; leukocytes from these patients fail to mobilize during inflammation, and as a consequence these patients suffer from recurrent life-threatening bacterial and fungal infections.

A. Nomenclature

LFA-1 is an acronym for lymphocyte function-associated antigen-1. Mac-1 is an abbreviation for macrophage antigen-1 and is also called Mo-1, OKM-1, and complement receptor type-3 (CR3). In some reports p150,95 is called complement receptor type-4 (CR4) and Leu M5. The Third International Workshop on Human Leukocyte Differentiation Antigens (McMichael, 1987) has designated the α subunits of LFA-1, Mac-1, and p150,95 to be CD11a, b, and c, respectively, and the common β subunit to be CD18.

The LFA-1, Mac-1, and p150,95 family has been called the LFA-1 family, leukocyte adhesion proteins, LeuCAM, and the leukocyte integrins. The homology of this family to other integrin receptors makes the latter name preferable.

B. Initial Characterization of LFA-1, Mac-1, and p150,95

Mac-1 was first defined by monoclonal antibodies (MAbs) as a marker for myeloid cells (Springer *et al.*, 1979). In contrast, LFA-1 was identified independent of Mac-1 by screening MAbs for the ability to inhibit cytotoxic T lymphocyte (CTL)-mediated killing of tumor cell targets (Davignon *et al.*, 1981a). Further analysis showed that the LFA-1 MAbs prevent the Mg^{2+}-dependent conjugate formation step, rather than the actual killing event (Davignon *et al.*, 1981b). Mac-1 and LFA-1 antigens are both high-molecular-weight $\alpha\beta$ heterodimers (Kürzinger *et al.*, 1981). Detailed immunochemical and physiochemical comparison of the two antigens showed that the α subunits of LFA-1 and Mac-1 are unique, but that the β subunit is identical in both proteins (Trowbridge and Omary, 1981; Kürzinger and Springer, 1982; Sanchez-Madrid *et al.*, 1983b). The remarkable structural similarity of LFA-1 and Mac-1 led to the hypothesis that Mac-1 would also function in adhesion. A function for Mac-1 was discovered when Mac-1 MAbs were found to inhibit Mg^{2+}-dependent binding of the C3bi fragment of complement by mouse and human myeloid cells, thus defining Mac-1 as the complement receptor type-3 (CR3) (Beller *et al.*, 1982; Wright *et al.*, 1983).

Analysis with a β-subunit-specific MAb led to the identification of a third heterodimeric protein, p150,95, which shares the common β subunit (Sanchez-Madrid *et al.*, 1983b).

Expression of all three leukocyte integrins is restricted to immune cells. LFA-1 is expressed by virtually all immune cells (Kürzinger and Springer, 1982; Krensky *et al.*, 1983), with the exception of some tissue macrophages (Kürzinger *et al.*, 1981; Strassmann *et al.*, 1985). Mac-1 has a more limited distribution (Springer and Unkeless, 1984); it is found on monocytes, macrophages, granulocytes, large granular lymphocytes, and immature and $CD5^+$ B cells (de la Hera *et al.*, 1988). The p150,95 protein has a similar distribution to Mac-1, although it is also expressed on some activated lymphocytes and is a marker for hairy-cell leukemia (Schwarting *et al.*, 1985; Miller *et al.*, 1986).

C. Leukocyte Adhesion Deficiency (LAD) Disease

Since 1974, a number of investigators have identified a class of immunodeficient patients who suffer from recurrent, life-threatening bacterial and fungal infections and who have neutrophils deficient in chemotaxis and phagocytosis (Anderson and Springer, 1987; Todd and Freyer, 1988; Fischer *et al.*, 1988; Anderson *et al.*, 1988; Kishimoto and Springer, 1988). Infected, necrotic lesions in these patients contain few leukocytes, despite the observation that these patients have chronic leukocytosis. One of the earliest reports described a possible actin dysfunction (Boxer *et al.*, 1974). However, Crowley *et al.* (1980) showed that neutrophils from these patients were deficient in a high-molecular-weight surface protein. They further proposed that defects in chemotaxis and phagocytosis were secondary to a defect in adhesion. In 1984, several groups used MAbs to demonstrate that the missing glycoprotein was actually the LFA-1, Mac-1, and p150,95 complex (Anderson *et al.*, 1984; Springer *et al.*, 1984; Beatty *et al.*, 1984; Dana *et al.*, 1984). In every case studied, expression of all three leukocyte integrins was found to be deficient. More recently, we have shown that LAD is due to heterogenous defects in the common β subunit (Kishimoto *et al.*, 1987b). Although LAD is a rare disease, the analysis of this disease has greatly increased our understanding of the biology of the leukocyte integrins and their role in inflammatory responses.

D. LFA-1, Mac-1, and p150,95 Are Members of the Integrin Family

LFA-1, Mac-1, and p150,95 are evolutionarily related to the integrin receptors that mediate cell adhesion to the extracellular matrix during development and wound healing (Fig. 1). There are three subfamilies

	LFA-1	Mac-1	p150,95	VLA-1	VLA-2	VLA-3	VLA-4	FNR(VLA-5)	VLA-6	VLA-7	IIb/IIIa	VNR
Primary Function	Immune cell adherence			Guiding morphogenesis and wound healing							Guiding morphogenesis and wound healing	
General Distribution	Leukocytes			Broad							Platelets	Broad
Structure	$\alpha_1\beta_1$			$\alpha_1\beta_1$							$\alpha_1\beta_1$	
Common β subunit	Yes			Yes							Yes	
Cleavage of α subunit upon reduction	No	No	No	No	No	Yes	No	Yes	Yes	?	Yes	Yes
Ligands	ICAM-1	iC3b Fibrinogen Factor X	(iC3b)	?	Collagen	Laminin Fibronectin Collagen	?	Fibronectin	Laminin	?	Fibronectin Fibrinogen Vitronectin von Willebrand factor	Vitronectin
Recognition Sequence								Arg-Gly-Asp			Arg-Gly-Asp	Arg-Gly-Asp
Interaction with cytoskeleton								Yes				

FIG. 1. A supergene family of adhesion proteins.

of integrins, each defined by a common β subunit which shares multiple distinct α subunits (Hynes, 1987; Kishimoto *et al.*, 1987a; Ruoslahti and Pierschbacher, 1987). The three β subunits are designated $\beta 1$, $\beta 2$, and $\beta 3$. The $\beta 1$ and $\beta 3$ subfamilies include receptors for extracellular matrix (ECM) components. The $\beta 1$ subunit is shared by at least six VLA antigens (VLA-1–VLA-6) (Hemler, 1988), which include the fibronectin receptor (VLA-5). VLA-3 has fibronectin and laminin-binding activity, while VLA-2 has recently been shown to be identical to platelet glycoprotein IaIIa, a collagen-binding receptor. The $\beta 2$ subunit is shared by the leukocyte integrins, LFA-1, Mac-1, and p150,95. The $\beta 3$ subunit is shared by the vitronectin receptor and platelet glycoprotein IIbIIIa.

The integrin family is ancient in origin. Homologous structures, termed position-specific (PS) antigens, have been implicated in guiding *Drosophila* development (Bogaert *et al.*, 1987; Wilcox and Leptin, 1985). The structure, function, and primary sequence of the integrins have been highly conserved in evolution. The integrins involved in cell matrix interactions recognize a sequence, Arg-Gly-Asp (RGD), which is embedded in numerous, unrelated matrix components (Ruoslahti and Pierschbacher, 1987). The ECM receptor integrins can show exquisite specificity for one matrix component or broad reactivity to multiple ligands. The leukocyte integrins are the first integrins known to mediate cell–cell interactions. The name integrin denotes that these are membrane receptors that integrate the extracellular environment (extracellular matrix or other cells) with the intracellular cytoskeletal network. The evolutionary and functional significance of LFA-1, Mac-1, and p150,95 as integrin proteins will be discussed.

II. Structural Features of the Leukocyte Integrins

A. Basic Structure

The leukocyte integrins are $\alpha_1\beta_1$ heterodimers (Kürzinger and Springer, 1982), in which the α subunit is noncovalently associated with the β subunit. The α subunits of LFA-1, Mac-1, and p150,95 are 180,000, 170,000, and 150,000 Da, respectively. The α subunits have been shown to be distinct by MAb reactivity, antigen-preclearing studies, and tryptic peptide mapping. In contrast, the β subunit, M_r = 95,000, has been shown to be identical in all three proteins by the same criteria (Trowbridge and Omary, 1981; Kürzinger and Springer, 1982; Sanchez-Madrid *et al.*, 1983b). Deglycosylation of the LFA-1, Mac-1, and p150,95 α subunits and the common β subunit reveal polypeptide backbones of 149,000, 137,000, 132,000, and 78,000 Da, respectively (Miller and Springer, 1987;

Kishimoto *et al.*, 1987b; Sastre *et al.*, 1986). Heterogeneity in the glycosylation of LFA-1 has been reported. N-Linked oligosaccharides of LFA-1 on T cells but not B cells or macrophages are sulfated (Dahms and Hart, 1985). Moreover, sialyation patterns of LFA-1 on B and T cells differ, with LFA-1 on B cells being more acidic (Takeda, 1987). Finally, only a subset of leukocyte integrins on neutrophils contain a lacto-*N*-fucopentaose II oligosaccharide moiety (Skubitz and Snook, 1987). The functional significance of this heterogeneity is unknown.

B. Biosynthesis

The α subunits of LFA-1, Mac-1, and p150,95 and the common β subunit are synthesized as distinct precursors of 165,000, 160,000, 146,000, and 89,000 Da, respectively (Sanchez-Madrid *et al.*, 1983b; Sastre *et al.*, 1986; Miller and Springer, 1987). The newly synthesized precursors contain high-mannose N-linked oligosaccharides (Sastre *et al.*, 1986). Association of the α subunit precursor and the β subunit precursor is required for further processing to complex-type N-linked oligosaccharides (Ho and Springer, 1983; Springer *et al.*, 1984). This oligosaccharide modification occurs in the Golgi apparatus (Kornfeld and Kornfeld, 1985) and is evident by a decrease in the electrophoretic mobility of the mature polypeptide and resistance to endoglycosidase H digestion. The mature proteins are then transported to the cell surface or, in some cases, to intracellular granules (Todd *et al.*, 1984; Miller *et al.*, 1987; Bainton *et al.*, 1987).

C. Primary Structure

1. β *Subunit Structure*

a. β Subunit cDNA. The cDNA encoding the common β subunit was isolated and characterized by us (Kishimoto *et al.*, 1987a) and independently by Law *et al.* (1987). The deduced 769-amino acid sequence (Fig. 2) has the characteristic features of an integral membrane protein, with a 677-amino acid extracellular domain containing six potential N-glycosylation sites, a 23-amino acid transmembrane domain, and a 46-amino acid cytoplasmic domain. A striking feature of the β subunit is the high cysteine content (7.4% overall). A cysteine-rich (20%) region of 186-amino acid contains a fourfold repeat of an unusual cysteine motif. The high cysteine content is predicted to give the β subunit a very rigid tertiary structure.

Northern blot analysis, Southern blot analysis, and peptide sequence data from the β subunit isolated independently from purified LFA-1, Mac-1, and p150,95 confirm immunochemical evidence that a single

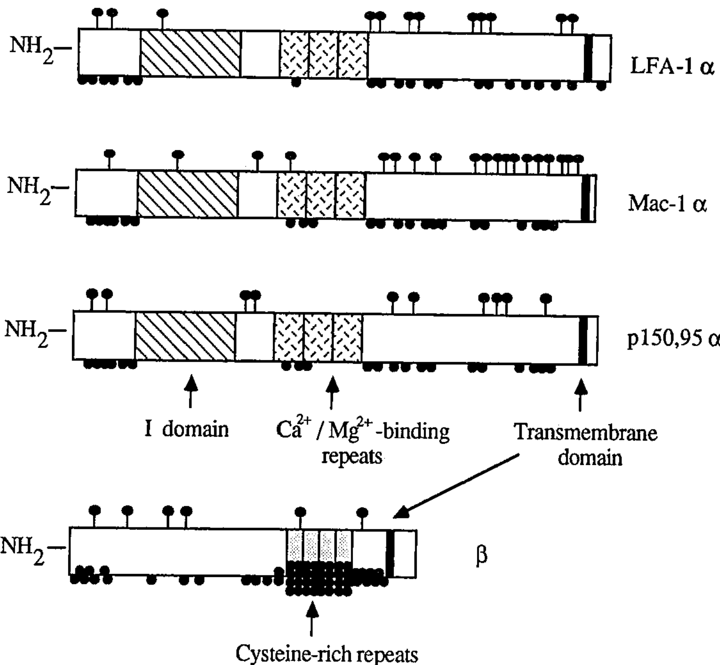

FIG. 2. Schematic representation of primary structure of the LFA-1, Mac-1, and p150,95 α subunits and common β subunit. Black lollipops and circles represent N-linked glycosylation and cysteines, respectively.

gene encodes the β subunit of all three leukocyte integrins (Kishimoto *et al.*, 1987a).

b. Homology to Other Integrin β Subunits. The three β subunits, which define the three integrin subfamilies, share 37–45% amino acid identity, with particularly high conservation of the cytoplasmic domain, the transmembrane domain, and a stretch of 241 amino acids in the extracellular domain (70, 47, and 64%, respectively) (Tamkun *et al.*, 1986; Kishimoto *et al.*, 1987a; Law *et al.*, 1987; Fitzgerald *et al.*, 1987). All 56 cysteine residues are conserved in each of the three β subunits, including the fourfold repeat of the unusual cysteine motif, first described for the chick $\beta 1$ subunit (Tamkun *et al.*, 1986). This high cysteine content probably gives the β subunits similar tertiary structures and may account for the observed differences in mobility in reducing versus nonreducing gels (Leptin, 1986; Ruoslahti and Pierschbacher, 1987). Interestingly, $\beta 1$ (Tamkun *et al.*, 1986) and $\beta 3$ (Fitzgerald *et al.*, 1987) have a consensus tyrosine phosphorylation sequence in the cytoplasmic domain,

which is not found in the leukocyte $\beta 2$ subunit (Kishimoto *et al.*, 1987a; Law *et al.*, 1987).

2. α Subunit Structures

a. α Subunit cDNAs. cDNAs encoding the α subunits of p150,95 (Corbi *et al.*, 1987), Mac-1 (Corbi *et al.*, 1988b; Pytela, 1988; Arnaout *et al.*, 1988), and more recently LFA-1 (Larson *et al.*, 1989) have been cloned and characterized. The deduced amino acid sequences (Fig. 2) define homologous integral membrane proteins with a long extracellular domain (αX, 1081 amino acids; αM, 1092 amino acids; αL, 1063 amino acids), a hydrophobic transmembrane domain (αX, 26 amino acids; αM, 26 amino acids; αL, 29 amino acids), and a short cytoplasmic domain (αX, 29 amino acids; αM, 19 amino acids; αL, 53 amino acids). Although the polypeptide backbones are of similar size, the differences in apparent molecular weight of the mature polypeptides may be due, in part, to differences in the number of potential N-glycosylation sites (αX, 10 potential sites; αM, 19 potential sites; αL, 12 potential sites).

A striking feature of the α subunits is that each contains three homologous repeats that have putative divalent cation-binding sites which are similar to the Ca^{2+}-binding "EF-hand loop" sequences of calmodulin, troponin C, and parvalbumin. These putative metal-binding sites may account for the Mg^{2+} dependency of leukocyte integrin-mediated adhesion. The concept that exogenous divalent cations stabilize the interaction of integrin α and β chains (Jennings and Phillips, 1982) has been used in the immunopurification of leukocyte integrins in functional form (Dustin and Springer, 1989; S. A. Stacker, M. S. Diamond, and T. A. Springer, unpublished). The stabilizing effect of Mg^{2+} and Ca^{2+} on leukocyte integrins is the most direct evidence for binding of divalent cations by these heterodimers.

The Mac-1 and p150,95 α subunits share 63% amino acid identity with each other but only 35% identity with the LFA-1 α subunit. The transmembrane domain and the three homologous repeats containing putative divalent cation-binding sites are the most highly conserved regions (88 and 87% amino acid identity, respectively, between Mac-1 and p150,95).

b. Homology to the Extracellular Matrix Receptor Integrins. The α subunits of the three subfamilies of integrins share 25–63% amino acid identity (Corbi *et al.*, 1987, 1988b; Pytela, 1988; Arnaout *et al.*, 1988; Larson *et al.*, 1989; Argraves *et al.*, 1987b; Poncz *et al.*, 1987; Suzuki *et al.*, 1987). The evolutionary relationships among integrin α subunits can be assessed by percent amino acid sequence identity (Fig. 3). The integrins may be divided into two functional groups, those which bind extracellular matrix ligands (ECM receptors) and those involved in cell–cell

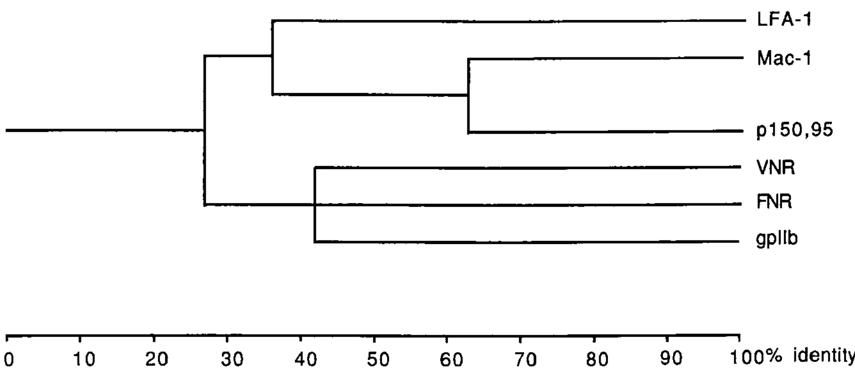

FIG. 3. Evolutionary relationship of integrin α subunits. A pathway of evolution is suggested by the percentage identity among α subunits, indicated by the scale above.

interactions and expressed on leukocytes (the leukocyte integrins). The ECM receptor integrins utilize both $\beta 1$ and $\beta 3$ subunits whereas the leukocyte integrins use the $\beta 2$ subunit. The α subunits of the leukocyte integrins are more similar to each other ($\bar{x} = 47\%$ identity) than to those of the ECM receptor integrins ($\bar{x} = 27\%$ identity). Moreover, the ECM receptor integrin α subunits are more related to one another ($\bar{x} = 42\%$ identity) than to the leukocyte integrins. The α subunits of the ECM integrins, VLA-3, VLA-5 (fibronectin receptor), vitronectin receptor, and glycoprotein IIb/IIIa (gpIIb/IIIa) share a sequence in the extracellular domain which is posttranslationally cleaved, with resulting fragments bridged by a disulfide bond (Ruoslahti and Pierschbacher, 1987). This sequence is not found in the α subunits of leukocyte integrins (Corbi *et al.*, 1987, 1988b; Pytela, 1988; Arnaout *et al.*, 1988; Larson *et al.*, 1989). This explains the increased electrophoretic mobility in reducing gels observed for the α subunits of the ECM receptor integrins but not the leukocyte integrins (Ruoslahti and Pierschbacher, 1987). The regions of highest conservation are the transmembrane domain and the putative metal-binding domains. Like leukocyte integrins, the ECM receptor integrins are dependent upon divalent cations for activity; however, the metal specificity is for Ca^{2+}, Mg^{2+}, or Mn^{2+}. Radioactive calcium has been shown to bind directly to the α subunit of gpIIb/IIIa (Fujimura and Phillips, 1983) and the fibronectin receptor (Gailit and Ruoslahti, 1988).

c. I Domain. All three leukocyte integrin α subunits have a 200-amino acid segment in the extracellular domain which has a counterpart in only one of five known ECM receptor integrin α subunit sequences (Takada and Hemler, 1989). This domain, termed the I domain for

inserted/interactive domain, is homologous to the three A domains of von Willebrand factor (vWF), to a domain in the complement cascade proteins C2 and factor B, and to two domains in the cartilage matrix protein (CMP) (Corbi *et al.*, 1988b; Arnaout *et al.*, 1988; Larson *et al.*, 1989; Pytela, 1988).

The relationships among the α subunits suggest the evolutionary scheme depicted in Fig. 3. A primordial α integrin gene duplicated and gave rise to at least two branches of integrin α subunits, the leukocyte integrins and the ECM receptor integrins. The primordial leukocyte integrin α subunit gene, containing an I domain, then duplicated and gave rise to LFA-1 and Mac-1/p150,95 primordial α subunit genes. Further duplication of the Mac-1/p150,95 primordial gene gave rise to the Mac-1 and p150,95 α subunits.

d. Expression. As a first step to uncover the structural basis for the adhesive activities displayed by LFA-1, Mac-1, and p150,95, their corresponding cDNAs (α and β subunits) have been inserted in the vector CDM8 (Seed, 1987) to obtain transient expression of the three heterodimers. After DEAE-dextran mediated cotransfection of α and β cDNAs, LFA-1, Mac-1, and p150,95 α/β complexes have been detected on the surface of COS cells by immunofluorescence (Larson *et al.*, 1990). Immunoprecipitation studies have demonstrated that the α and β subunits of LFA-1, Mac-1, and p150,95 are noncovalently associated. Moreover, the LFA-1 and Mac-1 heterodimers expressed by the transfected cells were functional. COS cells expressing LFA-1 bound to purified ICAM-1 absorbed to plastic, and this binding was inhibited by anti-LFA-1, anti-ICAM-1, and EDTA (R. S. Larson and T. A. Springer, unpublished). COS cells expressing Mac-1 specifically bound to erythrocytes sensitized with human or mouse C3bi, and this was inhibited with anti-Mac-1α OKM10 MAb and anti-β 60.3 MAb (A. L. Corbi and T. A. Springer, unpublished).

D. Relation of Structure to Ligand Binding

The three-dimensional structure of the leukocyte integrins and the individual contributions of the α and β subunits to ligand binding remain to be determined. The ECM receptor integrins recognize the RGD sequence and related sequences such as KQAGD within their ligands (Ruoslahti and Pierschbacher, 1986; Ruggeri *et al.*, 1986). Because the integrins within a given subfamily share the same β subunit but have distinct ligand-binding specificity, it seems reasonable to propose that the α subunits impart this specificity. The α subunits may influence recognition by changing the conformation of the β subunit to recognize different conformations of the RGD sequence present in different matrix proteins, or by binding to further sites on the matrix protein. Complementation studies with fragments of fibronectin suggest that a second site distinct

from RGD is required for binding to the fibronectin receptor (Obara *et al.*, 1988).

Chemical cross-linking studies show that although radiolabeled RGDS tetrapeptide binds predominately to a highly conserved region of the β subunit of gpIIb/IIIa, there is significant labeling of the α subunit as well (D'Souza *et al.*, 1988). The RGD binding site of the β subunit maps to the N-terminal portion of a highly conserved 241-amino acid segment of the extracellular domain. The RGD binding site of the α subunit has not been determined. One model is that divalent cations held in the metal-binding domains of the α subunits may help to stabilize interaction with the RGD sequence (Corbi *et al.*, 1987). X-Ray crystallography of the Ca^{2+}-binding EF-hand loops shows that amino acids with oxygen-containing side groups form the coordination axes for ligating the metal (Szebenyi *et al.*, 1981). The putative metal-binding domains of all integrins differ from these classical EF-hand loops in that a glutamic acid in the $-Z$ position of the coordination axes is missing from the former. This may leave the metal free to coordinate in the $-Z$ position with a residue on the ligand. It is tempting to speculate that the metal bound to the receptor may coordinate with the aspartic acid (D) of the RGD recognition sequence. This sequence appears suited for metal binding because GD and DG sequences appear frequently both in the EF-hand loop and in the metal-binding domains of the integrins.

The I domain appears as a functional unit in diverse proteins. The A1 domain of vWF binds glycoprotein Ib and heparin, while both A1 and A3 domains are involved in binding collagen (Girma *et al.*, 1987). Partial sequence data from the cartilage matrix protein reveals two I domainlike repeats separated by an epidermal growth factorlike sequence (Argraves *et al.*, 1987a). The I domainlike region in factor B and C2 is clearly demarcated on the N-terminal side by the cleavage site that gives the active Bb factor, and on the C-terminal side by the serine protease domain (Bently, 1986). Interestingly, both factor B and the homologous protein C2 bind C3b, whereas Mac-1 and p150,95 are receptors for the C3bi fragment of C3. It is tempting to speculate that the I domains of Mac-1 and p150,95 contribute to this specificity. The ECM integrins which recognize RGD do not contain I domains. The I domain may impart distinctive and additional recognition specificities to leukocyte integrins.

III. Chromosomal Localization

A. β Subunit Gene on Chromosome 21

The gene encoding the common β subunit was first mapped to chromosome 21 by gene complementation in somatic cell hybrids (Marlin *et al.*, 1986; Suomalainen *et al.*, 1983; Akao *et al.*, 1987). With the

availability of the β subunit cDNA, the gene has been further localized to band 21q22 by *in situ* hybridization to metaphase chromosomes (Corbi *et al.*, 1988a). More recently, the β subunit has been shown to be the most distal marker known on the long arm of chromosome 21 (21q22.3) by hybridization to a panel of chromosome 21 deletion mutants and linkage of restriction fragment length polymorphisms (Gardner *et al.*, 1988). The β subunit gene may be a very useful marker for analysis of trisomy 21 in Down's syndrome. In addition, band 21q22 has been identified as a breakpoint in chromosomal translocations [t(3;21)(q25;q22)] associated with the blast phase of chronic myelogenous leukemia (CML) (Rubin *et al.*, 1987). Hematopoietic progenitor cells in CML show abnormal adhesive interactions with bone marrow stroma (Gordan *et al.*, 1987). Further studies are required to determine if the β subunit gene is involved in this translocation and contributes to the progression of CML.

B. α Subunit Gene Cluster on Chromosome 16

The LFA-1 α subunit gene has been mapped to chromosome 16 by gene complementation in somatic cell hybrids (Marlin *et al.*, 1986). Subsequent Southern blot analysis of DNA from LFA-1 α hybrid cells containing human chromosome 16 has shown that the genes encoding Mac-1 and p150,95 are on the same chromosome (Corbi *et al.*, 1988a). Furthermore, *in situ* hybridization data show that all three α subunit genes are clustered between bands p11-p13.1 on chromosome 16, defining a gene cluster involved in cell adhesion (Corbi *et al.*, 1988a). The close proximity of the α subunit genes provides further support for their evolution by gene duplication.

Inversions [inv(16)(p13q22)] and translocations [t(16;16)(p13;q22)] involving this region of chromosome 16 are frequently observed in patients with acute myelomonocytic leukemia (Le Beau *et al.*, 1985). Further investigation is required to determine if the genes encoding the leukocyte integrin α subunits are involved in these rearrangements.

IV. The Leukocyte Integrins in Inflammation

A. The Role of Leukocytes in the Inflammatory Process

The acute inflammatory reaction provides a rapid host defense response to contain and eliminate infectious agents in extravascular tissues. The peripheral blood leukocytes constitute the recruitable force which infiltrates the infected tissues. Neutrophils and monocytes migrate in response to chemotactic factors released at the infection site. Chemotactic stimulation causes leukocyte cell polarization, granule release, and increased adhesiveness. Diapedesis requires the ability of leukocytes to

bind vascular endothelial cells, cross the basement membrane, and enter the infected tissues. Regulation of the inflammatory response must be exquisite, so that leukocytes only enter the infected area and do not damage healthy tissues. Until recently, the molecular mechanisms which regulate and mediate leukocyte extravasation have been largely unknown. The role of the leukocyte integrins in this process has been elucidated by the characterization of human leukocyte adhesion deficiency disease.

B. Insights from the Study of Leukocyte Adhesion Deficiency

The clinical hallmarks of LAD are recurrent necrotic and indolent infections of soft tissues, such as the skin, mucous membranes, and intestinal tract (Anderson and Springer, 1987). The infectious microbes include a wide spectrum of fungi and bacteria, but most commonly staphylococcal or gram-negative enteric bacteria. A key feature of LAD is that infected skin lesions are largely devoid of granulocytes, despite chronic peripheral blood leukocytosis (5–20 times normal levels). This failure of leukocytes to mobilize is also observed in Rebuck skin window assays. Transfused normal leukocytes are capable of mobilization to the infected tissues (Bowen *et al.*, 1982). Adhesion-independent responses, such as cell polarization in response to chemotactic factors, fMLP-binding, and respiratory burst from soluble stimuli, are normal. These observations demonstrate a direct involvement of the leukocyte integrins in the extravasation of leukocytes during an inflammatory response.

In vitro, neutrophils and monocytes from LAD patients show profound defects in adhesion-related functions (Anderson and Springer, 1987). Mac-1 and p150,95 are deficient both on the cell surface and in intracellular granules. Chemoattractants induce granule mobilization, but not up-regulation of leukocyte integrin expression or homotypic cell aggregation. Neutrophils and monocytes fail to adhere and spread on artificial substrates, such as glass and plastic, and on endothelial cell monolayers. As a consequence, leukocytes have impaired directed motility in response to chemoattractants. C3bi-coated particles fail to induce phagocytosis or subsequent respiratory burst. Antibody-dependent cellular cytotoxicity mediated by granulocytes and monocytes is also abnormal (Kohl *et al.*, 1984, 1986).

C. Functional Studies

1. *LFA-1 Function*

LFA-1 serves to mediate cellular adhesion events in a wide spectrum of both antigen-dependent and antigen-independent interactions of immune cells (Springer *et al.*, 1987; Martz, 1986). The role of LFA-1 in conjugate formation during CTL- and NK-mediated cytolysis has been

extensively reviewed (Springer *et al.*, 1987; Martz, 1986) and will not be discussed here. Lymphocyte localization to lymphoid organs, sites of inflammation, and grafts is dependent upon specific interaction with the vascular endothelium. T lymphocyte adherence to endothelial cells (Mentzer *et al.*, 1986b; Haskard *et al.*, 1986; Dustin and Springer, 1988), fibroblasts (Dustin *et al.*, 1986), epidermal keratinocytes (Dustin *et al.*, 1988), synovial cells (Mentzer *et al.*, 1988), and hepatocytes (Roos and Roossien, 1987) is inhibitable partially or totally by LFA-1 MAb. Typically adherence is greatly increased by stimulation of the nonhematopoietic cells with cytokines such as TNF, LPS, IFN-γ, and IL-1 and by activation of the lymphocytes.

T lymphocyte and lymphoblast adherence to cultured endothelial cells has both LFA-1-dependent and LFA-1-independent components (Haskard *et al.*, 1986; Dustin and Springer, 1988). Both pathways are increased upon stimulation of the endothelial monolayer with cytokines. LFA-1$^-$ lymphoblasts from LAD patients exhibit the LFA-1-independent pathway, but not the LFA-1-dependent pathway (Dustin and Springer, 1988). The impaired primary responses of LAD lymphocytes to specific antigen, but near-normal secondary responses (Krensky *et al.*, 1985; Mentzer *et al.*, 1986a), may reflect selective involvement of LFA-1-independent and LFA-1-dependent pathways. LAD patients display essentially normal delayed-type hypersensitivity responses. Inflammatory tissues, which are devoid of granulocytes, do contain lymphocytes. The nature of the LFA-1-independent pathway of adhesion to endothelium is unknown, but does not appear to be the CD2/LFA-3 pathway. In the case of lymphocyte binding to high endothelial venules of lymphoid organs, adherence is partially inhibitable by LFA-1 MAb (Hamann *et al.*, 1988); however, organ specificity is mediated by lymphocyte homing receptors (Butcher, 1986).

An *in vitro* model of LFA-1-dependent adhesion is the homotypic aggregation of lymphocytes and lymphoid cell lines in response to phorbol ester stimulation (Patarroyo *et al.*, 1985; Mentzer *et al.*, 1985; Rothlein and Springer, 1986). Time-lapse videomicroscopy (Rothlein and Springer, 1986) shows that phorbol ester-activated peripheral blood lymphocytes are motile and show uropod formation and extensive membrane ruffling. Contact between pseudopodia of adjacent cells leads to adhesion, followed by mass aggregation. This aggregation event is both inhibitable and reversible by LFA-1 MAbs. Moreover, cells from patients who are genetically deficient in LFA-1 expression do not aggregate (Rothlein and Springer, 1986). Although phorbol ester-induced aggregation is an *in vitro* phenomenon, it has been a useful model to study the cell biology of LFA-1 function and probably correlates with adhesion events *in vivo*.

The requirements for aggregation are remarkably similar to the adhesion phase of CTL conjugate formation. Both events are Mg^{2+}, energy, and temperature dependent and require an intact cytoskeleton (Martz, 1977; Patarroyo *et al.*, 1983; Rothlein and Springer, 1986). Aggregation-like cluster formation is also observed shortly after T helper cell activation by antigen presentation (Werdelin, 1980). Homotypic aggregation of activated lymphocytes may be an important step in extravasation of lymphocytes.

Improved isolation procedures have allowed functional studies on purified LFA-1 (Dustin and Springer, 1989). LFA-1 purified in the presence of Mg^{2+} remains associated in an $\alpha\beta$ complex and is functional because it mediates adhesion of several human cell lines when reconstituted into planar membranes or absorbed directly to plastic. This binding is blocked by pretreatment of cells with ICAM-1 MAbs, pretreatment of the monolayer with LFA-1 MAbs, and removal of divalent cations. The major difference between LFA-1-ICAM-1 mediated cell–cell adhesion and adhesion of cells to purified LFA-1 is that the former requires high temperature and metabolic activity, while the latter does not. In contrast, binding of cells to ICAM-1 monolayers is temperature and energy dependent (Marlin and Springer, 1987). This suggests that the energy and temperature requirements are met on the LFA-1-bearing cell side of the interaction, consistent with cellular regulation of LFA-1 avidity for its ligand.

2. *Mac-1 Function*

A functional role for Mac-1 was first demonstrated by the ability of Mac-1 MAbs to inhibit monocyte and granulocyte binding of C3bi-coated erythrocytes (Beller *et al.*, 1982). Thus Mac-1 is equivalent to the complement receptor type-3. Mac-1 can mediate phagocytosis and lysis of C3bi-coated erythrocytes (Rothlein and Springer, 1985) and contributes to elevated natural killer (NK) activity against C3bi-coated target cells (Ramos *et al.*, 1988). Mac-1 has also been implicated in the ability of macrophages to bind *Leishmania* promastigotes (Mosser and Edelson, 1985; Russell and Wright, 1988), *Escherichia coli* (Wright and Jong, 1986), and *Histoplasma capsulatum* (Bullock and Wright, 1987).

Recent evidence suggests that Mac-1, like LFA-1, may play a more general role in mediating adhesive interactions of myeloid cells. Activated neutrophils, like phorbol ester-stimulated lymphocytes, form homotypic aggregates *in vitro*. Neutrophil aggregation is inhibitable by Mac-1 MAbs, and not by LFA-1 MAbs (Anderson *et al.*, 1986). Neutrophil and monocyte chemotaxis (Anderson *et al.*, 1986; Dana *et al.*, 1986) and adherence to glass and plastic (Anderson *et al.*, 1986; Dana *et al.*, 1986)

and to endothelial (Wallis *et al.*, 1986) and epithelial (Simon *et al.*, 1986) monolayers also involve Mac-1. Differential MAb blocking of CR3 activity and general adhesion suggest that Mac-1 may be a multifunctional receptor (Anderson *et al.*, 1986; Dana *et al.*, 1986).

3. p150,95 Function

A role for p150,95 as a complement receptor was first discovered when both Mac-1 and p150,95 were found to elute specifically from a C3bi affinity column (Micklem and Sim, 1985; Malhotra *et al.*, 1986). The physiological relevance of this finding was tested with p150,95 on intact cells. The contribution of p150,95 to C3bi binding by neutrophils and monocytes could only be assessed after antibody blockade of CR1 and Mac-1, which are expressed in 10-fold excess of p150,95. The remaining C3bi-binding activity on these antibody-treated cells could be blocked with the p150,95 MAbs. These results suggest that p150,95, like Mac-1, may have some C3bi-binding activity, and it has thus been designated as CR4 (Myones *et al.*, 1988). However, p150,95 expressed in COS cells does not bind C3bi (J. Garcia-Aguilar, A. L. Corbi, and T. A. Springer, unpublished).

p150,95, like Mac-1, probably has a broad role as a general adhesion protein. Anderson *et al.* (1986) showed that p150,95 MAbs could partially inhibit the adhesion of neutrophils to substrates. However, Mac-1 appears to have a more important role. In contrast, Figdor and his colleagues reported that p150,95 is a major component of peripheral blood monocyte adhesion to substrates and endothelial cells, phagocytosis of latex particles, and chemotaxis (Keizer *et al.*, 1987b; te Velde *et al.*, 1987). These results were somewhat unexpected, because p150,95 is expressed only at low levels on blood monocytes but at high levels on tissue macrophages (Schwarting *et al.*, 1985).

Recently p150,95 expression has been reported on some activated lymphocytes and lymphocytic cell lines (Miller *et al.*, 1986; Keizer *et al.*, 1987a). The p150,95 MAb was found to inhibit conjugate formation by cytotoxic T lymphocytes (CTL) expressing comparable amounts of p150,95 and LFA-1 equally as well as LFA-1 MAbs (Keizer *et al.*, 1987a). The inhibitory effects of the p150,95 MAb and LFA-1 MAb are additive. One group (Lanier *et al.*, 1985) reported no effect of the p150,95 MAb on CTL activity. This discrepancy may reflect differences in p150,95 expression by CTL clones.

D. Functional Redundancy among the Leukocyte Integrins

All three leukocyte integrins appear to function as general adhesion proteins for immune cell function. MAbs directed to the α subunits have provided powerful tools to dissect the functions of the individual proteins.

While the relative importance of LFA-1, Mac-1, and p150,95 may vary with different systems, there is clearly some redundancy, particularly with neutrophil and monocyte functions. Comparative studies have shown that all three leukocyte integrins contribute to neutrophil and monocyte adhesion to endothelial cells and artificial substrates (Anderson *et al.*, 1986; te Velde *et al.*, 1987). These results are consistent with the finding that MAbs to the common β subunit are often the most potent inhibitors of adhesion-related functions. Moreover, none of the leukocyte adhesion deficiency patients examined to date have been found to have a selective deficiency in the expression of only one of the leukocyte integrins (reviewed in Anderson and Springer, 1987), suggesting that the immune system might be able to compensate for the loss of one but not all three proteins.

E. Animal Models

Whereas the study of human LAD has provided tremendous insight into the role of the leukocyte integrins in inflammation, the next stage of investigation—experimental manipulation *in vivo*—requires a suitable animal model. One possibility is a canine LAD model (Giger *et al.*, 1987), which appears completely analogous to human LAD. A serious limitation is the difficulty and expense of maintaining a stable colony.

A second approach has been to mimic the LAD state by *in vivo* administration of MAbs directed against the leukocyte integrins. An anti-β subunit MAb given intravenously to rabbits inhibits leukocyte extravasation and leukocyte-dependent plasma leakage in response to intradermal administration of chemotactic agents fMLP, leukotriene B4, and C5a (Arfors *et al.*, 1987). Intravital microscopy of the rabbit tenuissimus muscle revealed that the MAb inhibited adherence of leukocytes to the vascular endothelium but not rolling of leukocytes along the venule wall (Arfors *et al.*, 1987). In another system, mice given an intravenous injection of Mac-1 MAb fail to mobilize monocytes in response to thioglycolate-induced inflammation of the peritoneum (Rosen and Gordon, 1987). These results suggest that of the three leukocyte integrins, Mac-1 may play the major role in myeloid cell extravasation.

F. Potential Therapeutic Value of Leukocyte Integrin MAb

Inflammation is an integral part of the host defense to infection and injury of extravascular tissue. However, an inappropriate or uncontrolled inflammatory response may contribute to the pathogenesis of chronic disease states, such as arthritis, and acute ischemic shock followed by reperfusion. Leukocytes are thought to be a major culprit in promoting tissue damage following ischemia-reperfusion injury, perhaps by generating reactive oxygen metabolites, proteases, and phospholipases. Animals depleted of peripheral blood leukocytes show significantly reduced damage from myocardial ischemia and reperfusion.

Two recent studies have investigated the use of leukocyte integrin MAbs to reduce ischemia-reperfusion injury. In a dog model of myocardial infarction, arterial flow is interrupted, then reestablished. Reperfusion injury, measured as infarct size as a percentage of area at risk, was reduced twofold by *in vivo* administration of Mac-1 MAbs (Simpson *et al.*, 1988). Histological studies show that Mac-1 MAb-treated dogs had fewer neutrophils in the myocardium. A rabbit model of hemorrhagic shock and resuscitation showed similar protective effects of an anti-β subunit MAb against liver and gastrointestinal injury but not lung injury (Vedder and Harlan, 1988). All MAb-treated animals survived 5 days, compared to a 29% survival rate among control animals. These results suggest potential therapeutic value for leukocyte integrin MAbs in controlling tissue and organ injury following myocardial infarction, hemorrhagic shock, and other trauma that cause ischemia and are followed by reestablishment of normal circulatory flow. Of particular relevance is treatment of myocardial infarction with streptokinase or tissue plasminogen activator (TPA), both of which dissolve clots and allow circulation to be reestablished in affected areas of the heart. Administration of leukocyte integrin MAbs or ICAM-1 MAbs together with anticlotting agents promises to significantly reduce the amount of damaged cardiac tissue.

G. Regulation of Expression and Functional Activity

1. De novo Synthesis

Cell-surface leukocyte integrin expression is regulated both by *de novo* biosynthesis and by up-regulation of preformed material. Change in biosynthetic rate is a relatively slow and inefficient means of responding to rapid changes in the environment, such as in an infection, and is primarily associated with immune cell differentiation. LFA-1 is expressed during the differentiation of hematopoietic stem cells. In the B cell and myeloid lineages, LFA-1 expression is first detected in the cytoplasmic μ-chain-positive pre-B cells and late myeloblasts, respectively (Campana *et al.*, 1986). Mac-1 expression is associated with committed granulocyte and monocyte precursors in the bone marrow (Miller *et al.*, 1985). These observations concur with the *in vitro* differentiation of the promyeloblastic cell line HL-60. Undifferentiated HL-60 expresses only LFA-1. Differentiation to the monocytic lineage, induced with phorbol esters, or to the granulocytic lineage, induced with retinoic acid, results in expression of both Mac-1 and p150,95 (Miller *et al.*, 1986; Corbi *et al.*, 1987, 1988b). Peripheral blood monocytes express high levels of Mac-1 and low levels of p150,95. However, upon extravasation and maturation

to tissue macrophages, the pattern of expression is reversed to low levels of Mac-1 and high levels of p150,95 (Schwarting *et al.*, 1985). Work in the murine system shows that many peripheral monocytes lose expression of LFA-1 after differentiation to tissue macrophages (Kürzinger and Springer, 1982; Strassmann *et al.*, 1985). These results suggest some role for p150,95 in tissue macrophage function.

2. *Mobilization of an Intracellular Pool*

In contrast to the slow time course for up-regulation by *de novo* synthesis, Mac-1 (Todd *et al.*, 1984; Berger *et al.*, 1984; Springer *et al.*, 1984; Miller *et al.*, 1987a) and p150,95 (Lanier *et al.*, 1985; Springer *et al.*, 1986; Miller *et al.*, 1987) expression on phagocytic cells can be dramatically up-regulated in a matter of minutes. Most of the Mac-1 and p150,95 proteins are stored in intracellular pools of neutrophils (Todd *et al.*, 1984; Miller *et al.*, 1987; Bainton *et al.* 1987) and monocytes (Miller *et al.*, 1987). Electron microscopy shows that leukocyte integrins are associated with peroxidase-negative granules (Miller *et al.*, 1987; Bainton *et al.*, 1987). This latent pool is mobilized in response to chemotactic factors, including fMLP, C5a, and leukotriene B4, and results in up to a 10-fold increase in surface expression of Mac-1 and p150,95. LFA-1 is up-regulated twofold in monocytes and none in neutrophils. Up-regulation presumably aids in the rapid mobilization of monocytes and neutrophils to inflammatory sites.

3. *Receptor Activation*

The regulation of the functional activity of leukocyte integrins is more complex than changing the gross level of surface expression. Peripheral blood lymphocytes express LFA-1, but do not spontaneously adhere to each other. Phorbol ester stimulation induces a rapid homotypic aggregation event. Aggregation is inhibitable by MAbs against LFA-1 (Patarroyo *et al.*, 1985; Mentzer *et al.*, 1985; Rothlein and Springer, 1986) and ICAM-1, an LFA-1 ligand (Rothlein *et al.*, 1986). Aggregation is not associated with quantitative changes in the surface expression of either LFA-1 or ICAM-1. These results suggest that cell activation induces some change in either LFA-1 or ICAM-1 molecules.

Mac-1 functional activity appears to be similarly regulated on neutrophils. Neutrophils also form homotypic aggregates upon stimulation. Buyon *et al.* (1988) were able to dissociate up-regulation of the latent pool of Mac-1 from the homotypic aggregation event. Mac-1 MAbs can effectively block neutrophil aggregation. Freshly isolated neutrophils were pretreated with Mac-1 MAbs to coat surface Mac-1, washed, and then activated. Precoating the surface Mac-1 did not affect up-regulation

and expression of the latent pool; however, it did effectively inhibit neutrophil aggregation. Furthermore, up-regulation and aggregation were dissociated kinetically and pharmacologically. In contrast, Vedder and Harlan (1988) found that treating neutrophils with an anion channel-blocking agent, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), inhibited both Mac-1 up-regulation and neutrophil aggregation. Interestingly, DIDS did not inhibit Mac-1-dependent binding of neutrophils to endothelial cells.

Phorbol esters have a biphasic effect on the C3bi-binding activity of Mac-1 that is independent of up-regulation (Wright and Meyer, 1986). First, a rapid increase in C3bi-binding activity is observed. However, after 1 hour, binding activity is reduced below resting levels, despite the increased expression of Mac-1. Interferon-γ depressed C3bi-binding activity without affecting Mac-1 expression. Binding activity is restored by adherence to fibronectin-coated surfaces (Wright *et al.*, 1986). These results suggest that qualitative changes in Mac-1 are important for functional activity. The quantitative changes as a result of up-regulation of intracellular pools may augment the response.

a. Activation Epitopes. The molecular mechanisms which regulate functional activity are not clear. The simplest explanation is a conformational change which unmasks the ligand binding site. However most MAbs that block adhesion of activated cells also bind to leukocyte integrins on resting cells. One MAb against a novel epitope of LFA-1 has been shown to induce homotypic aggregation of lymphoid cells, which is inhibitable by other LFA-1 MAbs (Keizer *et al.*, 1988). This and several other MAbs appear to define activation epitopes on LFA-1 (Pircher *et al.*, 1986; Morimoto *et al.*, 1987).

b. Interaction with Other Receptors. Interaction of the leukocyte integrins with other membrane proteins may also influence binding activity. Pytowski *et al.* (1988) recently raised a MAb against a 157,000-Da cell-surface protein on neutrophils, which specifically inhibits the C3bi-binding activity of Mac-1. The antigen is distinct from the leukocyte integrins and is expressed on cells from LAD patients. Another report suggests that a subset of Mac-1 on monocyte cell surfaces is associated with the Fc receptor and shows no lateral diffusion in the membrane (Brown *et al.*, 1988). Antibody against the nondiffusable Mac-1 blocks IgG-mediated phagocytosis.

c. Receptor Clustering. Binding activity may also be regulated by cross-linking of receptors. Phorbol ester-induced C3bi-binding activity of Mac-1 correlates with aggregation of Mac-1 in small clusters on the cell surface. Prolonged exposure to phorbol myristate acetate (PMA) reverses C3bi-binding activity and results in dispersal of the Mac-1 clusters (Detmers *et al.* 1987).

d. Phosphorylation. Receptor activity is likely to be regulated by intracellular events. Phorbol esters, which induce homotypic aggregation, are known to activate protein kinase C. Phosphorylation of the LFA-1 β subunit is induced by phorbol ester stimulation of peripheral blood lymphocytes (Hara and Fu, 1986). Neutrophils loaded with thiophosphate, which renders phosphorylated proteins resistant to phosphatases, show enhanced C3bi-binding activity upon phorbol ester stimulation. Moreover, thiophosphate-loaded neutrophils do not display the characteristic deactivation of C3bi-binding activity after prolonged exposure to phorbol esters (Wright and Meyer, 1986). These results suggest that phosphorylation may play an important role in regulating leukocyte integrin function.

e. Interaction with the Cytoskeleton. Cell activation may also induce association of the leukocyte integrins with cytoskeletal elements. LFA-1-dependent adhesion is disrupted by cytochalasin B (Rothlein and Springer, 1986). Furthermore, LFA-1 and actin filaments colocalize to the site of contact between NK cells and target cells (Carpen *et al.*, 1986). More interestingly, the fibronectin receptor, an evolutionarily related integrin, has been shown to interact directly with talin, a cytoskeletal protein (Horwitz *et al.*, 1986). LFA-1 and talin also colocalize in activated, but not resting, lymphocytes (Burn *et al.*, 1988), and redistribute to sites of adhesion with specific antigen-bearing cells (Kupfer and Singer, 1988). Redistribution of LFA-1 and talin is one of the earlier events, preceding redistribution of the T cell antigen receptor and reorientation of the Golgi and microtubule organizing center. Redistribution of LFA-1 may be triggered by initial antigen receptor engagement, a mechanism earlier proposed to regulate adhesion strengthening via LFA-1 based on experiments with phorbol esters (Rothlein and Springer, 1986; Springer *et al.*, 1987). An unglycosylated intracellular protein of 86,000 Da has been found in association with cell-surface LFA-1 (van Agthoven *et al.*, 1985). The functional significance is unknown, although this protein may be part of a cytoskeletal linkage. Association and disassociation of the leukocyte integrins with the cytoskeleton would provide a mechanism for rapid adhesion and deadhesion events, as required for CTL-mediated cytolysis and other immune functions.

V. Ligand Molecules for the Leukocyte Integrins

A. LFA-1 Ligands

1. *ICAM-1*

a. Identification and Characterization of an LFA-1 Ligand. Phorbol ester-induced homotypic aggregation of lymphoid cells is an LFA-1-dependent phenomenon. Rothlein and Springer (1986) showed that

LFA-1$^-$ cells from LAD patients do not form homotypic aggregates; however, LFA-1$^+$ control cells can form aggregates with LFA-1$^-$ cells from LAD patients. These results suggested that LFA-1 binds a ligand distinct from itself, and that cells from LAD patients should express the ligand. Rothlein *et al.* (1986) raised MAb against LFA-1$^-$ cells from LAD patients and screened them for the ability to inhibit LFA-1-dependent homotypic aggregation, in the hopes of identifying the putative LFA-1 ligand. One MAb (RR1/1) defined a 76,000- to 114,000-Da heavily glycosylated molecule, ICAM-1, which fit these criteria.

The receptor–ligand relationship of LFA-1 and ICAM-1 was formally proved with purified ICAM-1 incorporated into planar lipid membranes (Marlin and Springer, 1987) and by gene transfection (Simmons *et al.*, 1988). All of these systems can mediate LFA-1-dependent adhesion of lymphoblasts, which is inhibitable by pretreatment of the lymphoblast with LFA-1 MAbs or pretreatment of the ICAM-1 surface with ICAM-1 MAbs. Moreover, LFA-1$^-$ cells from LAD patients do not bind to purified ICAM-1 (Marlin and Springer, 1987). Confirming studies with ICAM-1 cross-linked to artificial substrates extended these findings and demonstrated that three independent MAbs defined ICAM-1, one of which had been used to map it to chromosome 19 (Makgoba *et al.*, 1988b).

Reciprocal studies have been done using purified LFA-1 (Dustin and Springer, 1989). Binding of some cell types to purified LFA-1 in planar membranes was inhibited by ICAM-1 MAbs. Furthermore, purified LFA-1 binds to purified ICAM-1 when ICAM-1 is presented in planar membranes, confirming that LFA-1-dependent adhesion can be accounted for by direct interaction of LFA-1 with ICAM-1 and other ligands (see below).

ICAM-1 is a widely distributed molecule whose expression is highly regulated. Basal ICAM-1 expression on nonhematopoietic cells is normally low, but can be up-regulated by a variety of cytokines, including interleukin-1, tumor necrosis factor, and interferon-γ (Dustin *et al.*, 1986, 1988; Pober *et al.*, 1986, 1987; Dustin and Springer, 1988). ICAM-1 expression is prominent on cytokine-activated endothelial cells during inflammation (Dustin *et al.*, 1986; Cotran *et al.*, 1988). Increased ICAM-1 expression correlates directly with increased LFA-1-dependent adhesion of lymphoblasts to induced cells (Dustin *et al.*, 1986, 1988; Dustin and Springer, 1988). These results suggest that ICAM-1 provides dynamic "position-specific" information to guide lymphocyte and leukocyte localization during the course of the immune response. ICAM-1 is expressed only weakly on resting peripheral blood leukocytes, but expression is increased upon cell activation. A molecule identical to ICAM-1 (Makgoba *et al.*, 1988b) was defined as a B cell activation

molecule (Clark *et al.*, 1986). The LFA-1/ICAM-1 adhesion pathway has been implicated in lymphocyte adhesion to endothelial cells (Dustin and Springer, 1988), fibroblasts (Dustin *et al.*, 1986), epidermal keratinocytes (Dustin *et al.*, 1988), synovial cells (Mentzer *et al.*, 1988), and other lymphoid cells (Rothlein *et al.*, 1986; Makgoba *et al.*, 1988a).

ICAM-1 has been reported to localize to uropods of the T cell line HSB-2 (Dougherty *et al.*, 1988). By indirect immunofluorescence, ICAM-1 expressed in COS cells (D. E. Staunton and T. A. Springer, unpublished) and endothelial cells (Smith *et al.*, 1989) demonstrates a punctate staining. This localized cell membrane distribution may facilitate initial interaction with LFA-1. Spreading of cells on surfaces coated with extracellular matrix components or adhesion ligands is an indication of cytoskeletal association. A Reid-Sternberg cell line expressing high levels of ICAM-1 spreads dramatically on LFA-1 bearing planar membranes. This raised the possibility that at least in some cells, ICAM-1 is associated with the cytoskeleton. A truncated form of ICAM-1, which completely lacks a cytoplasmic tail was generated by oligonucleotide mutagenesis (Staunton *et al.*, unpublished). The truncated protein immunoprecipitated from transfected COS cells demonstrates a mobility on SDS-PAGE which is consistent with its predicted 3-kDa reduction in molecular weight. Resistance to cleavage by a phosphatidyl inositol (PI)-specific phospholipase C suggests that it does not acquire a PI membrane anchor. This truncated form of ICAM-1 still demonstrates its characteristic cell-surface localization. Thus, if localization is through cytoskeletal interaction, this must occur in the ICAM-1 transmembrane region, or by association of the extracellular domain with an anchored protein.

b. Gene Cloning. ICAM-1, which has recently been cloned and sequenced (Simmons *et al.*, 1988; Staunton *et al.*, 1988), has been shown to be a member of the immunoglobulin superfamily, with its closest relationship being to the neural cell adhesion molecule (NCAM) and myelin-associated glycoprotein (MAG), another neural adhesion protein. All three proteins contain five immunoglobulin domains. Interestingly, NCAM has been shown to participate in homophilic (like-like) interactions (Edelman, 1986), whereas ICAM-1 participates in heterophilic interactions. LFA-1 is the only known member of the integrin superfamily which binds to a member of the immunoglobulin superfamily. Members of the other two integrin subfamilies bind a conserved Arg-Gly-Asp (RGD) sequence in unrelated extracellular matrix proteins. ICAM-1 does not have an RGD sequence (Simmons *et al.*, 1988; Staunton *et al.*, 1988) and neither does ICAM-2 (see Section V,A,2; Staunton *et al.*, 1989), suggesting that leukocyte integrin binding specificity has diverged from that of other integrins. Preliminary results from mutational studies reveal

that at least two sites in ICAM-1 are involved in binding LFA-1 (Staunton *et al.*, unpublished).

2. *Other Ligands*

There is evidence that ICAM-1 is not the only ligand for LFA-1. Phorbol ester-induced homotypic aggregation of the SKW3 T cell line is inhibited by LFA-1 MAbs but not by ICAM-1 MAbs (Makgoba *et al.*, 1988a). Similarly, some heterotypic cell interactions, such as CTL adhesion to B lymphoblastoid cell lines, is inhibitable by LFA-1 MAbs but not by ICAM-1 MAbs (Makgoba *et al.*, 1988b). LFA-1-dependent T cell adherence to endothelial cells has both an ICAM-1-dependent and an ICAM-independent component (Dustin and Springer, 1988). The ICAM-1-dependent pathway is inducible with cytokines, such as tumor necrosis factor (TNF), whereas the ICAM-1-independent pathway is unaffected. These results suggest that multiple ligands may serve to mediate distinct adhesion requirements of lymphocytes during different stages of the immune response. Cell binding to purified LFA-1 in glass-supported planar membranes or immobilized directly on plastic also suggests the presence of other ligands. The most striking example of this is the T cell line SKW3, which expresses virtually no ICAM-1 yet binds strongly to purified LFA-1. This binding is blocked by LFA-1 MAbs but not by any single ICAM-1 MAb or combination of ICAM-1 MAbs (Dustin and Springer, 1989). Other cell lines show different degrees of inhibition with ICAM-1 MAbs, depending on the LFA-1 density of purified LFA-1 on the monolayer. With these cell lines, ICAM-1 MAbs are inhibitory at low LFA-1 density but not at high LFA-1 density, whereas LFA-1 MAbs are inhibitory at all densities. These results suggest that both ICAM-1 and the alternative LFA-1 ligand are present on these cells, and that interaction is stronger with ICAM-1 than with the alternative ligand. The only nonhematopoietic cells we have encountered that express ICAM-1 in the absence of other LFA-1 ligands are epithelial cells from several tissues and dermal fibroblasts (Dustin *et al.*, 1986, 1988). The existence of multiple ligands for a single integrin is not unprecedented. Other integrins, such as platelet glycoprotein IIb/IIIa, have as many as four distinct ligands.

Recently, a second LFA-1 ligand, designated ICAM-2, was cloned based on its functional properties (Staunton *et al.*, 1989). A cDNA expression library was screened for ability to confer on COS cells the ability to bind to purified LFA-1 coated on Petri dishes. Screening was in the presence of ICAM-1 MAb. ICAM-2 has two Ig-like domains, in contrast to ICAM-1, which has five, and these are 35% identical to the first two domains of ICAM-1. ICAM-1 and ICAM-2 are much more similar to one another than to other members of the Ig superfamily, and thus represent an Ig

subfamily specialized to interact with LFA-1. The functional cDNA isolation approach should have wide application for identifying other adhesion counterstructures.

B. Mac-1 AND p150,95 LIGANDS

1. C3bi

Both Mac-1 and p150,95 bind the C3bi fragment of complement (Micklem and Sim, 1985). A peptide fragment of C3bi which contains an RGD sequence binds to macrophages in a Mac-1 MAb-inhibitable fashion (Wright *et al.*, 1987). However, it is not clear whether the RGD sequence is critical for binding.

2. Other Ligands

The ability of Mac-1 to mediate a number of cell-cell interactions in which C3bi is not involved, such as neutrophil aggregation, suggests putative cell surface ligand(s) for Mac-1. The epitopes on Mac-1 involved in C3bi-binding activity and general adhesion have been distinguished by MAb-inhibition studies (Anderson *et al.*, 1986; Dana *et al.*, 1986; Sanchez-Madrid *et al.*, 1983a; Beatty *et al.*, 1983, 1984). Some MAbs show differential effects on neutrophil homotypic aggregation and adhesion to endothelial monolayers as compared to C3bi binding, while other MAbs block both.

VI. Molecular Basis of Leukocyte Adhesion Deficiency Disease

A. EARLY STUDIES

Every LAD patient analyzed to date has been found to be deficient in the expression of all three leukocyte integrins (Anderson and Springer, 1987). The simplest hypothesis is that a defect in the common β subunit could account for LAD. This hypothesis was tested in biosynthesis and human X mouse lymphocyte hybridization experiments.

Biosynthesis experiments utilized Epstein-Barr virus (EBV)-transformed B lymphocyte and mitogen-stimulated T lymphocyte cell lines, which, in healthy individuals, synthsize the LFA-1 $\alpha(\alpha L)$ subunit and the common β subunit and express the LFA-1 $\alpha\beta$ complex on cell surfaces. Early studies showed that patient cell lines synthesize an apparently normal αL subunit precursor, but that the αL precursor does not undergo carbohydrate processing, does not associate in an $\alpha\beta$ complex, and neither subunit is expressed on the cell surface (Springer *et al.*, 1984; Lisowska-Grospierre *et al.*, 1986). In these studies, the use of available anti-β subunit monoclonal antibodies (MAb) did not allow for the immunoprecipitation of β subunit precursors from either control cells or LAD patient cells.

In human X mouse lymphocyte hybrids, human LFA-1 α and β subunits from normal cells were shown to associate with mouse LFA-1 subunits to form interspecies $\alpha\beta$ complexes. Surface expression of the α but not the β subunit of patient cells can be rescued by the formation of interspecies complexes (Marlin *et al.*, 1986). These studies showed that the LFA-1 α subunit in genetically deficient cells is competent for surface expression in the presence of an appropriate mouse β subunit. Taken together, these results suggest that leukocyte adhesion deficiency is secondary to a defect in the common β subunit.

B. Identification of Heterogeneous Mutations in the Common β Subunit

The acquisition of two molecular probes, the β subunit cDNA (Kishimoto *et al.*, 1987a; Law *et al.*, 1987) and a rabbit antiserum that recognizes the precursor form of the β subunit (Kishimoto *et al.*, 1987b; Dana *et al.*, 1987), allowed analysis of the β subunit from LAD patients. Five phenotypes of β subunit expression and structure were identified by Kishimoto *et al* (1987b). One class of mutations resulted in no detectable mRNA or protein precursor. Southern analysis of genomic DNA from these patients showed no gross deletions of the β subunit gene. A second class of mutation is represented by a moderately deficient patient, whose cells synthesized trace amounts of the β subunit precursor and low levels of mRNA message. Dimanche *et al.* (1987) studied two patients with no apparent β subunit precursor synthesis, and this may fall into one of these two classes; however, further analysis at the RNA level is required.

Two other classes of mutations affect the structure of the common β subunit. In one, the patient synthesizes an aberrantly large β subunit precursor. However, after endoglycosidase H digestion, the protein backbone appears about normal in size. One hypothesis is that a point mutation causes an amino acid change, which creates a novel consensus N-glycosylation site (Asn-X-Ser/Thr). In the other class of mutation, four moderately deficient patients, who are all related, synthesized an aberrantly small precursor that was degraded. The pedigree analysis of 14 members of this kindred show that inheritance of the aberrant precursor correlates with the expected disease state and surface expression of LFA-1. Endoglycosidase H digestion of N-linked carbohydrates from the precursor shows that the defect is in the protein backbone rather than in glycosylation.

Finally, three unrelated patients studied by us (Kishimoto *et al.*, 1987b), a group of four patients studied by Dana *et al.* (Dana *et al.*, 1987), and one patient studied by Dimanche *et al.* (Dimanche *et al.*, 1987), synthesized both a normal size β subunit precursor and a normal

size α subunit precursor. Neither subunit was processed or transported to the cell surface. Although it is likely that there is a point mutation in the β subunit, we cannot exclude the possiblity of α subunit mutations.

C. Molecular Basis of the Severe and Moderate Deficiency Phenotypes

Heterogeneity in the defect causing LAD disease was first observed in the extent of the leukocyte integrin deficiency at the cell surface. Patients are classified as severely deficient ($<0.5\%$ normal levels of expression) and moderately deficient (3–10% normal levels of expression) (Anderson *et al.*, 1985). Survival of LAD patients is greatly influenced by the extent of deficiency of the leukocyte integrins. Severely deficient patients often die in the first 2 years of childhood, while moderately deficient patients are less prone to life-threatening infections and can survive to adulthood. However, even those patients who survive to adulthood can suddenly die of complications from severe infections.

The molecular basis for this heterogeneity is unclear. In the case of deficiency of β subunit mRNA and protein precursor, the extent of deficiency correlates with moderate and severe phenotype (Kishimoto *et al.*, 1987b). Two severely deficient patients had no detectable β subunit mRNA expression or protein precursor synthesis, while one moderately deficient patient had low levels of mRNA expression and precursor synthesis (Kishimoto *et al.*, 1987b). Apparently, this low level of expression is sufficient to account for the moderate phenotype and less severe clinical complications. However, among 13 patients synthesizing normal quantities of mRNA and protein precursor, it has been unclear why some are of the moderate and some of the severe deficiency phenotype (Kishimoto *et al.*, 1987b; Dimanche *et al.*, 1987; Dana *et al.*, 1987) (Wardlaw and T. A. Springer, unpublished).

We have analyzed four related patients with the moderate deficiency phenotype (Kishimoto *et al.*, 1989). The predominant form of the β subunit precursor synthesized by these patients is several thousand daltons smaller than normal and is degraded before transport to the Golgi apparatus. However, ^{125}I cell-surface labeling shows that the small amount of LFA-1 that reaches the surface contains a normal size β subunit. At the RNA level, S1 nuclease protection studies define a 90-nt deletion in the β subunit message from these patients. The Taq polymerase chain reaction (PCR) was adapted to amplify the aberrant mRNA. Sequence analysis shows an in-frame 90-nt deletion in the region encoding the extracellular domain. This 90-bp region is also shown to be encoded on a separate exon in both the normal and patient genome. Sequence analysis of genomic DNA from these patients shows a single

G-to-C substitution in the sequence of the 5′ splice site, suggesting aberrant RNA splicing. A small amount of normally spliced message, detected by Sl nuclease protection analysis and Taq PCR, encodes a normal size β subunit and accounts for the low levels (3% of normal) of cell-surface expression of the leukocyte integrins observed in these patients, hence the moderate deficiency phenotype. The 30 amino acids encoded by the deletion region share 63% amino acid identity with the corresponding region of the fibronectin receptor. The high conservation suggests some functional significance of this region, perhaps in $\alpha\beta$ subunit association.

VII. Future Avenues of Research

The first leukocyte integrin to be functionally characterized was LFA-1, in 1981 (Davignon *et al.*, 1981a). Rapid progress in recent years in the study of leukocyte adhesion reflects an ever-increasing appreciation of the diverse functions of leukocyte integrins. The leukocyte integrins, like other members of the integrin superfamily, play a dynamic role in transmitting positional information and linking the extracellular environment with intracellular processes. The interaction of LFA-1 and ICAM-1 represents one of the few defined heterotypic receptor–ligand relationships of cell-surface molecules. LFA-1-dependent adhesive interactions are exquisitely regulated at both the level of receptor and ligand. We have an understanding of the role of leukocyte integrins *in vivo* in both immune and nonimmune inflammatory responses. The recent advances hold promise for many new avenues of research.

Precise structure–function relationships of the leukocyte integrins and ICAM-1 can now be directly tested by *in vitro* mutagenesis of the appropriate cDNAs. Domain-swapping among the leukocyte integrin α subunits may allow dissection of LFA-1, Mac-1, and p150,95 functional specificity. Domains that are defined as functionally important can be synthesized and tested both *in vitro* and *in vivo*. Similar mutagenesis approaches may reveal how integrin receptor activation occurs, what kinds of signals are transmitted to the cell by leukocyte integrins, and the nature of cytoskeletal interaction. In addition, the acquisition of the leukocyte integrin and ICAM-1 cDNAs allows analysis of the basis for gene regulation by a variety of cytokines.

There is also substantial evidence that other ligands for LFA-1, Mac-1, and p150,95 exist. Rational strategies must be designed to identify these ligands and to assess their contributions in different phases of the immune response. Multiple ligands may provide quite distinct signals and positional information to leukocytes.

The answers to these questions of structure, function, and regulation of receptor activity have profound therapeutic implications, as suggested

by recent animal models of ischemia and reperfusion injury. The control of pathologic tissue damage by neutrophils during inflammation can be approached by inhibition of receptor activation, modulation of ICAM-1 expression, or by using MAbs or fragments of leukocyte integrins or their ligands to block acute adhesive events.

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Structure and Function of the Complement Receptors, CR1 (CD35) and CR2 (CD21)

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I. Introduction

A simplified view of complement holds that its primary function is to mediate the uptake of foreign macromolecules by cells of the immune system. Uptake promotes removal of foreign material either directly through the process of phagocytosis or indirectly by recruitment of other recognition mechanisms, such as those employed by B and T cells. This function of complement requires two components: ligands that can be attached to foreign macromolecules, and cellular structures that bind the ligands. The former are represented by C3 and C4, the covalent binding functions of which are activated by the classical and alternative pathways, and the latter are the cellular receptors specific for fragments of C3 and C4. Analysis of major aspects of the two activating pathways was completed during the 1960s and 1970s, and in the 1980s attention has been directed to elucidating the structure and function of complement receptors. This review reports recent findings regarding two of these receptors, complement receptor type 1 (CR1; CD35) and complement receptor type 2 (CR2; CD21), which bind the cleavage fragments of C4 and C3 that are covalently attached to activators of the complement system.

II. Complement Receptor Type 1

The studies of CR1 will be discussed under three major headings relating to its structure, tissue distribution and biosynthesis, and function, respectively.

A. Structure of CR1

The complete primary structure of CR1 is now known through molecular cloning studies (Klickstein *et al.*, 1987, 1988). The most common allotype of CR1, the F (Wong *et al.*, 1983) or A allotype

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(Dykman *et al.*, 1983a), is a polypeptide of 2039 residues: a 41-amino acid signal peptide, an extracellular domain of 1930 residues, a 25-amino acid transmembrane region, and a 43-amino acid cytoplasmic domain. The extracellular portion of the receptor is composed exclusively of 30 short consensus repeats (SCRs). Each SCR contains 60 to 70 amino acids, six of which are found in all SCRs: four cysteines, a single tryptophan between the third and fourth cysteine, and a glycine after the second cysteine. There is a relatively high content of glycine and proline, accounting for the high probability of β-turn formation and low probability of α-helical formation. Overall, approximately half of the residues are found in at least 50% of all SCRs of CR1.

A shorter form of CR1 having SCR1-8 has been proposed based on finding a polyadenylated mRNA formed by use of an alternative polyadenylation site in the intron separating the two exons encoding SCR-9. A protein product of this transcript has not been found *in vivo* (Hourcade *et al.*, 1988).

The SCR is predicted to have a triple loop structure, as shown in Fig. 1, that is maintained by disulfide linkages between cysteines-1 and -3, and -2 and -4. This prediction is based on observations of the roles of the half-cysteines in two other proteins having SCRs, β_2-glycoprotein I (Lozier *et al.*, 1984) and C4-binding protein (C4-bp) (Janatova *et al.*, 1988). A single SCR has been calculated to be 38 × 30 Å based on electron microscopic measurement of the subunits of C4-bp which have eight SCRs and dimensions of 3000 × 38 Å (Perkins *et al.*, 1986).

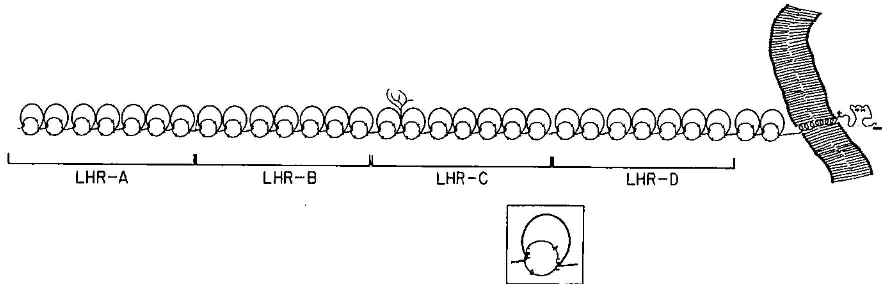

FIG. 1. Proposed secondary structure of human CR1. The extracellular domain of CR1 consists of 30 short consensus repeats (SCRs), and the amino-terminal 28 SCRs comprise four long homologous repeats (LHRs), which are indicated by brackets. The inset is an enlargement of the triple-loop structure of the SCR, which is maintained by disulfide bonds. The branched structure within LHR-C represents one of 17 potential sites for N-linked glycosylation (Klickstein *et al.*, 1987).

Every eighth SCR is a highly homologous repeat, ranging from approximately 60 to 100% identity (Fig. 2), so that seven SCRs constitute a long homologous repeat (LHR) of which the F allotype has four; only the carboxy-terminal SCR-29 and -30 are not part of an LHR. The amino-terminal three LHRs, termed LHR-A, -B, and -C, have independent binding sites for C3b and C4b (Klickstein *et al.*, 1988), as will be discussed below, so that the LHR can be considered as a structural and a functional unit.

Although the LHR is unique to CR1, the basic structural element of the SCR has been found in other proteins (Table I), most of which have C3/C4 binding function, indicating that the SCR motif has been repeatedly used during evolution of the many C3/C4-binding proteins of the complement system. However, some SCR-containing proteins, such as the interleukin-2 receptor, β_2-glycoprotein, and factor XIIIb, have not been demonstrated to have any function in the complement system, so that this struction–function correlation does not always obtain and

LONG HOMOLOGOUS REPEAT	SHORT CONSENSUS REPEAT 1	2	3	4	5	6	7
A	a	a	a	a	a	a	a
	56%	66%	99%	100%	100%	100%	100%
B	b	b	a	a	a	a	a
	100%	98%	97%	100%	78%	81%	75%
C	b	b	a	a	c	c	c
	57%	56%	66%	60%	95%	87%	93%
D	d	d	d	d	c	c	c
	(60%)	(56%)	(66%)	(60%)	(77%)	(83%)	(73%)

FIG. 2. Homology among CR1 short consensus repeats (SCRs). The 28 SCRs of CR1, which are contained within long homologous repeats (LHRs)-A, -B, -C, and -D, consist of four different types: a, b, c, and d. These designations are based upon amino acid sequence homology. The percentages shown indicate the relatedness of the SCRs immediately above and below, except for the numbers in parentheses at the bottom of each column, which reflect a comparison of the SCRs in LHR-D with those in LHR-A.

TABLE I
SCR-CONTAINING PROTEINS

Protein	Number of SCRs	Reference
CR1	30	Klickstein *et al.* (1988)
CR2	15, 16	Weis *et al.* (1988); Moore *et al.* (1987)
β_2-glycoprotein I	4	Lozier *et al.* (1984)
B	3	Morley and Campbell (1984)
Factor XIIIb	10	Ichinose *et al.* (1986)
Clr subunit of Cl	2	Leytus *et al.* (1986)
C4-binding protein (C4-bp)	8	Chung *et al.* (1985)
Haptoglobin	1	Kurosky *et al.* (1980)
IL-2 receptor (IL2R)	2	Leonard *et al.* (1985)
H	20	Ripoche *et al.* (1988)
Fragment b of C2 (C2b)	3	Bentley (1986)
Decay-accelerating factor	4	Caras *et al.* (1987); Medof *et al.* (1988)
Membrane cofactor protein	4	Lublin *et al.* (1988)
Vaccinia virus 35-kDa protein	4	Kotwal and Moss (1988)
Endothelial leukocyte adhesion molecule	6	Bevilacqua *et al.* (1989)
Mouse lymph node homing receptor	2	Siegelman *et al.* (1989)
Mouse CRY	5	Paul *et al.* (1989)

members of other plasma and membrane protein systems have adopted the SCR for presumably different functional purposes.

The transmembrane and intracellular domains of CR1 are less unusual. The former is comprised of 25 hydrophobic amino acids having a tendency for α-helix formation (Fig. 3); no charged residues are present that might favor membrane protein–protein interactions. The size of the cytoplasmic region, 43 amino acids, is too limited to constitute an enzyme, although this part of the receptor presumably mediates interaction of CR1 with the cytoskeleton of phagocytic cell types (Jack and

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CR1 LEGSPWSQCQADDRWDPPLAKCTSRAHDA|LIVGTLSGTIFFILLIIFLSWIII|LKHRKGNNAHENP KEVAIHLHSQGGSSVHPRTLQTNEENSRVLP
CR2 LEGSPQSQCQSDHQWNPPLAVCRSRSLAP|VLCGIAAGLILLTFLIVVTLYVI|SKHRARNYYTDTSQKE AFHLEAREVYSVDPYNPAS

     3' EXTRACELLULAR           TRANSMEMBRANE                 CYTOPLASMIC
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FIG. 3. Amino acid sequence homology between the carboxy-terminal domains of CR1 and CR2. An asterisk is placed between identical residues. Potential sites for phosphorylation in the cytoplasmic domains are in boldface (serine/threonine) or underlined (tyrosine).

Fearon, 1985; Jack *et al.*, 1986) and serves as a substrate for protein kinase C (Changelian and Fearon, 1986). With respect to the latter reaction, a candidate site for phosphorylation is the six-amino acid sequence, VHPRTL, which is homologous to the sequence, VRKRTL, a site of protein kinase C phosphorylation in the epidermal growth factor receptor and the *erbB* oncogene product (Hunter *et al.*, 1984; Davis and Czech, 1985).

B. Allotypes and Genomic Structure of CR1

Four allotypic forms of CR1 have been identified that differ by molecular weight (Table II) (Dykman *et al.*, 1983a,b, 1984, 1985; Wong *et al.*, 1983; van Dyne *et al.*, 1987). In addition to the common A allotype are the larger S, or B, allotype, that occurs with a frequency of 0.18, and two rare forms that are larger and smaller than the B and A allotypes, respectively. Although the primary structure of only the A allotype is known through cDNA sequence analysis, five observations suggest that the size differences may be accounted for by the presence of variable numbers of LHRs. First, the allotypic differences have been shown not to be altered by removal of N-linked oligosaccharides (Wong *et al.*, 1983) or by biosynthesis in the presence of tunicamycin (Lublin *et al.*, 1986). Second, the allotypic forms differ by increments of 30,000 to 50,000 in M_r, which is comparable to the M_r of an LHR. Third, the high-performance liquid chromatography (HPLC) profile of tryptic peptides of ^{125}I-labeled CR1 allotypes were indistinguishable (Nickells *et al.*, 1986), suggesting that the segments of the protein that were involved in the allotypic differences represented conserved regions common to

TABLE II
Characteristics of CR1 Allotypes

Nomenclature	M_r	mRNA (kb)	Frequency
F[a] (A)[b]	250,000[c] (190,000)[d]	7.9, 9.2[e] (8.6, 10.0)[f]	0.82
S (B)	290,000 (220,000)	9.2, 10.7 (10.0, 11.6)	0.18
F′ (C)	210,000 (160,000)	6.5, 7.9 (7.3, 8.6)	<0.01
(D)	(250,000)	(11.6, 12.8)	<0.01

[a] Nomenclature of Wong *et al.* (1983).
[b] Nomenclature of Dykman *et al.* (1983a).
[c] The calculated molecular weight of the unglycosylated polypeptide of this allotype is 220,000.
[d] M_r of unreduced CR1.
[e] From Wong *et al.* (1986).
[f] From Holers *et al.* (1987).

all allotypes. Fourth, the mRNAs for the different allotypes differed by increments of 1.3 to 1.4 kb, a size that could encode an LHR (Wong *et al.*, 1986; Holers *et al.*, 1987). Fifth, and most informative, was the identification of an additional *Bam*HI restriction fragment that hybridized with CR1 cDNA probes on genomic blots of DNA from almost all persons expressing the B allotype and in none lacking the form of CR1 (Wong *et al.*, 1986). Southern blot analysis with a noncoding probe derived from the *Bam*HI fragment hybridized under stringent conditions not only to this fragment but to two others, and these additional *Bam*HI fragments were also present in individuals homozygous for the A allotype (Wong *et al.*, 1986). Thus, an intervening sequence in a *B* allele-specific region was highly homologous to sequences in two other regions of the CR1 gene, suggesting that the CR1 gene, like the cDNA, was composed of highly conserved, repetitive regions that perhaps corresponded to genomic segments containing exons encoding LHRs and that the *B* allele arose from the *A* allele by duplication of such a genomic segment.

Direct evidence in support of this proposal was obtained by isolating and mapping overlapping phage and cosmid clones from a genomic library prepared with DNA from an individual homozygous for the *B* allele (Wong *et al.*, 1989). The gene encoding the *B* allele was shown to extend approximately 160 kb and could be divided into nine regions: the 5′ region containing untranslated and leader sequences; five regions of 18 to 30 kb having exons hybridizing to cDNA probes encoding SCRs comprising the LHRs; a short region containing the two 3′ SCRs that are not included within an LHR; a segment having exons encoding the transmembrane and cytoplasmic regions; and 3′ untranslated sequences. Four of the five genomic regions corresponding to the LHR cDNA sequences were readily identified by hybridization with cDNA probes specific for LHR-A, -B, -C, and -D, respectively. The fifth region was 20 kb in length, resided between the LHR-A and -B genomic segments, and was lacking in overlapping genomic clones isolated from a library prepared with DNA from an AA homozygote. The restriction map of the 5′ half of this genomic region was identical to that of the 5′ half of the LHR-B genomic region, and the map of the 3′ half was indistinguishable from that of the corresponding region of the LHR-A genomic region. Furthermore, CR1 cDNA probes specific for the nearly identical 5′ regions of LHR-B and -C hybridized to the 5′ exons of the additional *B* allele segment, whereas 5′ cDNA probes for LHR-A and -D did not, and cDNA probes specific for the almost identical 3′ sequences of LHR-A and -B hybridized to exons in the 3′ portion of this genomic segment. Thus, the *B* allele has an additional genomic segment that

appears to encode an additional LHR resembling a chimera of the 3′ region of LHR-A and the 5′ region of LHR-B. This allele most likely arose through an homologous recombination event involving the LHR-A and -B regions, and similar mechanisms of duplication or deletion by homologous recombination with unequal cross-over may account for the rarer CR1 allotypes.

These mapping studies also revealed that at least 42 exons are present within the gene for the B allotype. Among these are distinct exons encoding the leader sequence, the transmembrane and cytoplasmic sequences, and most of the SCRs of the extracellular segment of the receptor. Interestingly, the second SCR of each LHR is encoded by more than one, and probably two, exons, perhaps indicating special function for this SCR. In keeping with this possibility is the finding that the second SCR of factor H also is encoded by two exons (Vik *et al.*, 1988) and, as will be discussed below, the ligand binding sites of the A allotype of CR1 have been localized to the first two SCRs of LHR-A, -B, and -C (Klickstein *et al.*, 1988). The ligand binding site of factor H for C3b also has been determined to be in the NH_2-terminal region (Alsenz *et al.*, 1985).

The human gene for CR1 (*CR1*) has been shown to be linked to the genes for CR2 (*CR2*) (Weis *et al.*, 1987), decay accelerating factor (*DAF*) (Rey-Campos, Jr. *et al.*, 1987; Lublin *et al.*, 1987), C4-binding protein (*C4-bp*) (Rodriguez de Cordoba *et al.*, 1984, 1985), factor H (*H*), and membrane cofactor protein (*MCP*) (Lublin *et al.*, 1988) in a locus termed the Regulator of Complement Activation (or RCA) gene cluster. All members of this gene family are C3/C4-binding proteins composed entirely or in part of SCRs. The RCA locus has been identified at or near band q32 of chromosome 1 by *in situ* hybridization and analysis of somatic cell hybrids (Weis *et al.*, 1987). Recently, three reports using the technique of pulsed-field gel electrophoresis have provided physical maps of up to 1500 kb of DNA encompassing five of the members of the RCA locus (Rey-Campos *et al.*, 1988; Carroll *et al.*, 1988; Bora *et al.*, 1989) (Fig. 4). The order of the genes is *MCP, CR1, CR2, DAF,* and *C4-bp*. The inability to link physically the *H* gene to this cluster is consistent with the mapping of this locus to 6.9 cM from the *C4-bp/CR1* locus (Rodriguez de Cordoba and Rubinstein, 1984). Perhaps related to the distal position of the *H* gene is the lesser sequence homology between *H* and the other members (Fig. 5). It is not clear what functional inferences should be drawn from this observation of the clustering of these genes encoding C3/C4-binding proteins, just as the significance of the presence of the genes for C4A, C4B, factor B, and C2 in the Major Histocompatibility Complex is not certain. However,

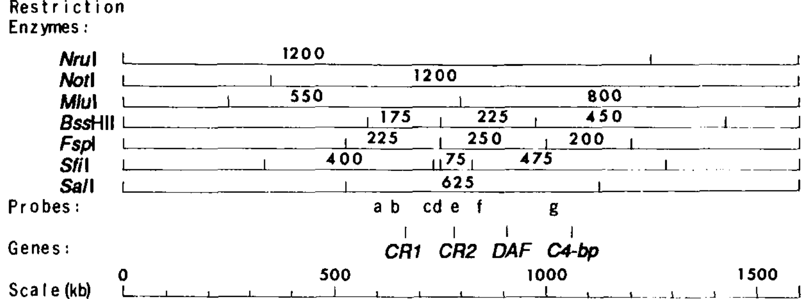


FIG. 4. Organization of the genes that encode CR1, CR2, DAF, and C4-bp within the RCA locus on chromosome 1. The restriction map was prepared by pulsed-field gradient gel electrophoresis and Southern blot analysis of human genomic DNA hybridized with probes specific for CR1 (a, b, c, and d), CR2 (e), DAF (f), and C4-bp (g). The factor H gene lies outside of this 1500-kb region (Carroll *et al.*, 1988). The *MCP* locus has recently been mapped to within 100 kb of the 3′ end of the *CR1* gene (Bora *et al.*, 1989).

the grouping cannot simply be based on the common presence of the SCR, as other complement proteins having this basic structural unit, such as C2, factor B, and Clr, reside on other chromosomes.

C. Tissue Distribution and Biosynthesis of CR1

The following cell types in man have been demonstrated to express CR1 by structural and functional criteria: erythrocytes, B lymphocytes, a subset of T lymphocytes, monocytes, and *in vitro* cultured macrophages, neutrophils, eosinophils, glomerular podocytes, and follicular dendritic cells (Fearon, 1980; Wilson *et al.*, 1983; Reynes *et al.*, 1985; Gelfand *et al.*, 1976; Kazatchkine *et al.*, 1982). The rank order of cells based on their numbers of receptors has been determined for several cell types: peripheral blood neutrophils = monocytes = B lymphocytes (20,000 to 40,000 CR1/cell) > T lymphocytes (1000 to 5000 CR1/cell) > erythrocytes (100 to 1000 CR1/cell). A soluble form of CR1 also has been found in plasma in picomolar concentrations that sedimented as a broad peak with other plasma proteins on ultracentrifugation (Yoon and Fearon, 1985). This form of CR1 was capable of binding C3b, was indistinguishable from erythrocyte CR1 on SDS-polyacrylamide gel electrophoresis, and its concentration correlated with the number of CR1 molecules present on erythrocytes. Whether it is shed in an intact form from erythrocytes, or has had its transmembrane/cytoplasmic domain cleaved, or is directly secreted is not known.

The wide variation of CR1 number on erythrocytes is, at least in part,

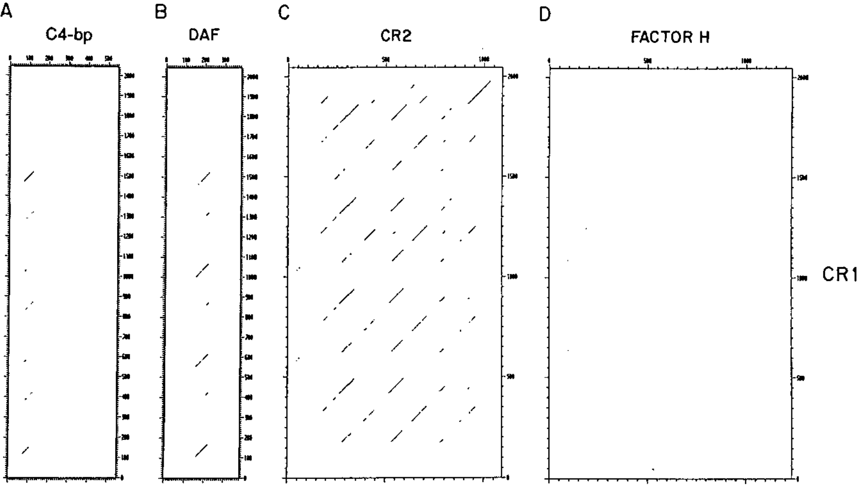


FIG. 5. Dot matrix analysis of the CR1 cDNA sequence compared with those of other SCR-containing proteins. A dot was placed if there was at least a 40-bp of 90-bp match.

a genetically regulated trait (Wilson *et al.*, 1982). A restriction fragment length polymorphism involving allelic *Hin*dIII fragments of 7.4 and 6.9 kb that hybridizes with CR1 cDNA probes correlates with the quantitative expression of CR1 on E (Wilson *et al.*, 1986). Individuals homozygous for the 7.4 kb *Hin*dIII fragment had an average of 661 sites per cell to which monoclonal anti-CR1 bound; persons homozygous for the 6.9-kb *Hin*dIII fragment had 156 sites per cell, and heterozygotes had an intermediate number, 455 sites per cell. The polymorphism is caused by an additional *Hin*dIII site in the allele containing the 6.9-kb fragment that has been created by a single base change in an intron located in the region of the CR1 encoding LHR-D (Wong *et al.*, 1988). As the base change cannot account for the altered expression of CR1, it presumably is in linkage disequilibrium with another polymorphic locus controlling biosynthesis or stability of the receptor on erythrocytes. No quantitative variation of CR1 expression has been observed with neutrophils or B lymphocytes, although unequal expression of CR1 structural allotypes in these cells occurs, perhaps indicating some effect of the *Hin*dIII-linked regulatory locus on CR1 expression by these cells (Wilson *et al.*, 1986).

A mechanism for rapidly up-regulating the plasma membrane expression of CR1 that involves intracellular pools of preformed receptor is

utilized by neutrophils and monocytes (Fearon and Collins, 1983). In their "resting" states as found in peripheral blood of normal individuals, these cells express only 5% to 10% of total cellular CR1 at the plasma membrane, the rest residing within an intracellular compartment that can be elicited to the plasma membrane. Agents that have been shown to up-regulate CR1 include chemotactic peptides, such as C5a and formyl-methionyl-leucyl-phenylalanine (f-MLP), endotoxin, certain cytokines, calcium ionophores, and phorbol esters (Fearon and Collins, 1983; Berger *et al.*, 1985, 1988; O'Shea *et al.*, 1985b; Changelian *et al.*, 1985; Davis *et al.*, 1987). The intracellular pool of CR1 has not been identified, but it is not specific-granule associated, as is a portion of intracellular CR3 (O'Shea *et al.*, 1985a), and may be identical to the non-granule-associated vesicles described as a site for CR3 (Bainton *et al.*, 1987). Up-regulation of CR1 by chemotactic peptides would prepare cells for encountering opsonized microorganisms during and after their motile response leading to localization in infected tissue. That such up-regulation occurs *in vivo* has been demonstrated in patients undergoing hemodialysis with complement-activating membranes that cause the generation of C5a (Lee *et al.*, 1984), and in burn patients in whom increased CR1 expression may be caused (1) by inappropriate systemic activation of neutrophils by C5a derived from the interaction of complement with thermally injured tissue (Moore *et al.*, 1986) or (2) by cytokines, such as tumor necrosis factor, derived from macrophages at sites of tissue damage.

Studies of the biosynthesis of CR1 by induced HL-60 cells (Atkinson and Jones, 1984) and lymphoblastoid cells transformed with Epstein–Barr virus (Lublin *et al.*, 1986) have indicated that the four allotypes of the receptor are initially synthesized as precursor molecules having high-mannose oligosaccharides that are converted to complex-type oligosaccharides. The M_r of CR1 on SDS-polyacrylamide gel electrophoresis was decreased approximately 25,000 by treatment with endoglycosidase F (Wong *et al.*, 1983), consistent with the presence of approximately six to eight oligosaccharide moieties per receptor, which had been predicted also by analysis of the glucosamine content of purified CR1. The receptor lacks O-linked oligosaccharides (Lublin *et al.*, 1986). A function for the N-linked oligosaccharides was indicated by the findings that nonglycosylated CR1 had impaired ligand binding activity, and was inserted into the plasma membrane with decreased efficiency relative to normally glycosylated CR1 (Lublin *et al.*, 1986). Although the small M_r difference between neutrophil CR1 and receptor of other cell types (Dykman *et al.*, 1983a) is caused by unspecified differences in glycosylation, no functional correlates have been reported. Finally, mature peripheral blood neutrophils continue to synthesize CR1, as shown by

incorporation of [^{35}S]methionine, and to contain CR1 mRNA (Jack and Fearon, 1988). This residual biosynthetic capacity of the circulating neutrophil may indicate a capacity for an adaptive response to its microenvironment, a view that has been supported by the observation that granulocyte/macrophage colony-stimulating factor increased CR1 synthesis by neutrophils twofold during *in vitro* cultivation (Neuman and Jack, 1988).

The appearance of CR1 during the ontogenic development of B lymphocytes, myelomonocytic cells, and glomerular podocytes has been studied (Tedder *et al.*, 1983; Appay *et al.*, 1985). B cells gradually acquired the ability to express CR1 during maturation: 15% of large pre-B cells, 35% of small pre-B cells, 60% to 80% of immature B cells having membrane IgM but not IgD, and essentially all mature B cells expressed CR1 detectable by immunofluorescent staining with specific antibody. Plasma cells did not have CR1. The relatively early presence of CR1 during the ontogeny of B cells and its uniform expression by mature B cells are consistent with some role for CR1 in critical B cell functions, although, as will be reviewed, this role has not been fully defined.

In contrast to B lymphocytes, myelomonocytic cells expressed CR1 relatively late in development (Tedder *et al.*, 1983). Whereas all segmented neutrophilic leukocytes and 75% of band forms were stained with anti-CR1, only 4% of metamyelocytes and less than 1% of myelocytes expressed the receptor. These findings refined earlier results obtained with rosetting assays (Ross *et al.*, 1978) and are consistent with CR1 expression temporally correlating with the functional development of this cell type into a competent phagocyte at which time the endocytic function of the receptor can be utilized by the cell.

In ontogenesis of the glomerulus, CR1 was one of the earliest markers expressed by resident glomerular cells (Appay *et al.*, 1985). The receptor on glomerular epithelium was detected as early as the late S-body stage of nephron differentiation, where it was present on the basolateral part of primitive podocytes following acquisition of the latter's capacity for synthesis of a basal lamina. A function of CR1 in the kidney has not been demonstrated and thus the consequences of its early appearance are not known.

D. Function of CR1

1. *Ligand Binding Sites of CR1*

The molecular cloning of CR1 enabled the construction of eukaryotic expression vectors containing cDNA inserts representing the entire CR1 coding sequence and various deletion mutants (Klickstein *et al.*, 1988).

The expression of these recombinant forms of CR1 and the analysis of their ligand binding function has led to the identification of distinct C3b and C4b recognition sites. These functional domains have been assigned to specific structural elements of the receptor, the SCR and the LHR. A conserved *Bsm*I site present midway through the coding sequence of the first SCR of each LHR corresponded closely to the boundaries of the LHRs and maintained the open reading frame and the appropriate positions of the four cysteines necessary for the cys-1/-3, cys-2/-4 disulfide bonds within each SCR. Therefore, by partially digesting the full-length cDNA with *Bsm*I, and religating the appropriate fragments, four deletion mutants were formed which were inserted into the expression vector, CDM8, downstream of a cytomegalovirus promotor. Transfection into COS cells and transient expression of the constructs, termed piABCD, piAD, piBD, piCD, and piD, permitted the assessment of the C3/C4-binding functions of each LHRs.

COS cells expressing the recombinant full-length CR1 formed rosettes with erythrocytes bearing either methylamine-treated C3 [EC3(ma)] or C4 [EC4(ma)], comparable to the known ligand specificities of wild-type CR1 (Table III). Among the deletion mutants, only the piBD and piCD constructs directed expression of receptor capable of binding EC3(ma), indicating the presence of C3 recognition sites in LHR-B and -C, but not LHR-A or -D (Table III). With respect to C4-binding function, the recombinant receptor encoded by the piAD construct was most effective in mediating rosette formation of the COS cells with EC4(ma), suggesting that LHR-A contains the primary C4 recognition site (Table III). Two other deletion mutants, which had been prepared by digesting the CR1 cDNA construct at other restriction sites, further localized this C4-binding site in LHR-A. The constructs, piA/D (which encoded SCR-1 through -5 of LHR-A and the COOH-terminal three SCRs of LHR-D) and piE-2 (which encoded SCR-1 and -2 of LHR-A, the COOH-terminal five SCRs of LHR-C, and all of LHR-D), directed expression of recombinant proteins that demonstrated preferential binding of EC4(ma). Thus, the primary recognition site in CR1 for C4 was shown to require the NH_2-terminal two SCRs of LHR-A. The finding that the C4b and C3b recognition sites in CR1 were distinct is consistent with an earlier study in which soluble C4b was unable to inhibit the formation of rosettes between C3b-bearing erythrocytes and human B lymphoblastoid cell lines (Gaither *et al.*, 1983).

Although comparable deletion mutants dissecting LHR-B or -C were not constructed, the SCRs within these LHRs that were essential for binding C3 become evident on analysis of the amino acid sequences of CR1 (Fig. 2). Because LHR-A and -B differ only in their NH_2-terminal

TABLE III
FORMATION OF ROSETTES BETWEEN COS CELL TRANSFECTANTS TRANSIENTLY EXPRESSING RECOMBINANT FORMS OF CR1 AND SHEEP E BEARING C3(ma) OR C4(ma)

	% Transfectants forming rosettes / % Transfectants fluorescent with anti-CR1	
COS cell transfectant	EC3(ma)	EC4(ma)
piABCD[a]	109	62
piAD	8	107
piBD	107	12
piCD	127	32
piD	0	0
piA/D[b]	11	83
piE-2[c]	0	102

[a] Nomenclature refers to LHRs coded for by the cDNA insert in the CDM8 vector.

[b] piA/D encodes a hybrid LHR with the five NH_2-terminal SCRs of LHR-A linked to the three COOH-terminal SCRs of LHR-D.

[c] piE-2 encodes a mutant CR1 containing SCR-1 and -2 of LHR-A, SCR-17 through -30, and the transmembrane and cytoplasmic domains.

two SCRs, the other five being greater than 99% identical, SCR-8 and -9 are predicted to contain the C3 recognition site within LHR-B and, as these are 100 and 98% identical, respectively, to the NH_2-terminal two SCRs of LHR-C, SCR-15, and SCR-16, the latter are presumed also to have this function in LHR-C. The sequence divergence in the NH_2-terminal two SCRs of LHR-D may be the basis for the absence of C3- or C4-binding function in the recombinant encoded by piD, although some accessory function cannot be excluded by these studies in which all functional constructs contained LHR-D.

The presence of three distinct ligand recognition sites in CR1, two for C3b and one for C4b, suggests that each receptor molecule can interact multivalently with complexes containing multiple C4b and C3b molecules. In fact, the low K_a of 1×10^5 for the binding by CR1 of monomeric C3b suggests that a multivalent ligand–receptor interaction is required. Therefore, the capacity of the classical and alternative pathways to attach many molecules of C4b and C3b per activating complex, and of C4b and C3b to form covalent homo- and heterodimers, is essential, as these reactions generate multivalent ligands for CR1.

The finding of ligand binding sites in LHR-A, -B, and -C also indicates that CR1 allotypes may vary in their number of C3b- and C4b-binding

sites. For example, the B allotype, in which the genomic organization suggests that the NH_2-terminal portion of the additional LHR is LHR-B-like (Wong *et al.*, 1989), may have three C3b-binding sites. Conversely, the smaller C allotype, in which an LHR may have been deleted, may be lacking one of the C3b-binding sites, diminishing its capacity for binding of immune complexes bearing this ligand. As CR1 on erythrocytes may have a role in the clearance of C3b-bearing immune complexes, it is of interest that there may be an association between the expression of the C allotype and the occurrence of systemic lupus (van Dyne *et al.*, 1987).

The presence of highly homologous repeats in CR1 raised the possibility that epitopes binding anti-CR1 antibodies also may be repetitive, and this likelihood was confirmed by the demonstration that a monoclonal anti-CR1, YZ1, bound to the full-length recombinant CR1 encoded by piABCD, and to the deletion mutants encoded by piAD, piBD, and piCD, but not to the product of piD. Therefore, the epitope for YZ1 was present three times, in LHR-A, -B, and -C, respectively, and previous studies assuming a 1:1 molar ratio between the number of cell-bound monoclonal anti-CR1 antibodies and CR1 molecules probably have overestimated the number of receptor molecules present on various cell types. Monoclonal antibodies also may vary in the number of epitopes to which they bind, leading to apparently inconsistent quantitative analyses (Edberg *et al.*, 1987). Even studies employing C3b as the ligand in which each C3b-binding site was presumed to represent a molecule of CR1 probably overestimated by a factor of two the numbers of CR1 molecules. However, the low numbers of CR1 sites on erythrocytes of patients with systemic lupus erythematosus found by all investigators cannot be accounted for by abnormal expression of epitopes, as structural studies of patients' CR1 molecules, including determination of the frequency of the A and B allotypes, have revealed no differences from CR1 of healthy individuals. In any event, it is evident that each monoclonal antibody must be characterized with respect to the number of epitopes to which it binds on each CR1 allotype prior to the use of that antibody for absolute rather than relative measurements of CR1.

2. *Complement Regulatory Functions of CR1*

CR1 was the first membrane protein to be characterized as having an inhibitory effect on enzymes involved in complement activation (Fearon, 1979). CR1 can regulate alternative pathway activation by three mechanisms: impairing uptake of factor B by C3b, displacing Bb from the C3b,Bb C3 convertase, and promoting the cleavage of C3b to iC3b,

C3c, and C3dg by factor I. Similarly, CR1 inhibits the classical pathway by impairing uptake of C2 by C4b, displacing C2a from the C4b,2a C3 convertase and from the C4b,2a,3b C5 convertase, and promoting the cleavage of C4b to C4c and C4d by factor I (Iida and Nussenzweig, 1981). These capabilities of CR1 combine the functions of several other regulatory plasma and membrane proteins of the complement system (Table IV). For example, whereas CR1 is effective in regulating both the classical and alternative pathways, the plasma proteins, C4-bp and factor H, selectively inhibit the classical and alternative pathways, respectively. Furthermore, CR1 is more active on a molar basis than either of these plasma proteins (Table IV), and, unlike factor H, is not diminished in its capacity to bind to C3b present on an alternative pathway activating surface. CR1 also has a wider range of functions than does DAF in that the latter cannot serve as a cofactor for cleavage of C4b and C3b by factor I, this function being exercised by the membrane protein, MCP. However, despite its potent inhibitory activities, CR1 probably does not have a major role in preventing complement activation either in plasma or on a cell membrane: the plasma concentrations of soluble CR1 are 1000- to 10,000-fold less than those of C4-bp and factor H, and the rate of lateral diffusion of CR1 in the plasma membrane and its tissue distribution, relative to the characteristics of the glycolipid-anchored DAF, may be too limited. Rather, its cofactor function may be important for releasing receptor-bound immune complexes from erythrocytes (Medof *et al.*, 1982), and possibly in the generation of the C3dg ligand for CR2.

TABLE IV
COMPARISON OF REGULATORY FUNCTIONS OF CR1, H, C4-bp, AND DAF

	Inhibition of classical and alternative pathway C3 convertase (M)[a]				
	Fluid-phase enzyme		Particle-bound enzyme		
	C4b,2a	C3b,Bb	C4b,2a	C3b,Bb	C3b,Bb-desialated
CR1	2×10^{-9}	1×10^{-8}	5×10^{-11}	1.7×10^{-11}	1.7×10^{-11}
H	$>2 \times 10^{-6}$	1×10^{-8}	–	2×10^{-10}	7×10^{-10}
C4-bp	2.6×10^{-9}	$>1 \times 10^{-6}$	1×10^{-9}	–	–
DAF	7.5×10^{-9}	4×10^{-9}	–	–	–

[a]Data summarized from Fearon (1979); Iida and Nussenzweig (1981); and Seya *et al.* (1985).

3. *The Erythrocyte–CR1 Immune Complex Clearance System*

Although relatively fewer CR1 molecules are present on erythrocytes than on peripheral blood neutrophils, monocytes, and B lymphocytes, the predominance of erythrocytes in blood makes this cell type the major locus of intravascular CR1. A special function for erythrocyte–CR1 was found in experiments that traced the clearance of model immune complexes injected into the ascending aorta in baboons and rhesus monkeys (Cornacoff *et al.*, 1983). Within seconds, the majority of the injected immune complexes became bound to erythrocytes in a complement-dependent reaction. The complexes remained erythrocyte bound until the cells traversed the portal circulation, when the radiolabeled complexes were found in the liver rather than on circulating erythrocytes. Thus, either complexes were removed from the cells, perhaps by CR1-dependent cleavage of the C3b on the complexes to C3dg, or those cells bearing large numbers of complexes were removed from the circulation by adherence to reticuloendothelial cells. In either event, the critical functional consequence of uptake of the immune complexes by CR1 on erythrocytes was the prevention of nonspecific trapping of complexes elsewhere by diffusion into tissues. A primary role for erythrocyte–CR1 in this clearance reaction also has been shown by studies in man in which the uptake of model tetanus–antitetanus complexes by erythrocytes directly correlated with the number of CR1 molecules per erythrocyte among different individuals, and inversely with the percentage of complexes trapped in sites other than the liver (Schifferli *et al.*, 1988).

4. *The Endocytic Function of CR1*

One of the earliest functions of CR1 to be recognized was that of participating in the phagocytosis of C3b-coated particles by neutrophils and monocytes (Gigli and Nelson, 1968). This function of CR1 occurs in two guises: enhancement of the Fc receptor-mediated phagocytosis of particles bearing C3b and IgG (Mantovani *et al.*, 1972), and direct phagocytosis of the C3b-bearing particle by CR1 independent of Fc receptors. Enhancement of phagocytosis involves the capacity of CR1 on the phagocyte to promote adherence of the C3b-coated target, a process for which the multivalent CR1 may be especially suited. Also, capping of CR1 induced by cross-linking with anti-CR1 causes cytoskeleton-dependent cocapping of Fc receptors (Jack and Fearon, 1985); thus, ligation of CR1 by target-bound C3b may direct Fc receptors to sites where interaction with target-bound IgG is more likely to occur, especially if the C3b is covalently attached to the IgG (Fries *et al.*, 1987), as may occur under physiologic circumstances. This function of CR1 may reflect

its capacity to interact with the microfilamentous network of myelomonocytic cell types (Jack *et al.*, 1986). Ligation of CR1 also may transmit a signal to the cell that alters the capacity of other receptors to mediate phagocytosis.

Direct phagocytosis by CR1, in the absence of other ligand-receptor interactions, apparently does not occur with "resting" neutrophils or monocytes. However, treatment of these cells with T cell-derived cytokines, with intercellular matrix proteins, such as laminin, serum amyloid P component, and fibronectin, or with the chemotactic peptide f-MLP alters CR1 function so that ingestion of C3b-coated particles may occur (Griffin and Griffin, 1979; Wright and Silverstein, 1982; Wright *et al.*, 1983; Pommier *et al.*, 1983; Bohnsack *et al.*, 1985). The biochemical basis for this response has been studied with the use of protein kinase C-activating phorbol esters. Treatment of neutrophils with phorbol esters increases CR1 expression by translocating intracellular receptor to the plasma membrane (Changelian *et al.*, 1985). At relatively high concentrations of phorbol esters, up-regulation of CR1 is followed by ligand-independent internalization (Changelian *et al.*, 1985; O'Shea *et al.*, 1985b). Based on these effects of phorbol esters, and the observation that they also induce phosphorylation of CR1 in neutrophils, monocytes, and eosinophils, which have no detectable constitutive phosphorylation of the receptor, it has been suggested that the activated state of CR1 for phagocytosis may correlate with its phosphorylation (Changelian and Fearon, 1986). In this regard, it is interesting that activation of protein kinase C in erythrocytes and B lymphocytes, two nonphagocytic cell types, does not induce phosphorylation of CR1 (Changelian and Fearon, 1986). It is not known whether the cytoplasmic domain of CR1 differs among these cell types, although cDNA clones from tonsillar and neutrophil libraries encoding this portion of the receptor have identical sequences (T. Bartow, L. B. Klickstein, and D. T. Fearon, unpublished observation).

Soluble, highly multivalent ligands are capable of inducing endocytosis of CR1 by neutrophils and monocytes through clathrin-coated pits in the absence of cellular activation (Abrahamson and Fearon, 1983; Fearon *et al.*, 1981). However, the extent of cross-linking of receptors required for this adsorptive endocytic reaction has not been quantitatively addressed, and some studies have observed that endocytosis of soluble ligand by CR1 is modest, even when multivalent attachment has occurred (Mehta *et al.*, 1986).

Endocytosis through CR1, whether of soluble ligand or of particles, is not associated with rises in free intracellular calcium concentration, granule secretion, or a respiratory burst. The metabolic fate of natural

ligand internalized via this receptor has not been studied, although anti-CR1/ferritin conjugates were observed to enter lysosomal structures in neutrophils and monocytes (Abrahamson and Fearon, 1983). Thus, it is possible that internalization of microorganisms by this route may not be an effective mode of intracellular killing. This consideration may be relevant to the finding that *Leishmania*, an intracellular pathogen for macrophages, exhibits the highest rate of intracellular survival when it utilizes the alternative pathway to become coated with C3b and CR1 on human macrophages for gaining entry into the cells without triggering the generation of H_2O_2 (Mosser and Edelson, 1987; da Silva *et al.*, 1988).

5. Immunoregulatory Function of CR1

Despite the presence of CR1 on all mature B cells, some T cells, and several types of cells having antigen-presenting capabilities, the function of this receptor in the afferent phase of the immune response has not been as well assessed as has its role in other cellular reactions. A technical difficulty is that the natural ligand, C3b, is subject to proteolytic processing to iC3b and C3dg, which interact with CR2 and CR3. Thus, studies in which depletion of C3 in mice impaired the generation of memory B lymphocytes (Klaus and Humphrey, 1977), although indicative of an important role for complement in the immune response, did not identify the receptors involved in the C3-dependent event. The impaired secondary humoral immune response to a T cell-dependent antigen in C4- and C2-deficient guinea pigs (Bottger *et al.*, 1985), respectively, and in a single C4-deficient patient with systemic lupus (Jackson *et al.*, 1979), also suggest a function for products of C3 activation in the immune response, but do not begin to provide a molecular explanation. Therefore, only those studies that have employed anti-CR1 as a ligand will be reviewed.

Cross-linking CR1 does not increase the intracellular concentration of calcium (Carter *et al.*, 1988) or induce proliferation or differentiation of B cells (Tedder *et al.*, 1986). However, cross-linking the receptor with monoclonal anti-CR1 and goat antimouse antibody does induce the formation of caps; CR1 also cocaps with cross-linked membrane immunoglobulin when bound by monoclonal anti-CR1 alone (Tsokos *et al.*, 1988). In an assay of the production of immunoglobulin by lymphocytes stimulated with pokeweed mitogen, the addition of monospecific rabbit $F(ab')_2$ anti-CR1 augmented IgM, IgG, and IgA synthesis in a 6-day culture of peripheral blood B and T cells (Daha *et al.*, 1984). The effect could be reduced by the simultaneous addition of purified CR1, which neutralized the antibody. The augmenting effect of the

anti-CR1 was greater when added to B cells having relatively high rather than low numbers of CR1, and when $F(ab')_2$ rather than Fab' anti-CR1 was used. No effect was observed when optimal doses of pokeweed mitogen were employed in this or another study in which monoclonal anti-CR1 was added to the lymphocyte culture (Tedder *et al.*, 1986).

Another study examining possible functions of CR1 on B cells reported that the production of anti-TNP by human peripheral blood B cells in the presence of T cells or T cell-derived soluble factors and a sub-optimal dose of TNP–polyacrylamide beads was enhanced twofold to fourfold by low concentrations of either of two monoclonal anti-CR1 antibodies (Weiss *et al.*, 1987). No effect of these antibodies was observed when an optimal amount of TNP–polyacrylamide was used, when saturating concentrations of anti-CR1 were present, or when T cells or their soluble factors were absent.

These studies suggest that CR1 has a role in the differentiation of B cells into plasma cells, but the inherent complexity of B cell differentiation and the use of unfractionated supernatants of activated T cells make difficult a molecular explanation of these effects. It is difficult to determine whether the two studies are assessing the same effect of CR1, or whether even the same cell types bearing CR1 are responsible for the apparently common effects. For example, in the former study, the enhancing effect of anti-CR1 was shown to correlate with the presence of CR1 on B cells, suggesting that this cell type was responding to antibody, whereas in the latter study the possibility was not excluded that another cell type expressing CR1, such as the monocyte which expresses CR1, may have been responsible for the enhancing effect. Further evaluation of this function of B cell CR1 will require the use of highly purified cells and interleukins.

III. Complement Receptor Type 2

A. Structure of CR2

The primary protein structure of CR2 has been determined through analysis of the nucleotide sequences of clones isolated from human tonsillar (Weis *et al.*, 1988) and Raji lymphoblastoid cell line (Moore *et al.*, 1987) cDNA libraries. Four of the five clones obtained from the tonsillar library encode a protein composed of 1032 amino acids with a M_r of 112,716, consistent with an estimated M_r of 111,000 for CR2 synthesized by lymphoblastoid cells in the presence of tunicamycin (Weis and Fearon, 1985). These clones encode a 20-amino acid signal peptide, a 954-residue extracellular domain, a 24-amino acid transmembrane

region, and a 34-amino acid cytoplasmic domain. The extracellular domain is composed entirely of 15 tandem short consensus repeat sequences which are homologous to those described in CR1 and other C3/C4-binding proteins, as well as in some membrane proteins which do not share this function (see CR1 structure). Each SCR of CR2 contains from 57 to 74 amino acids, including one glycine, one tryptophan, and four cysteine residues which are invariant and characteristic of this family of SCR-containing transmembrane proteins. One of the five clones isolated from the tonsillar library and the single clone obtained from Raji cells encoded a sixteenth SCR (10a) composed of 59 amino acids inserted between SCRs 10 and 11 of the 15-SCR form of CR2. It is not clear whether this is an allelic polymorphism, a product of alternative splicing, or both. Northern analysis of mRNA from both SB and Raji lymphoblastoid cell lines with a CR2-specific probe detects a single band of approximately 5 kb; however, RNase protection assays are able to distinguish these two forms (Kalli and Fearon, unpublished observations). The CR2 coding sequence spans 25 kb within the RCA gene cluster located on chromosome 1, band q32 (Weiss *et al.*, 1987; Weis *et al.*, 1988; Fujisaku *et al.*, 1989) (see Section II,B).

Earlier studies had determined that posttranslational modifications of CR2 included both glycosylation (Weis and Fearon, 1985) and probable serine/threonine phosphorylation (Changelian and Fearon, 1986). Subsequent sequence analysis confirmed the presence of 11 Asn-X-Ser/Thr sites for potential glycosylation within the extracellular domain of the 15-SCR form, and another two sites within SCR 10a, consistent with the 8–11 N-linked oligosaccharides predicted from biosynthetic studies which compared high-mannose-containing precursor and nonglycosylated forms of CR2 (Weis and Fearon, 1985). The cytoplasmic domain contains three serine, two threonine, and four tyrosine residues, including the sequence -TSQK- (Fig. 3). This site, in which a basic amino acid is carboxy terminal to the serine/threonine, is the best candidate for a protein kinase C substrate (Kishimoto *et al.*, 1985). Although the cytoplasmic domain of CR2 is too small to encode a tyrosine kinase, its potential to serve as a substrate for tyrosine phosphorylation is suggested by the presence of the sequence -EAREVY- in which a tyrosine is carboxy terminal to two acidic residues, a pattern that resembles the consensus sequence for tyrosine kinase substrates (Hunter and Cooper, 1985). A potential function for these tyrosines might also be predicted because CR1, the receptor that most resembles CR2 in the extracytoplasmic regions and which is 35% identical to CR2 in the overlapping portions of their cytoplasmic domains, has no tyrosines in its 43-amino acid cytoplasmic domain (Fig. 3) (Klickstein *et al.*, 1987).

There are no LHRs within CR2 as there are in CR1, however, dot matrix analysis reveals a less conserved repeating pattern of homology involving every fifth SCR, such that 1, 5, 9, and 12 are homologous, as are 2, 6, 10, 13; 3, 7, 10a, 14, and 4, 8, 11. SCR 15 is unique and not part of this repeating pattern. Based upon the shared SCR motif, the tertiary structure of CR2 would be expected to consist of an extended array of triple loops maintained by disulfide bonds, similar to that predicted for CR1 (Fig. 1).

B. Tissue Distribution and Biosynthesis of CR2

Human CR2 has been found on mature B lymphocytes and some B lymphoblastoid cell lines (Ross *et al.*, 1973; Eden *et al.*, 1973; Iida *et al.*, 1983; Weis *et al.*, 1984; Nadler *et al.*, 1981; Tedder *et al.*, 1984), on human thymocytes (Tsoukas and Lambris, 1988), on rare T lymphoblastoid cell lines such as Molt-4, Jurkat, and HPB-ALL (Menezes *et al.*, 1977; Tatsumi *et al.*, 1985; Fingeroth *et al.*, 1988), on pharyngeal (Young *et al.*, 1986) and cervical (Sixbey *et al.*, 1987) epithelium, and on follicular dendritic cells (Reynes *et al.*, 1985). Quantitation of CR2 expression on various cell types has determined that the Burkitt B lymphoma cell lines JY, SB, and Raji express 24,000–63,000 receptors per cell as compared with the T cell leukemia line Molt-4, which expresses 8000 per cell (Fingeroth *et al.*, 1984).

A C3d-binding glycoprotein with M_r 72,000 that may be related to CR2 was isolated from a Raji cell culture supernatant (Lambris *et al.*, 1981). Polyclonal rabbit antiserum raised against this purified 72-kDa C3d-binding protein immunoprecipitated a molecule of this size from cells that were either surface iodinated or biosynthetically labeled with [^{3}H]leucine, but did not precipitate a membrane protein resembling CR2. This antiserum bound only to immunoglobulin-positive B lymphocytes and not to T cells, and blocked the formation of rosettes between B cells and erythrocytes bearing iC3b or C3d, a CR2-dependent function.

There is one report of low CR2 expression on human platelets as detected by specific binding of the anti-CR2 monoclonal antibodies, HB-5 and OKB-7, and immunoprecipitation of a 140-kDa platelet component using polyclonal anti-CR2 (Nunez *et al.*, 1987). Other investigators did not detect specific binding of HB-5 to platelets, which did, however, bind C3dg via CR4 (Vik and Fearon, 1987), and CR2 was not detected in the eluate of iC3b-Sepharose that had been preincubated with a detergent-solubilized platelet preparation (Yu *et al.*, 1986).

Ontogenic analysis of CR2 expression during human B cell differentiation, using the anti-CR2 monoclonal antibody HB-5 (Tedder *et al.*, 1984), demonstrated that pre-B and immature B cells from fetal bone

marrow and liver did not express CR2, although 25% of B cells in fetal spleen were CR2 positive. Approximately half of the B cells in adult bone marrow expressed CR2, as did 76 and 95% of newborn and adult peripheral blood B cells, respectively, and 72-97% of the B cells in adult spleen, tonsil, and lymph node. Plasma cells rarely expressed CR2, although plasma cells were derived from the HB-5-positive population. Thus, CR2 expression is most characteristic of the mature B lymphocyte, although in contrast to the normal human B cell lineage, EBV-transformed Burkitt B lymphoma cell lines arrested at the pre-B cell stage express CR2 (Cohen *et al.*, 1987).

The expression of CR2 on human epithelium is also regulated during cellular differentiation, as demonstrated by indirect immunofluorescence staining with the anti-CR2 monoclonal antibodies HB-5 and B2 (Young *et al.*, 1986). Primary cultures of ectocervical epithelial cells contained from 1 to 9% CR2-positive cells (Sixbey *et al.*, 1986), and ultrastructural studies indicated that these were the metabolically active, less differentiated cells. Similarly, stratified squamous epithelia from both human tongue and ectocervix expressed CR2 in the less differentiated basal and parabasal layers, with no detectable membrane fluorescence on the most superficial layers (Young *et al.*, 1986; Sixbey *et al.*, 1987). In contrast, epithelia from the soft palate, oropharynx, inner cheek, and fossa of Rosenmuller (where nasopharyngeal carcinoma is thought to arise) were shown to express CR2 in all strata, from the most basal and undifferentiated to the most superficial and differentiated cells. Expression of CR2 appeared to be greater in stratified squamous tissue from several anatomical sites than in the pseudostratified columnar epithelium of nasal and tracheal sites in which only basal cells were positive.

Studies of CR2 biosynthesis performed in the SB lymphoblastoid cell line have characterized three forms of the receptor: an unglycosylated form, a glycosylated precursor form, and a mature glycosylated form (Weis and Fearon, 1985). Pulse-chase experiments of CR2 biosynthetically labeled with [^{35}S]methionine demonstrated the presence of a precursor of M_r 134,000 containing high-mannose N-linked oligosaccharides sensitive to endoglycosidase H, which was processed, presumably in the Golgi, to the mature M_r 145,000 form of CR2 containing complex-type N-linked oligosaccharides that could be released by endoglycosidase F. In the presence of tunicamycin, a nonglycosylated polypeptide of M_r 111,000 was synthesized containing no N- or O-linked carbohydrate. Although glycosylation was not required for expression of the receptor at the plasma membrane, CR2 lacking N-linked oligosaccharides underwent accelerated catabolism such that its half-life was 2.8 hours as

compared with 13.8 hours for normally glycosylated CR2. Glycosylation was not required for receptor–ligand interaction, because all three forms of CR2 were capable of binding to C3-Sepharose.

C. Functions of CR2

The functions of CR2 have been more difficult to define fully than has its structure. The experimental evidence to date suggests that this single transmembrane glycoprotein may have several important immunoregulatory roles in addition to being a primary determinant of the narrow tissue tropism of Epstein–Barr virus.

1. *Immunoregulation*

CR2 binds to the d fragment of C3, which is most accessible on iC3b, C3dg, and C3d, such that the rank order of binding of Raji or normal B lymphocytes to erythrocytes coated with C3 intermediates is C3d = iC3b > C3b (Weis *et al.*, 1984). Binding studies have shown that CR2 recognizes a M_r 8600 CNBr fragment of C3d which represents residues 1199–1274 of the C3 cDNA sequence (Lambris *et al.*, 1985). Further localization of the C3d-binding domain was achieved with the use of a series of overlapping synthetic peptides which spanned this region. The peptides which bound to CR2 shared the sequence -LYNVEA-. These residues are contained within a particularly hydrophilic span of amino acids, from 1209–1236, which by secondary structure analysis (Chou and Fasman, 1974) is predicted to contain a strong β turn.

The covalent attachment of these terminal C3 fragments to activators of complement, such as immune complexes and bacterial particles, suggests that CR2 functions to enable B cells to bind C3–antigen complexes at sites distant from inflammation. There is evidence to suggest that B cells may have a major role in presenting antigen to T cells *in vivo* (reviewed in Ashwell, 1988), and that this function is enhanced by the uptake of antigen by membrane immunoglobulin. The presence of CR2 raises the possibility that antigen which is covalently bound to C3 intermediates also could be taken up by B lymphocytes for presentation to T cells.

There is also evidence to suggest that cross-linking CR2 provides a T cell- and antigen-independent stimulus for B cell proliferation. Lipopolysaccharide-activated murine spleen cells, enriched for B cell blasts, were stimulated to proliferate and mature into immunoglobulin-secreting cells after incubation with Sepharose-bound or glutaraldehyde-cross-linked human C3b or 3d, as measured by [^{3}H]thymidine incorporation and formation of IgM plaque-forming cells (Erdei *et al.*, 1985; Melchers *et al.*, 1985). There was no response to soluble C3, which was

shown actually to block this effect, and resting spleen cells were refractory to stimulation. The observation was also made that C3dg-coated microspheres as well as monomeric C3dg had no mitogenic effect upon unseparated human T and B cells (Nemerow *et al.*, 1985a). However, stimulation of these same cell suspensions with anti-CR2 monoclonal antibody resulted in a 50-fold to 200-fold dose-dependent stimulation of DNA synthesis. The greatest effects were produced with either OKB-7 (IgG2a) (Mittler *et al.*, 1983) or AB-1 (IgG_{2a}) (Wilson *et al.*, 1985). OKB-7 recognizes an epitope at or very near the C3d-binding site on CR2, as indicated by its unique ability among anti-CR2 monoclonal antibodies to block rosetting of human tonsillar B cells with erythrocytes bearing C3d without the addition of a second antibody (Rao *et al.*, 1985). A slight effect was observed with B2, while there was no effect with HB-5 (Tedder *et al.*, 1984). Each of these two antibodies is able to block C3d rosetting only in the presence of a second antibody (Iida *et al.*, 1983; Weis *et al.*, 1984). These effects were shown to be T cell dependent and were observed when either intact OKB-7 IgG or $F(ab')_2$ fragments were used. However, experiments performed with subsequent preparations of OKB-7 failed to reproduce these findings (Cooper *et al.*, 1988). Similar results were obtained by Wilson *et al.*, who observed T cell-dependent stimulation of DNA synthesis in human peripheral blood B cells which were cultured in the presence of anti-CR2 MAbs AB1, AB2, AB3, AB5, or B2 (Wilson *et al.*, 1985). This response was seen with addition of as little as 1% irradiated T cells, suggesting that a soluble factor was responsible. Alternatively, the anti-CR2 MAb AB5 was shown by others to inhibit B cell responsiveness to the T cell-derived lymphokine B cell growth factor in a dose-dependent manner (Perri *et al.*, 1986).

Four other anti-CR2 MAbs, IC8, IF8, 2G7, and 6F7, recognize three distinct epitopes (IF8 and 2G7 recognize the same site) on the same M_r 28,000 trypsin-generated fragment of CR2, yet none of them is capable of inhibiting EC3d rosette formation with Raji cells in the absence of a second antibody (Petzer *et al.*, 1988). All four, however, induced B cell proliferation in the presence of a T cell-derived "BCGF-like" factor. Rabbit polyclonal $F(ab')_2$ anti-CR2 added to cultures of human B cells also enhanced the stimulatory effect of partially purified supernatants from cultured T cells (Frade *et al.*, 1985c). However, intact polyclonal anti-CR2 IgG was inhibitory in the same assay. Tedder *et al.* found no effect of $F(ab')_2$ HB-5, HB-5 coupled to Sepharose beads, or soluble C3dg upon T cell-dependent B cell proliferation or differentiation (Tedder *et al.*, 1986). Furthermore, these reagents did not enhance anti-IgM-induced B cell proliferation, nor did cross-linking of CR2 with HB-5 and second antibody. Recently, C3dg has been shown to have a

dose-dependent stimulatory effect upon low-density cultures of Raji cells grown in a serum-free defined medium (Hatzfeld *et al.*, 1988). Native C3 and monomeric C3dg, but not C3c, were observed to induce Raji cells to proliferate but had no effect upon the T lymphoblastoid cell line JM or the monocytic cell line U937, which are CR2 negative. This mitogenic effect could be blocked with either $F(ab')_2$ anti-C3d or OKB-7, but not with anti-C3c. This CR2-mediated stimulation of Raji cell growth in serum-free medium is triggered not only by C3dg but also by the monoclonal antibodies 1C8, 2G7, and 6F7, which recognize CR2 epitopes distinct from the C3dg binding site (Pernegger *et al.*, 1988).

Whereas the T cell-dependent signal for B cell proliferation transduced by CR2 is antigen independent, there are data to suggest that CR2 is also capable of mediating an antigen-specific signal for B cell proliferation. Carter *et al.* have determined that there is a synergistic interaction between CR2 and membrane IgM on B lymphocytes (Carter *et al.*, 1988). In these studies (Fig. 6), tonsillar B cells were loaded with the calcium-sensitive fluorescent probe indo-1, and were stimulated by cross-linking

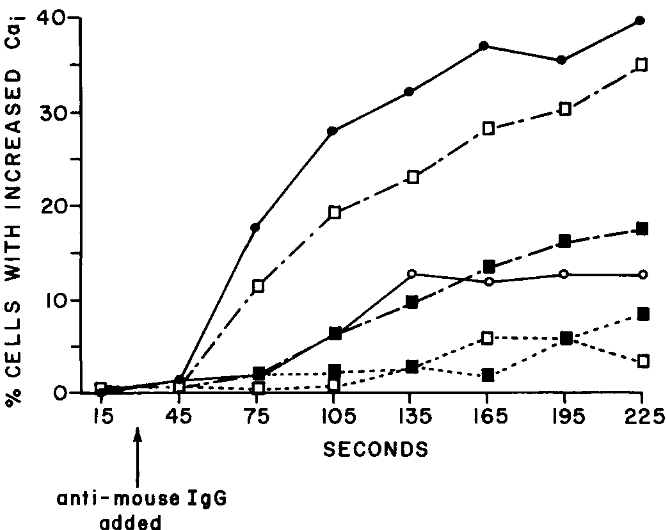


FIG. 6. Synergistic interaction between CR2 and membrane IgM on B lymphocytes. The percentage of B cell-enriched tonsil cells with elevated intracellular calcium was determined at 30-second intervals after addition of goat antimouse IgG to cells preincubated with Yz-1 anti-CR1, HB-5 anti-CR2, or DA4.4 antihuman IgM alone or in various combinations; 2.0 μg/ml HB-5 (□----□); 2.0 μg/ml Yz-1 (■----■); 0.06 μg/ml DA4.4 (○—○); 5.0 μg/ml DA4.4 (●—●); 2.0 μg/ml HB5 + 0.06 μg/ml DA4.4 (□-·-□); or 2.0 μg/ml Yz-1 + 0.06 μg/ml DA4.4 (■-·-■) (Carter *et al.*, 1988).

membrane IgM, CR1, or CR2 with MAbs DA4.4, Yz-1, or HB-5, respectively, followed by goat antimouse IgG. There was a direct dose-dependent response between the proportion of cells with increased free intracellular calcium concentration (Ca_i) and the amount of cell-bound Fab′ DA4.4. No such effect was observed after cross-linking CR1 or CR2 alone. However, when suboptimal doses of DA4.4 were used, addition of HB-5 but not Yz-1 was shown to have a synergistic effect, resulting in Ca_i equivalent to that obtained with optimal doses of DA4.4 alone. There was also a dose-related synergistic effect upon B cell proliferation produced with Sepharose coupled to HB-5 in the presence of suboptimal concentrations of DA4.4; substitution of Yz-1 anti-CR1 for HB-5 abolished the synergistic response. Of significance in this regard, Tanner *et al.* have demonstrated that CR2 is capable of cocapping with membrane Ig (Tanner *et al.*, 1987). Thus, cross-linking of CR2 and membrane IgM by complexes which contain cleavage fragments of C3 bound to antigen could enable B cells to respond to otherwise substimulatory doses of antigen.

CR2 has also been shown to interact independently of membrane IgM to prime B cells for subsequent stimulation through the antigen receptor (Carter and Fearon, unpublished observations). In these studies, preculture of tonsillar B cells with polymeric but not monomeric C3dg caused a dose-dependent enhancement of proliferation and accelerated entry into S phase after addition of anti-IgM. Polymeric C3dg alone did not induce proliferation, and its continued presence during stimulation by anti-IgM was not required. Aggregated C3dg, latex-bound C3dg, OKB-7, and UV-inactivated EBV, but not HB-5 or monomeric C3dg, have been shown to synergize with PMA and enhance the transition of tonsillar B cells from G1 to the S phase, although those reagents do not induce B cells to enter the cell cycle or synergize with low molecular weight B cell growth factor in triggering B cell mitogenesis (Bohnsack and Cooper, 1988).

The differential functional properties of CR1 and CR2 despite their homologous relationship may be related to an earlier observation that protein kinase C activation through stimulation with phorbol ester results in phosphorylation of B cell CR2 but not CR1 (Changelian and Fearon, 1986). Thus, serine/threonine phosphorylation of CR2 secondary to kinase C activation by membrane IgM may account for the acquisition of B cell-activating function by the receptor. Other studies of the phosphorylation of CR2 have been performed with a cell-free system and CR2 isolated from Raji cells (Delcayre *et al.*, 1987). It was determined that CR2 could be phosphorylated in a cell-free system in which the phosphorylated form of CR2 (1) contained both phosphoserine and

phosphotyrosine, (2) could be found in Raji nuclei, and (3) controlled the phosphorylation of a 120-kDa nucleoprotein with which it could be coprecipitated. The relationship of these events to the cell biology of CR2 is not known.

Follicular dendritic cells are nonphagocytic accessory cells found in B cell-enriched germinal centers of peripheral lymphoid tissue. They are characterized by extensive cytoplasmic processes and are unique in their expression of CR1, CR2, and CR3 (Reynes *et al.*, 1985). The density of CR2 as detected by optical and electron immunohistochemistry is greater on the plasma membranes and cytoplasmic extensions of follicular dendritic cells found in both tonsil and lymph nodes than on peripheral blood B cells (Reynes *et al.*, 1985). Follicular dendritic cells retain antigen on the cell surface for prolonged periods of time in an antibody- and C3-dependent manner (Klaus *et al.*, 1980), and this process of antigen localization is thought to be critical to the generation and maintenance of memory B cells. Therefore, through expression of CR2 (in addition to CR1 and CR3), these accessory cells may be capable of binding to antigen which is coupled with any of the C3 cleavage fragments.

A role for CR2 in the activation of the alternative complement pathway was suggested by the observation that B lymphoblastoid cells, such as Raji, were lysed when exposed to human serum or to purified components of the alternative pathway, and the capacity to activate complement correlated with CR2 expression (Theofilopoulos *et al.*, 1974; Baker *et al.*, 1977; Schreiber *et al.*, 1980; Ramos *et al.*, 1985). Subsequent studies using purified components of the alternative pathway demonstrated that CR2 from Raji cells was a strong activator of the alternative pathway (Mold *et al.*, 1988). CR2 bound C3b both covalently and noncovalently during activation of the alternative pathway, and this interaction could not be blocked by preincubation with OKB-7, indicating that it occurred at a site distinct from the C3dg-binding site. The biologic significance of complement activation by CR2 on lymphoblastoid cells, including the CR2-positive T lymphoblastoid cell line Molt-4, has yet to be determined, although normal peripheral blood B lymphocytes do not (Budzko *et al.*, 1976; Praz and Lesavre, 1983). Whether these differential activating properties reflect structural differences between receptors among these cell types is not known.

CR2 has been shown also to regulate the complement cascade by serving as a cofactor for factor I-mediated cleavage of membrane-bound iC3b into C3dg and C3c (Mitomo *et al.*, 1987), but not of C3b to iC3b (Weis *et al.*, 1986). As this cofactor activity was blocked by preincubation with OKB-7, it was concluded that CR2 rather than CR1 was responsible for the effect. The biologic consequences of iC3b to C3dg conversion

would be to exclude reactivity of C3-bearing complexes with cells expressing CR3, such as phagocytes.

2. *Determination of Epstein–Barr Virus Tropism*

Epstein-Barr virus (EBV) is an oncogenic herpesvirus which causes acute infectious mononucleosis and is implicated in the pathogenesis of Burkitt's lymphoma and nasopharyngeal carcinoma. Jondal *et al.* first noted a correlation between the presence of receptors for EBV and those for C3d on B lymphocytes (Jondal *et al.*, 1976), and several subsequent observations led to the conclusion that CR2 is the receptor for both C3d and EBV. First, treatment of peripheral blood B lymphocytes with HB-5 anti-CR2 followed by goat antimouse IgG (Fingeroth *et al.*, 1984), or with OKB-7 alone (Nemerow *et al.*, 1985b), or with rabbit polyclonal anti-CR2 (Frade *et al.*, 1985a), inhibited binding of both C3d-coated sheep erythrocytes and EBV. Second, complexes of CR2/HB-5/staphylococcal protein A bound both C3d and EBV. Third, the rank order for binding of EBV to four lymphoblastoid cell lines was identical to that for binding of HB-5 (Fingeroth *et al.*, 1984). Fourth, CR2 purified from detergent extracts of Raji cells could be reconstituted into lipid membranes with maintenance of ability to bind both C3d and EBV (Mold *et al.*, 1986). Fifth, the tissue distribution of CR2 correlates with the tissue tropism of EBV:B lymphocytes and certain epithelial cells (Sixbey *et al.*, 1987).

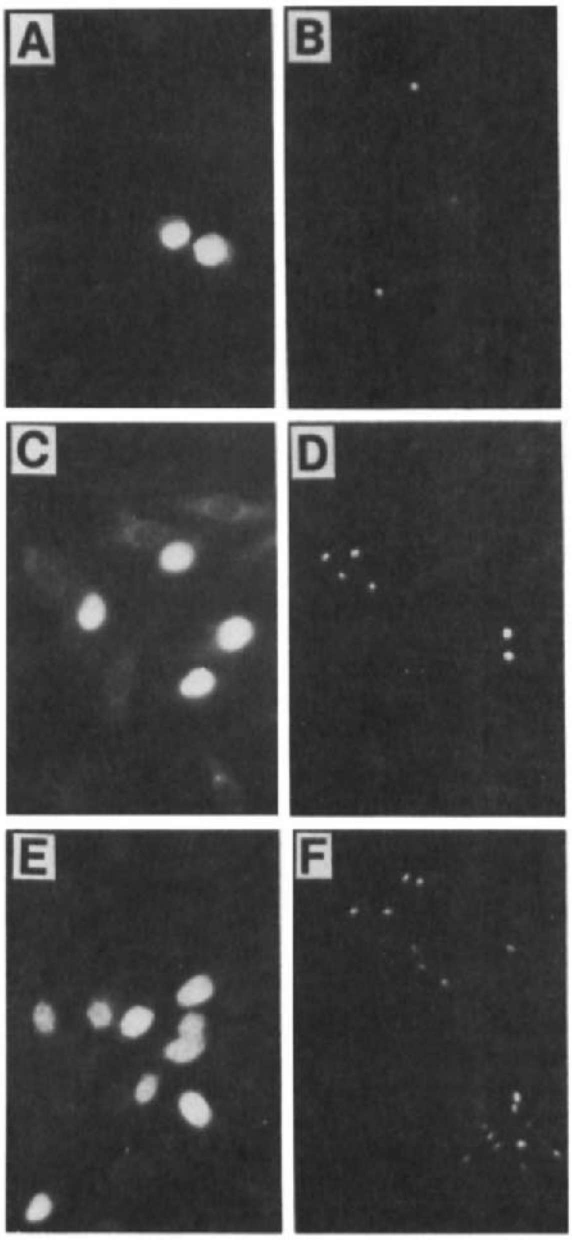
The full role of CR2 in EBV infection has not been determined, but it certainly includes the cellular uptake of the virus. Characterization of the early events of virus internalization by B lymphocytes has shown that at 4°C, 92% of virions remained on the cell surface, whereas at 37°C virions were internalized within 2–5 minutes, presumably a consequence of CR2 internalization (Nemerow and Cooper, 1984). After 15 minutes, 50% were found in 300- to 500-nm thin-walled vesicles, and after 60–90 minutes the viral nucleocapsids were found near the cell nucleus. In contrast to these observations with normal B cells, virus internalization with Raji B lymphoblastoid cells occurred through direct fusion of receptor-bound virus with the outer cell membrane, as CR2 probably does not enter these cells (Tedder *et al.*, 1986). These alternative modes of virus internalization, endocytosis versus membrane fusion, may relate to differences in membrane fluidity and cytoskeletal variability among normal and malignant B cells.

The EBV glycoprotein gp350 has been shown to mediate binding to CR2 (Nemerow *et al.*, 1987; Tanner *et al.*, 1987), and a comparison of the gp350/220 and C3d amino acid sequences has identified three regions of homology. One of these, which resides at the N-terminus of gp350/220

(EDPGFFNVE), corresponds to the region of C3d (EDPGKQLYNVE) that was predicted to bind CR2 (Lambris *et al.*, 1985). Soluble gp350/220 deletion mutants (Tanner *et al.*, 1988) and synthetic peptides (Nemerow *et al.*, 1989) have recently been employed to demonstrate that this is the primary CR2 binding site for EBV.

Despite the sequence homology between gp350/220 and C3d and the capacity of the monoclonal OKB-7 to block binding by B cells of C3d and EBV (Rao *et al.*, 1985; Nemerow *et al.*, 1985b), there is reason to suspect that these two ligands interact with distinct sites on CR2. Barel *et al.* have recently described a panel of nine IgG1 MAbs, MR.1-MR.9, of which four preferentially blocked 18-64% of EBV binding to Raji cells but had no effect upon C3d binding, and four others blocked 58-90% of C3d binding but did not affect EBV binding (Barel *et al.*, 1988). Analysis of the ligand-binding sites of CR2 by deletion mutagenesis, as has been accomplished for CR1, will provide more definitive evidence for the location of these sites (see note added in proof).

Although these studies have established that CR2 is capable of specifically binding EBV, they did not determine whether cellular expression of CR2 was sufficient for infection. For example, certain cell lines that express CR2, such as Molt-4, bind EBV but are not infected by the virus (Menezes *et al.*, 1977). The isolation of cDNA clones encoding human CR2 has recently enabled a direct assessment of whether this molecule may alone be sufficient to permit infection of any cell type that is normally not within the host range of the virus. Murine L cells were stably transfected with pMT.CR2.neo.1, a eukaryotic expression construct containing a full-length human CR2 cDNA encoding the 15-SCR form of the receptor downstream from a mouse metallothionein I promoter (Ahearn *et al.*, 1988). The transfectants expressing high levels of CR2 having an M_r identical to wild-type receptor formed rosettes specifically with erythrocytes coated with iC3b or C3d and bound EBV. Coculture of these cells with EBV for 60 hours led to infection of approximately 0.5% of the cells, as assessed by staining for Epstein-Barr nuclear antigen (EBNA) (Fig. 7). No fluorescent staining of cells was observed with monoclonal antibodies to the early antigen complex or to gp350/220, indicating that the infection was latent. Further support for latency was the observation that although the number of infected foci remained constant in a particular culture over time, the number of EBNA-positive cells per focus approximately doubled with each cell division of the L cells. The finding that expression of recombinant human CR2 renders murine L cells susceptible to stable, latent infection by EBV indicates that this single membrane protein can serve as one of the determinants of the narrow tissue tropism of this virus.


A
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F

The low efficiency of infection observed in CR2-positive L cells as compared with the B lymphoblastoid Ramos cell indicates that additional factors are involved in determining EBV tropism. Furthermore, the T lymphoblastoid cell line Molt-4 expresses approximately 8000 CR2 molecules per cell (Fingeroth *et al.*, 1984) and binds EBV, but cannot be infected. Although this level of receptor expression is lower than that of normal B cells, B lymphocytes in patients with common varied agammaglobulinemia, having undetectable numbers of CR2, have been infected with EBV (Schwaber *et al.*, 1980). Further, the recent finding of EBV-positive T lymphoma cells in patients with chronic EBV infection (Jones *et al.*, 1988) indicates that at least some T cells can be infected. Thus, CR2 alone may be responsible for the susceptibility of a cell to EBV infection, but other factors such as receptor density, membrane fluidity, cytoskeletal structure, and possibly cell-specific trans-acting transcription factors may modify this function of CR2.

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FIG. 7. Expression of CR2 renders murine fibroblasts susceptible to infection with EBV. The CR2-expressing mouse L cells were cocultured with EBV and were stained for Epstein-Barr nuclear antigen by indirect immunofluorescence. Fluorescence photomicrographs were taken of representative fields at original magnification of 100× (A, C, and E) and 10× (B, D, and F) after 24 hours (A and B), 48 hours (C and D), and 96 hours (E and F) (Ahearn *et al.*, 1988).

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NOTE ADDED IN PROOF. In recent experiments, we have localized the ligand binding sites and monoclonal epitopes in CR2 by expressing CR2 deletion mutants and CR2/CR1 chimeric receptors in COS cells. OKB-7, C3dg, and EBV all bound to the amino terminus of CR2 and required both SCR-1 and SCR-2 to do so. The HB-5 epitope was mapped to SCRs 3 and 4, and the B2 binding site was localized to a fragment containing SCRs 12 and 13 (C. A. Lowell, D. T. Fearon and J. M. Ahearn, unpublished observations).

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ADVANCES IN IMMUNOLOGY, VOL. 46

The Cellular and Subcellular Bases of Immunosenescence

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I. Introduction

The effect on the immune system of increasing age has been clearly documented. Advancing age is accompanied by a decline in most cell-mediated and humoral immune responses. Thus proliferation to mitogenic lectins and alloantigens, the generation of cytolytic effector cells, delayed-type hypersensitivity, and primary and secondary antibody responses are diminished in the aged. This review will not catalogue the responses that have been compared among young and aged individuals, but instead will focus on the cellular and intracellular basis for impaired immunologic activity. The activation of B and T cells, transit through the cell cycle, and differentiation present many points which might be age sensitive. Three possible mechanisms leading to, or contributing to, the decline in immune function will be considered: a shift in lymphocyte subset distribution with age, alterations in suppressor mechanisms, and changes in the maturation of B and T lymphocytes. In addition, the activity of the mucosal immune system will be discussed, as this lymphoid system differs from the peripheral, in that it appears to be resistant to the deleterious effects of aging.

II. B and T Lymphocyte Activation

A. Introduction

The effective generation of a vigorous immune response, be it cellular or humoral, requires the activation of antigen-responsive T and B lymphocytes and their entry into cell cycle with subsequent expression of differentiated function. Cell cycle entry and traverse have been examined in aged human and murine systems to ascertain whether the deficiency of responsiveness which accompanies aging specifically affects one or multiple points in the cycle.

The entry and transit of the cell cycle in T cells is thought to require delivery to the cell surface of several signals, triggering the sequential

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expression of several new proteins. This scheme involves the perturbation of the T cell receptor (TcR), activation of protein kinases, phosphorylation of target proteins, new mRNA synthesis, and protein synthesis, leading to the acquisition of receptors for interleukin 2 (IL-2) and increased expression of the receptor for transferrin. Interleukin 2 is synthesized and secreted by specific subpopulations of T cells. Interaction between IL-2 and its high-affinity receptor and internalization appear necessary to drive T cells from G_{1b} into S phase. The specific function of IL-2 after internalization has not been elucidated. However, it is likely that IL-2 does not operate via the protein kinase C (pkC) pathway or involve the influx of Ca^{2+} (Mills *et al.*, 1985; Larsen *et al.*, 1986), as recent reports indicate that T cell mutants lacking pkC can be triggered to cell cycle with IL-2 (Mills *et al.*, 1985, 1988; Valge *et al.*, 1988). The signal requirements for subsequent cell cycles are less well defined; however, continued stimulation ultimately leads to a loss of high-affinity receptors for IL-2, which are reacquired only upon a secondary challenge (Cantrell and Smith, 1983, 1984). Several of these processes have been examined in aged cells to determine at which site (or sites) the activation sequence is altered by the aging process.

B. Cell Cycle Analysis

Several investigations of the proliferative response have concluded that the diminished aged response is due to a limited number of cells entering cell cycle (Abraham *et al.*, 1977; Callard and Basten, 1977; Sohnle *et al.*, 1982; Gershon *et al.*, 1979). In a recent study of T cell activation by anti-CD3 reagents, a detailed analysis was made of the number of cells entering cell cycle and cell cycle transition, and of the expression of three early activation-associated antigens (Ernst *et al.*, 1989). It was demonstrated that the rate of cell cycle traverse is similar in aged and young populations. Examining the appearance of RL388,[1] IL-2 receptors, and transferrin receptors, an initial increase was observed in both age groups, followed by a rapid augmentation in the mean fluorescence intensity (MFI) of these markers in the young population, while a much smaller increase in the aged population occurred. The maximum change in MFI achieved was the same in the aged and young populations, but this level was attained by a lower percentage of the aged cells. Other cell cycle analyses have determined that aged lymphocytes enter cell cycle in fewer numbers, may exhibit delayed transit times, and undergo fewer repeat cycles relative to similarly stimulated young lymphocytes

[1] An early activation antigen thought to be the murine homologue of the human 4F2 antigen (Bron *et al.*, 1986).

(Tice *et al.*, 1979; Inkeles *et al.*, 1977; Staiano-Coico *et al.*, 1984; Kubbies *et al.*, 1985).

C. Early Activation Events

Both T and B lymphocytes use similar intracellular mechanisms for translocating a membrane event to an intracellular one. Perturbation of the membrane antigen receptor [either membrane IgM (mIgM) or TcR] leads to a rapid activation of phospholipase C, which initiates the hydrolysis of phosphatidylinositol-4,5-bisphosphate. The products of this reaction, diacylglycerol and inositol trisphosphate, in turn activate pkC and trigger the release of intracellular stores of Ca^{2+}. This sequence of events has been the object of intense recent investigation and has been reviewed by numerous investigators (Coggeshall *et al.*, 1986; Chen *et al.*, 1986; Mizuguchi *et al.*, 1986; Nel *et al.*, 1986; Ransom and Cambier, 1986; Ransom *et al.*, 1986; Cambier and Ransom, 1987; Cambier *et al.*, 1987; DeFranco *et al.*, 1987; Isakov *et al.*, 1987).

A second major signaling mechanism, involving the cyclic nucleotides cAMP and cGMP, is also utilized by lymphocytes. DeFranco *et al.* (1987) have described a B cell line, WEHI-233, which is growth inhibited by activation through mIgM, which triggers the phospholipid hydrolysis pathway. Lipopolysaccharide (LPS) is antagonistic to the effects of anti-IgM, blocking the growth inhibition. LPS, in this cell line, apparently activates the Gi protein, which in turn activates adenylate cyclase, suggesting that the cAMP/cGMP pathway is antagonistic in B lymphocytes to the phosphoinositol pathway. Similarly, in T lymphocytes, activation of adenylate cyclase is inhibitory to the pathway in which activation by the mitogen, Con A, induces pkC activation and phospholipid hydrolysis. Thus, although early work focused on the requirement for cAMP or cGMP changes for the successful activation of lymphocytes, recent work has defined the role of cAMP as a down-regulator. cAMP has been demonstrated to inhibit at several levels the activation of B and T cells induced by mitogenic lectins or antireceptor antibodies (Bismuth *et al.*, 1988; Holte *et al.*, 1988; Beckner and Farrar, 1986; Knudsen *et al.*, 1987; Lerner *et al.*, 1988). Phosphoinositide hydrolysis, Ca^{2+} increases, cell volume increases, expression of activation antigens, and transcription of c-*myc* are all sensitive to down-regulation by cAMP. Other, later events must also be cAMP regulatable, because of the sensitivity of proliferation to the late addition of cAMP. Alterations in these early activation events may influence the functional activity of aged lymphocytes, as suggested by several investigations, reviewed in the subsequent sections.

1. *Membrane Composition and Cytoskeleton*

Composition changes in the plasma membrane and the cytoskeleton accompany the aging process in humans and mice (Rivnay *et al.*, 1978, 1983; Rao, 1982; Allalouf *et al.*, 1988). Capping of mitogen and surface immunoglobulin (sIg) receptors has been demonstrated to be altered in aged humans and rodents (Rao, 1982; Rosenberg *et al.*, 1982; Whisler *et al.*, 1985; Gilman *et al.*, 1981), as well as the sensitivity of capping to the microtubule-disrupting agent, colchicine. Studies on ligand-induced decrease in G-actin content have revealed that this process is less pronounced in aged human peripheral blood lymphocytes (PBLs) (Rao, 1986). Rivnay *et al.* (1978, 1983) have examined the effect of altering the viscosity of the plasma membrane on subsequent activation to proliferation. These studies indicate that aging is accompanied by an increase in the viscosity of the plasma membrane, which may prevent the activation of these cells. These results are further confirmed by the findings of Woda *et al.* (1979) in which the lateral diffusion of cell membrane-associated antigens was quantitated and found to be reduced in aged rat lymphocytes.

2. *Phospholipid Hydrolysis*

No studies have been carried out on the activation of aged B lymphocytes to examine the intracellular events associated with phospholipid breakdown. However, recently a number of studies appeared which reported on the effect of age on T cell responsiveness and these early activation events.

Two studies have assessed the hydrolysis of phospholipids and the activation of protein kinase C in mitogen-activated murine T lymphocytes. In one study (Proust *et al.*, 1987), the total protein kinase C activity in resting T cells was found to be slightly higher in aged cell populations. However, upon the addition of Con A, young cells responded by translocation of pkC to the membrane, in amounts 2–2.5 times higher than is seen in aged cells. Direct activation of pkC with phorbol myristate acetate (PMA) resulted in equal levels of pkC translocation in the aged and young cell populations. These data suggested that the defect in the aged may reside in the activation of pkC by diacylglycerol or Ca^{2+}. Additional studies on the generation of the hydrolysis products, inositol-1-phosphate (IP1), inositol-1,4-bisphosphate (IP2), and inositol-1,4,5-trisphosphate, were performed. As was found with pkC, the resting levels of these metabolites were higher in the aged lymphocytes. Upon stimulation with mitogens, however, the young cells displayed significantly greater increases in these products. Analysis in the presence of LiCl (which inhibits inositol-1-phosphatase) ruled out the possibility that aged cells have a higher

turnover rate of these compounds, thus the nature of the greater resting levels of these compounds is still unexplained. These results were not confirmed in another study (Lerner *et al.*, 1989). In this report the synthesis of IP1, IP2, and IP3 was no different among the aged and young cells, nor were there any differences in the baseline values for these compounds. The reason for the disparate results in these two studies is not clear.

3. Ca^{2+} Mobilization

It has been determined in two studies on murine T lymphocytes that the magnitude of the increase in intracellular Ca^{2+} in aged cell populations following mitogen activation was lower than is seen in similarly stimulated young cells (Miller *et al.*, 1987; Proust *et al.*, 1987). In one of these studies it was determined that the level of intracellular Ca^{2+} was slightly higher in the resting aged T cell, but the mitogen-induced increase was lower than that seen in the young cell population (Proust *et al.*, 1987). Miller *et al.* (1987) further addressed the issue of whether this observed decrease in the Ca^{2+} was due to a lowered Ca^{2+} increase in the same number of reactive cells or due to a decreased number of responding cells. Using flow cytometric analysis, these investigators demonstrated that fewer aged T cells responded to Con A activation with an increase in Ca^{2+}. Approximately one-third fewer aged cells increase Ca^{2+} than do young cells at the peak response, which is consistent with the data of Ernst *et al.* (1989), indicating that fewer aged T cells enter the cell cycle or express early activation markers following stimulation.

Studies using human peripheral blood lymphocytes demonstrated that Ca^{2+} influx, as assessed by radioactive calcium uptake, was identical in aged and young cell populations (Kennes *et al.*, 1981a,b). However, the proliferation of aged cells was more sensitive to inhibition by lowering extracellular Ca^{2+} concentration, and to drugs which blocked or inhibited Ca-dependent processes. Uptake of Ca^{2+} into mitogen-activated murine cells was shown to be only one-third that of young cells, although the baseline rate of influx is slightly higher in the aged cells (Miller *et al.*, 1987). Taken together, these data indicate that mitogen activation of aged T cells is defective at the earliest steps, and problems can be identified at the levels of Ca^{2+} mobilization, phosphatidylinositol phosphate (PIP) hydrolysis, and pkC activation. Furthermore, the ability of PMA to effectively activate the pkC of aged cell populations suggested that a simple decrease in pkC content cannot explain the defect in activation.

To determine the impact of these diminutions in pkC activation and Ca^{2+} flux upon activation of aged lymphocytes, use was made of agents (phorbol myristate acetate) which directly activate pkC or translocate

Ca^{2+} across the plasma membrane (calcium ionophores). The ability of PMA and the Ca^{2+} ionophores (A23187 or ionomycin) to activate murine and human T cells has been examined as well as the ability of these two reagents to improve Con A activation of murine lymphocytes (Miller, 1986; Chopra *et al.*, 1987; Thoman and Weigle, 1988). Miller (1986) was able to trigger proliferation by aged murine T cells equal to that found in young cell populations with the combination of PMA and ionophore. However, although triggering significant proliferation in human PBLs and murine splenocytes, neither Chopra *et al.* (1987) nor Thoman and Weigle (1988) were able to achieve equal levels in young and aged cell populations with PMA and ionophore. All these studies revealed an increased requirement for Ca^{2+} ionophore by the aged cell population relative to the young, to achieve the maximal response. These data were interpreted to indicate a defect in the Ca^{2+}-dependent early activation events. Chopra *et al.* (1987) have examined human PBL T cells, in which PMA and A23187 are able to induce proliferation by the aged cells to a level equal to about half that found in the young cell populations. IL-2 production by the aged cells was also low, but the expression of the IL-2 receptor was not significantly different among the aged and young cells.

In an attempt to identify whether Ca^{2+} or pkC-dependent events were responsible for the Con A activation defects in aged murine lymphocytes, spleen cells were activated by a combination of Con A and PMA or ionophore (Thoman and Weigle, 1988). Proliferation, IL-2 synthesis, and IL-2 receptor expression were monitored. Ionomycin was particularly effective in reconstituting Con A-induced "normal," young levels of IL-2 synthesis and IL-2 receptor levels by aged spleen cells. PMA was also active, but to a lesser degree. However, although these two critical parameters of T cell activation were improved, the effect on proliferation was minimal (Thoman and Weigle, 1988).

4. Cyclic Nucleotides

Cyclic nucleotide-generating enzyme activity, cyclic nucleotide levels, and protein kinase activity have been examined in aged human and murine lymphocytes, with a degree of conflicting results (Tam and Walford, 1978, 1980; Kraft and Castleden, 1981; Krall *et al.*, 1981, 1983, 1985; Abrass and Scarpace, 1982; Mark and Weksler, 1982; Halper *et al.*, 1984). As summarized in Table I, basal cAMP levels in human PBLs have been reported as unchanged or decreased in the aged (Mark and Weksler, 1982; Tam and Walford, 1980; Halper *et al.*, 1984). One murine study shows lower basal cAMP levels in the aged (Tam and Walford, 1978). Similarly, the activity of adenylate cyclase has been reported as

TABLE I
STUDIES ON CYCLIC NUCLEOTIDE METABOLISM IN THE AGED

Experimental system	Compound	Change in activity in age	Reference
Mouse	Basal cAMP	↓	Tam and Walford (1978)
	Basal cGMP	↑	Tam and Walford (1978)
Human	Basal cAMP	=	Mark and Weksler (1982);
		↓	Tam and Walford (1980); Halper *et al.* (1984)
	Adenylate cyclase activity	=	Kraft and Castleden (1981);
		↑	Abrass and Scarpace (1982);
		↑	Krall *et al.* (1983); Tam and Walford (1980)
	cAMP-dependent protein kinase	=	Krall *et al.* (1985)
	cGMP	=	Mark and Weksler (1982);
		↑	Tam and Walford (1980)
	Guanylate cyclase activity	↓	Tam and Walford (1980)

decreased, unchanged, or increased in aged PBLs (Abrass and Scarpace, 1982; Krall *et al.*, 1983; Kraft and Castleden, 1981; Tam and Walford, 1980) and one study of cAMP-dependent protein kinase activity in aged PBLs reports that it is unchanged from that measured in young lymphocytes (Krall *et al.*, 1985).

D. APPEARANCE OF ACTIVATION PROTEINS

Transition of cells from G_{1a} to G_{1b} involves the synthesis of several "activation" proteins, including the protooncogene product, c-*myc*, RL388 (described previously), IL-2, IL-2 receptors, and the transferrin receptor. The synthesis of several of these products has been examined in the aged.

1. c-myc

The role of the protooncogene products in driving cell cycle progression is not clear, but c-*myc* expression appears necessary for at least T cell proliferation, and the presence of c-*myc* bypasses the requirement for growth factors (Buckler *et al.*, 1988). Two studies have addressed the issue of c-*myc* RNA synthesis and degradation in the aged (Buckler *et al.*, 1988; Deguchi *et al.*, 1988). The results indicate that perturbations in RNA metabolism are associated with the aging process, although human

and murine T lymphocytes apparently differ in the mechanisms. Buckler *et al.* (1988) quantitated c-*myc* mRNA in Con A-activated murine spleen cells and demonstrated a 60% decrease in aged cells. This decrease was not due to a diminution in the rate of transcription or an increase in degradation rate. In contrast, Deguchi *et al.* (1988) found no change in the level of c-*myc* mRNA in PMA- and ionomycin-activated human PBL T cells. The rate of degradation of this RNA was diminished in aged lymphocytes, however. Both in human (Deguchi *et al.*, 1988) and murine (Ono *et al.*, 1986) lymphocytes the degree of c-*myc* gene methylation was altered in aged individuals, suggesting that the regulation of this gene could be affected. The consequences of these alterations in chromatin structure and in posttranslational processing are not clear, but must be considered as potential contributors to the immunosenescent process.

2. *Interleukin 2 Synthesis*

The synthesis of interleukin 2, a necessary "second signal" for driving T cell entry into DNA synthesis, is low in both aged humans and aged experimental animals (Gillis *et al.*, 1981; Miller and Stutman, 1981; Thoman and Weigle, 1981, 1982; Chang *et al.*, 1982; Effros and Walford, 1983; Kennes *et al.*, 1983; deWeck *et al.*, 1984; Rabinowich *et al.*, 1985; Doria *et al.*, 1987; Iwashima *et al.*, 1987). The mechanisms underlying this defect have been examined. Because IL-2 synthesis requires the activation of the producer cell with mitogen or antigen and the accessory cell product, interleukin 1 (IL-1) (Smith *et al.*, 1980), several studies have examined the production of IL-1 in the aged. IL-1 synthesis by aged human peripheral blood monocytes and murine peritoneal macrophages has been found to be reduced relative to that produced by young cells (Bruley-Rosset and Vergnon, 1984; Inamizu *et al.*, 1985; Iwashima *et al.*, 1987). However, Canonica *et al.* (1986) have reported normal IL-1 production by aged human adherent peripheral blood cells. Attempts to improve IL-2 synthesis in cultures of aged lymphocytes with IL-1 supplementation and with young splenic macrophages were without effect (Thoman, 1985, 1986). Therefore, the role of IL-1 in the lowered IL-2 synthesis demonstrated by the aged cells is not understood, but it is most likely a relatively minor role. Likewise, suppressor cells appear to play an insignificant role in limiting IL-2 synthesis in the aged cells. Removal of various suppressor cells often can increase the amount of IL-2 present in the culture supernatants, but the degree of increase is not different among aged and young cultures (Thoman, 1985, 1986). The most significant element controlling the quantity of IL-2 produced by aged lymphocytes appears to be the number of precursors. Miller (1984), in a

limiting-dilution analysis, determined that the number of precursor cells for IL-2-producing cells was greatly reduced in aged animals. The quantity of IL-2 produced per precursor was the same whether derived from an aged or young animal. As detailed above, the defect in activating IL-2-producing cells may be related to the Ca^{2+}-sensitive steps, as IL-2 synthesis can be markedly enhanced in the aged cells by addition of the Ca ionophore, ionomycin, in addition to Con A (Thoman and Weigle, 1988), a conclusion also reached by Miller (1986). Molecular studies have confirmed that aged lymphocytes contain fewer copies of mRNA for IL-2 than do similar populations of activated young lymphocytes (Wu *et al.*, 1986; Nagel *et al.*, 1988). Fong and Makinodan (1988), utilizing *in situ* hybridization techniques, have demonstrated that fewer aged lymphocytes contain IL-2 mRNA than is seen in activated young populations, but also that a proportion of the aged IL-2 message-containing cells may express fewer message copies than that found in the young. This finding supports the results of Canonica *et al.* (1988), who reported that aged peripheral blood T cells produced less IL-2/cell than did young PBLs.

3. *Interleukin 2 Receptor Expression*

Expression of the IL-2 receptor is also deficient in aged animals and humans (Vie and Miller, 1986; Nagel *et al.*, 1988; Thoman and Weigle, 1988). This receptor is synthesized and expressed during the late G_{1a}–G_{1b} portion of the cell cycle. Early work suggested that IL-2 receptor expression was deficient in the aged, as evidenced by the inability of activated aged cells to effectively absorb and remove IL-2 activity from supernatants (Gillis *et al.*, 1981; deWeck *et al.*, 1984). As soon as the reagents became available, fluorescent analysis was done and confirmed the reduction of IL-2 receptor expression. Both the data of Vie and Miller (1986) and Thoman and Weigle (1988) indicate that fewer aged murine cells become receptor positive, while the fluorescence intensity, indicative of the number of receptors per cell, is relatively the same in aged and young cell populations. In contrast, the data of Nagel *et al.* (1988) suggests that fewer IL-2 receptors are expressed on activated aged human cells, and that fewer aged cells become activated to receptor expression. These data confirm and extend the hypothesis that age-associated functional changes are more often due to diminished numbers of reactive cells rather than to a decline in the activity of all cells.

The disadvantage of fluorescence studies has been the inability of the reagents to distinguish between high- and low-affinity IL-2 receptors. Low-affinity binding is mediated by a 50- to 55-kDa polypeptide (p50/55). High-affinity receptors are composed of both a 50- to 55-kDa and a 70- to 75-kDa polypeptide. The latter polypeptide alone also binds

IL-2 with an intermediate affinity. Internalization of IL-2 is mediated by both the 70- to 75-kDa polypeptide (p70/75) (the intermediate-affinity receptor) and the high-affinity receptor complex. Recently, other techniques have been employed to directly quantitate the expression of p50/55 and p70/75 on aged cells. Hara *et al.* (1988) cross-linked ^{125}I-labeled IL-2 to PHA-activated human PBLs and quantitated p50/55 and p70/75 by autoradiography after polyacrylamide gel electrophoresis. Lesser amounts of both polypeptides were detected from aged populations relative to young cells. The ratio of p50/55 to p70/75 was not altered by aging, however. These studies cannot distinguish whether individual aged cells display fewer receptors, or whether fewer cells express receptors in normal levels.

The situation may differ in mice. Proust *et al.* (1988) examined the IL-2-binding properties of Con A-activated murine T cells. Early after activation (16 hours), aged cells displayed far fewer high-affinity receptors than did young cells. However, at 40 hours after culture initiation, aged cell populations displayed the same number and type of IL-2 receptors as the young cells. This increase in the number of IL-2 receptors on the aged cells was concluded to be largely due to the selective survival of high-affinity IL-2 receptor-bearing cells. Functional studies of IL-2-driven proliferation suggested that fewer aged cells acquired high-affinity receptors, although the number of low-affinity sites (as determined by binding assays) was normal on aged cells. This might suggest that the ratio of p50/55 to p70/75 expression in aged murine cells differs from that of young cells in contrast to the situation described for human lymphocytes.

E. Secondary Signal Reception and Response

As discussed in the previous section, the G_{1b} phase of the lymphocyte cell cycle is characterized by the expression of new proteins, including lymphokine receptors. Transit from the G_{1b} into S phase requires the binding of appropriate lymphokines to these receptors. Although few studies have been done, the available data suggest that here, too, both B and T lymphocyte cell cycle transit can be affected by age.

1. T cells: IL-2 Response

The intracellular signaling mechanisms that ultimately trigger DNA synthesis in T cells following IL-2 binding and internalization are unknown. It is believed that protein kinase C activation is not involved (Mills *et al.*, 1986, 1988; Valge *et al.*, 1988). The ability of aged lymphocytes, which bear the IL-2 receptors, to proliferate in response to

IL-2 has been assessed by Negoro *et al.* (1986) and by M. L. Thoman (unpublished). Both groups have data which would suggest that an additional defect may exist that limits aged cell responsiveness to IL-2 at sites distal to receptor expression. Negoro's group purified IL-2 receptor-expressing human peripheral blood cells by rosetting with IL-2-coupled red cells, whereas we have purified activated murine cells by fluorescence-activated cell sorting. These latter cells were then cultured with increasing amounts of IL-2, and [^{3}H]thymidine incorporation was assessed 24 hours later. Cells derived from aged animals in general incorporate much less [^{3}H]thymidine than do similarly activated young cells. The data of Proust *et al.* (1988) suggest that perhaps the murine IL-2 receptor-positive cells purified by fluorescence-activated cell sorting express fewer high-affinity receptors, while possessing normal levels of low-affinity receptors.

2. *B Cells: Lymphokine Responses*

B cell activation with antigen or anti-μ reagents initiates a cascade of reactions similar to that described in activated T cells, and, like T cells, B cells also require interaction with lymphokines for cell cycle transit and further differentiation (Cambier and Ransom, 1987; Coffman *et al.*, 1988). Somewhat in contrast to the situation described for T cells, the exact sequence of lymphokines required for these processes is not as yet completely worked out, and some of the confusion may be due to differences in the signaling requirements for different B cell subsets, or multiple activation pathways may be available to B cells. At least five T cell-derived lymphokines have been characterized which influence B cell growth and differentiation, IFN-γ, IL-2, IL-4, IL-5, and IL-6. These lymphokines (most particularly IL-4 and IL-5) may act at several different stages in the B cell activation process, and may also have functional activity on other cell lineages, which has been reviewed elsewhere (Coffman *et al.*, 1988; Yokota *et al.*, 1988; Sidera *et al.*, 1988) and will not be further considered here.

Resting (G_0) B cells express receptors for IL-4 (Park *et al.*, 1987a,b). Interaction of IL-4 with the resting B cell causes the hyperexpression of Ia antigens (Noelle *et al.*, 1984) and potentiates the subsequent entry of the cell into the S phase (Rabin *et al.*, 1985), although IL-4 alone does not initiate cell cycle transit. IL-4 may also act later in the B cell activation process, causing a shift in isotype expression, increasing IgG_1 and IgE synthesis (reviewed in Coffman *et al.*, 1988; Howard and Paul, 1983; Paul *et al.*, 1986).

IFN-γ antagonizes the action of IL-4 on B cells, inhibiting IL-4 activation of increased Ia expression, proliferation, and IgG_1 and IgE

production. IFN-γ may function through the activation of the cAMP pathway, increasing the activity of the cyclic nucleotide kinases (Coffman *et al.*, 1988).

Like IL-4, IL-5 was originally described as having B cell growth and differentiating properties, and also influences isotype distribution, causing increases in IgA synthesis. Unlike IL-4, it has no apparent effect on resting B cells (Yokota *et al.*, 1988).

The role of IL-2 in B cell cycle transit and differentiation is still somewhat controversial. Activated B cells express IL-2 receptors (Ernst *et al.*, 1988; Zubler *et al.*, 1984; Lowenthal *et al.*, 1985; Muraguchi *et al.*, 1985) and IL-2 has been described as both driving B cell proliferation and enhancing differentiation to immunoglobulin-secreting cells. Other investigators have disputed these conclusions (Julius *et al.*, 1987), suggesting that the IL-2 receptors on activated B cells are nonfunctional and the [^{3}H]thymidine incorporation detected in these cultures is due to contaminating T cells and the increase in Ig secretion due to secondary products produced by the IL-2-stimulated T cells.

The effect of aging on the synthesis and activity of these lymphokines has been studied very little to date. As shown in Table II, the synthesis of IL-2 is severely reduced by aging in both humans and mice, but the information concerning the other lymphokines is much more tentative. IL-1 production has been reported to be reduced in three strains of mice; IL-3 production by aged mice has been reported to be both lower (Chang *et al.*, 1988) and higher (Iwashima *et al.*, 1987). IL-4 production by aged mice may be low, although it was not directly assayed (Winchurch *et al.*, 1987). Human B cell factors, defined as B cell growth factors to describe their ability to support B cell colony formation in soft agar, have been examined. Aged T cells are less supportive of young B cell colony formation than are young T cells, and were concluded to be deficient in factor production (Whisler and Newhouse, 1986).

The responsiveness of aged B cells to the action of these lymphokines has not been examined to any extent, although M. L. Thoman (unpublished observations) has preliminary results on studies of the effect of IL-4 on aged BALB/c splenic B cells. Resting B cells from 20- to 24-month-old animals, when cultured with IL-4, demonstrate increased Ia expression. However, the change in mean fluorescence intensity is much less than that displayed by young B cells. Costimulation of IL-4 with anti-μ to induce proliferation in aged B cells indicated that most aged mice contained B cells able to respond vigorously to IL-4 and anti-μ. However, because of the greater [^{3}H]thymidine incorporation in aged cells activated by anti-μ alone, the degree of synergy between anti-μ and IL-4 was lower in the aged cells relative to the young B cells. In a third

TABLE II
LYMPHOKINE SYNTHESIS IN THE AGED

Lymphokine	Experimental model	Synthesis	Inducing agent	Reference
IL-1	Murine	Low	LPS	Inamazu *et al.* (1985); Bruley-Rosset and Vergnon (1984); Iwashima *et al.* (1987)
		Normal	LPS	Doria *et al.* (1987)
	Human	Normal	LPS/Staph A	Whisler *et al.* (1985)
IL-2	Murine	Low	Con A	Iwashima *et al.* (1987)
			Con A, alloantigen	Thoman and Weigle (1981, 1982)
			Alloantigen	Miller and Stutman (1981)
			Virus	Effros and Walford (1983)
			Con A	Doria *et al.* (1987)
			Con A	Chang *et al.* (1982)
	Human	Low	PHA	Nagel *et al.* (1988)
			PHA	Kennes *et al.* (1983)
			Mitogen	Rabinowitch *et al.* (1985)
			PHA, Con A	Gillis *et al.* (1981)
			αT3	Canonica *et al.* (1986)
IL-3	Murine	Low	Con A	Chang *et al.* (1988)
		High	Mitogen	Iwashima *et al.* (1987)
	Human	Not tested		—
IL-4	Murine	Low	Con A	Winchurch (1987)
	Human	Not tested		—
Interferon	Murine	High	Con A	Heine and Adler (1977)
		Low	Virus	Rytel and Kilbourne (1971)
	Human	Low	Virus	Rytel *et al.* (1986); Shiozawa *et al.* (1986); A. Miller (1980)
		Normal	PHA	Canonica *et al.* (1986)
			Virus	Cantell *et al.* (1968)
B cell growth factor	Human	Low	Staph A	Whisler and Newhouse (1986)
B cell differentiation factor	Human	High	Staph A	Hara *et al.* (1987)

assay, IgE production by LPS and IL-4-stimulated B cells was lower in cultures derived from aged animals compared to cultures derived from young animals.

F. Chromatin Structure and Repair

Several studies have indicated that while the initial cell cycle may not be impaired in the aged lymphocyte, subsequent cycles might be significantly limited in aged cells. One possible explanation for the impaired ability of the aged lymphocytes to undergo repeat cycles might be damage to the chromatin structure (Dutkowski *et al.*, 1985; Harris *et al.*, 1986). Mutational frequency has been estimated to increase 1.6%/year in human lymphocytes, while DNA repair mechanisms are, in general diminished (Licastro and Walford, 1985). Structural changes in the chromatin increase with age, such as DNA demethylation and abnormal, small circular DNA forms (Kunisada *et al.*, 1985; Yamagishi *et al.*, 1985; Shmookler Reis *et al.*, 1983). Increased sensitivity to X irradiation and [^{3}H]thymidine-induced damage has also been associated with aging (Harris *et al.*, 1986; Staiano-Coico *et al.*, 1983), and these chromatin defects may limit the ability of these cells to undergo subsequent rounds of division.

G. Housekeeping Functions

Changes in the "housekeeping" activities of lymphocytes have been associated with increased age. Basal levels of sugar and ion transport are reduced in the aged, as is protein synthesis (Tollefsbol and Cohen, 1985). Aged murine lymphocytes display a depolarized state, which is due in part to alteration in the activity of the Na^{+},K^{+}-ATPase (Witkowski and Micklem, 1985; Witkowski *et al.*, 1985). The activation of lymphocytes is accompanied by an increase in the activity of glycolytic enzymes, sugar and ion transportation, and NAD levels (Tollefsbol *et al.*, 1981; Verity *et al.*, 1983; Chapman *et al.*, 1983; Segal, 1986; Von Zglinicki and Bimmler, 1987). Aged lymphocytes show diminished increases in all of these processes.

H. Conclusions

Summary of B and T Cell Activation

These data indicate that aged lymphocytes display a number of defects that prevent normal cell cycle entry and transit, which are summarized in Table III. The inability of aged T cells to enter and progress through

TABLE III
SUMMARY OF CELL CYCLE EVENTS AFFECTED BY THE AGING PROCESS

Cell cycle stage	Characteristic	Change
G_0	Membrane composition	Lipid composition changes
	Membrane potential, ion and sugar transport	Na^+, K^+-ATPase activity changes (rate decreased)
	Cytoskeleton	Actin polymerization state changes
G_0-G_{1a} Transition	Phosphatidylinositol hydrolysis	Lower
	Protein kinase C activation	No change
	Cyclic nucleotide generation	Conflicting data
G_{1a}-G_{1b} Transition	Transcription and translation of new proteins	Low mRNA for IL-2 and IL-2 receptors; alterations in c-*myc* message stability
	Appearance of "activation" proteins	Low RL388, transferrin receptor, IL-2 receptor
G_{1b}-S Transition	Lymphokine-mediated signals	Low IL-2-mediated [^{3}H]thymidine incorporation by IL-2R^+ cells
G_2M-G_1 Transition	Cycle reentry impaired	Accumulated chromatin damage; low DNA repair mechanism

the cell cycle is not due to a single defect, but a number of different deficiencies impact this process. The initial binding of the mitogen or antigenic determinant appears normal, however, the translocation of this signal may be impaired due to alterations in the cell membrane composition, or the cytoskeletal interaction with the plasmalemma. Very early events, such as ion translocation, particularly Ca^{2+} flux and protein kinase activation, also are impaired with age. Synthesis of lymphokines and of receptors for these lymphokines is also decreased by the aging process, further impacting the number of cells activated in aged populations. Even after successfully expressing receptors and interacting with the appropriate lymphokines, the aged T cell may not be able to effectively respond to the IL-2 signal by progression from the G_{1b} to the S and M phases of the cell cycle. The impact of aging on these activation processes in the B cell compartment has not been assessed. However, the available data suggest that some early events, such as Ia

hyperexpression, as well as some later responses, such as immunoglobulin gene switching, may be defective in aged murine B cells. The synthesis by T cells of lymphokines known to influence B cell activity may also be impaired by aging.

In addition to revealing multiple sites at which aging affects the activation sequence, these data also indicate that only a portion of the lymphoid population is affected. That is, aged lymphocytes are a mosaic of normal, active cells, and those which are defective. The mechanisms which result in this mosaic pattern of lymphocyte responsiveness are not clearly understood. In the following sections several possibilities will be considered: Is there evidence that changes in the distribution of functional subsets occurs with age? Are there changes in the maturation of lymphocytes, or do suppressor regulatory elements operate in the aged?

III. Age-Associated Alterations in Cell Subset Distribution and Marker Density

One hypothesis put forward to explain the decline of immunologic vigor with age is that advancing age is accompanied by a loss in specific, functional, subsets of lymphocytes. Numerous studies have addressed this issue, with somewhat contradictory results.

A. Quantitation of Lymphocyte Subsets

1. *Murine*

The number of B and T lymphocytes appears to be unchanged by aging (Callard and Basten, 1977; Callard *et al.*, 1977; Stutman, 1972; Sidman *et al.*, 1987). However, changes in subset distribution might be affected by aging and several studies in mice have examined the number and percentage of T cells bearing the Ly-1, Ly-2, and L3T4 cell-surface antigens, in a variety of lymphoid tissues (Chang *et al.*, 1982; Crowley *et al.*, 1983; Utsuyama and Hirokawa, 1987; Wade *et al.*, 1988; Sidman *et al.*, 1987). A study by Crowley *et al.* (1983) examined eight different lymphoid tissues, concluding that the effect of aging on the relative proportion of Ly-1^+ and Ly-2^+ cells varied considerably depending on tissue origin, and that the mucosal and systemic lymphoid systems were differentially affected by the aging process. The systemic lymphoid system (bone marrow, spleen, and thymus) displayed a relatively consistent increase in the proportion of Ly-1^+ cells with a concomitant decrease in Ly-2^+ cells with advancing age. The mucosal immune tissues, Peyer's patches (PP), bronchial (mediastinal) lymph nodes

(BLNs), intraepithelial lymphocytes (IELs), and lamina propria lymphocytes (LPLs) were more variable, with PPs, IELs, and LPLs showing a decrease in the proportion of Ly-2^+ cells in the aged. These studies are supported by preliminary studies performed in our laboratory (D. Ernst and M. L. Thoman, unpublished observations), in which it was found that the mucosal system differs from the systemic with respect to effects of age on T cell subsets. The percentage of splenic lymphocytes positive for L3T4 declined slightly with age. In contrast, the percentage of L3T4^+ cells in PP increased with age, while in both tissues the percentage of Ly-2^+ cells was largely unchanged by aging. Utsuyama and Hirokawa (1987) also reported a decline in the percentage of spleen cells bearing Ly-1 and Ly-2 in aged mice, however, in these studies the ration of L3T4^+/Ly-2^+ increased with age, implying no change with age in the proportion of L3T4^+ cells. In contrast to these results, no change in the percentage of splenic cells expressing Ly-1, Ly-2, or L3T4 antigens in young versus aged animals was reported by Sidman *et al.* (1987). These studies all use similar techniques, reagents, and mouse strains, so no simple explanation is apparent to explain the discrepancy in results. However, changes in the density of marker expression (which will be discussed at greater length in a subsequent section) may contribute to the divergent results.

Although murine and human T lymphocytes have been divided by expression of L3T4 (CD4) and Ly-2 (CD8) into "helper" and "cytotoxic/suppressor" subsets, recently the helper subset has been the focus of new studies utilizing antigen-specific T cell clones. Helper T cell clones have been further classified with respect to the spectrum of lymphokines which they produce and their function. Mosmann *et al.* (1986) were able to classify a panel of murine helper T cell clones into those which produced IL-2 and IFN-γ versus those which produced IL-4 and IL-5. Those which produced IL-2 (Thl) are less able to help B cells in an antigen-specific immunoglobulin response, while the IL-4-producing Th2 are more efficient in this function. Thl, but not Th2, cells are competent in transferring delayed-type hypersensitivity (DTH). A great deal of work has focused on identifying antigenic differences among these two subsets, and attention has recently focused on the T200 family of molecules (CD45). Differential expression of the CD45 apparently can distinguish subsets of CD4^+ cells, and the CD45 high appears to define the Thl subset (Bottomley, 1988; Powrie and Mason, 1988).

As yet, no studies have directly examined CD45 expression in the aged, nor have any studies directly addressed the question of relative Thl/Th2 activity in aged individuals. Certainly it can be inferred that both subsets

are deficient, as the loss of the ability to synthesize IL-2 by aged splenocytes is well documented (Thoman and Weigle, 1981, 1982; Miller and Stutman, 1981; Effros and Walford, 1983). Although production of IL-4 and IL-5 has not formally been analyzed, one report exists suggesting lowered IL-4 activity in Con A-induced supernatants generated from aged animals (Winchurch *et al.*, 1987). While suggestive of a functional loss of Th1 and Th2 cells, no direct studies have been done.

2. *Human*

Similar studies performed on human peripheral blood lymphocytes quantitating age-related changes in the relative proportion of T cell subsets have generated divergent results, summarized in Table IV (Cobleigh *et al.*, 1980; Birkeland, 1981; Nagel *et al.*, 1981, 1983; Mascart-Lemone *et al.*, 1982; Van deGriend *et al.*, 1982; Hallgren *et al.*, 1983; Schwab *et al.*, 1983; Traill *et al.*, 1985; Ligthart *et al.*, 1985; Brill *et al.*, 1987; Negoro *et al.*, 1987; Bender *et al.*, 1988; Hallgren *et al.*, 1988). Several groups report a decline in the absolute number and proportion of T lymphocytes in aged PBLs (see Table IV), while others detect no change. Declines in both the CD8$^+$ and CD4$^+$ subsets have been reported, as well as increases in the proportion of these subsets. However, a majority of the reports record a decrease in the proportion of T lymphocytes with a decrease in both CD4$^+$ and CD8$^+$ cells. The magnitude

TABLE IV
SUMMARY OF REPORTED CHANGES IN HUMAN T CELL SUBSETS DETECTED IN THE AGED

Surface phenotype			
OKT3 (Leu-4)	OKT4 (Leu-3a)	OKT8 (Leu-2a)	Reference
↓	=	↓	
=	=	=	Hallgren *et al.* (1983)
↓	↓	↓	Ligthart *et al.* (1985); Brill *et al.* (1987); Traill *et al.* (1985)
=	↑	↓	Schwab *et al.* (1983)
↓	↑	=	Van deGriend *et al.* (1982)
↓	=	↓	Nagel *et al.* (1981, 1983); Bender *et al.* (1988)
↓	↓	↑	Mascart-Lemone *et al.* (1982)
=	=	=	

of the differences between aged and young populations is less than 20%, which is much less than the degree of functional decline which accompanies aging, and is therefore not the sole mechanism responsible for immunosenescence among these two groups. In fact, several groups have reported that they found no correlation between the percentage of $OKT4^+$ or $OKT8^+$ cells and the proliferative response of these cells to mitogens.

Almost exclusively these studies have examined the T cell population. Only one study on PBL B cells indicates that surface immunoglobulin-bearing cells are lower by 20% in the aged population (Cobleigh *et al.*, 1980).

B. Marker Density

Although the data reviewed in the previous section indicate that subtle changes occur in the relative proportion of cells representing various subsets, another contributing factor to the poor activation of aged lymphocytes may be a reduction in the number of cell-surface sites through which the cells may be triggered. The density of some cell-surface antigens does appear to be altered by the aging process. In one large panel of individuals, the staining intensity of the aged PBL for OKT8 and OKT4 was lower than that observed for the young population (Traill *et al.*, 1985). Another study identified a population of T cells derived from aged individuals expressing low levels of T3 (Hallgren *et al.*, 1985). In aged mice the density of Thy expression has been reported to decrease (Brennan and Jaroslow, 1975), although others do not confirm this finding (Sidman *et al.*, 1987; Utsuyama and Hirokawa, 1987). The density of Ly-2 on aged murine splenocytes has also been reported to be low (Utsuyama and Hirokawa, 1987), although this too has not been confirmed by the results of Sidman *et al.* (1987). A loss in expression of W3/13, W3/25, and Thy on aged rat T lymphocytes was reported (Gilman *et al.* 1981). Furthermore, a large increase in the number of "null" cells in the blood of aged humans has also been reported (Ligthart *et al.*, 1985). These results suggest that reduced receptor numbers may contribute to the ineffective triggering of aged lymphocytes.

In contrast to the reports of diminished antigen expression, MHC antigen expression on murine splenic lymphocytes has been shown to increase with advancing age (Sidman *et al.*, 1987). The functional significance of this increase is not completely understood, but aged cells were shown to stimulate two to eight times as much proliferation as young cells when used as stimulators for allogeneic spleen cells or for self-reactive anti-I-A cell lines.

C. Summary of Age-Related Alterations in Cell Subset Distribution

In conclusion, the numerical shifts in cell subsets which occur with age are subtle and cannot fully explain functional changes. The possibility that changes in T lymphocyte subset distribution may not be a biomarker of aging but rather a reflection of an underlying disease process has been suggested by two studies. In an interesting longitudinal study, Boersma *et al.* (1985) found that a sharp decline in the proportion of Thy^+ PBLs in mice of the CBA strain was predictive of a short remaining life span. A human study, comparing young subjects with healthy aged individuals and with aged individuals with current "nonimmunologic" health problems demonstrated that altered surface marker expression was characteristic only of the latter, nonhealthy, group. These results suggested that these surface marker alterations occurred as a consequence of disease rather than aging (Hallgren *et al.*, 1988). The significance of the changes in marker density with age are unknown, but may contribute to the impairment in the activation of aged lymphocytes.

IV. Hematopoietic Stem Cells and Differentiation of Precursors

The maturation of functional T and B cells from hematopoietic precursors provides another site which aging might impact. Two aspects of this question have been examined, the quality and quantity of the stem cells and the effectiveness of the aged environment to support stem cell maturation. The cellular composition of the bone marrow does change with age. The cellularity of the bone marrow increases with age (Chen, 1971), as well as the number of differentiated lymphocytes, identified by the presence of sIg and Thy-1 (Farrar *et al.*, 1974; Eren *et al.*, 1988). However, the number of pre-B cells expressing B220 (a B lineage surface marker) but not sIg is lower in the aged bone marrow (Eren *et al.*, 1988).

A. Pluripotent Stem Cells

1. *Quantitation of Hematopoietic Stem Cells*

The number of hematopoietic pluripotent stem cells in the bone marrow of aged animals appears normal; however, their proliferative potential may be somewhat reduced relative to those from young animals. The total number of bone marrow pluripotent stem cells, characterized by their ability to form colonies in the spleen (colony-forming unit, spleen, CFU-S) when transferred to lethally irradiated recipients, is unaffected by aging in most strains of mice (Chen, 1971; Davis *et al.*, 1971; Tyan, 1976, 1982; Gozes *et al.*, 1982; Schofield *et al.* 1986). The proliferative

capacity of these stem cells may be reduced in the aged, as suggested by the data of Albright and Makinodan (1976). In this study, the incorporation of ^{125}I-labeled UdR was slightly decreased in spleens of irradiated animals receiving bone marrow derived from aged mice as compared to those injected with young bone marrow, as well as the number of cells in the spleen colonies. These data indicated that the aged CFU-S underwent between one and four fewer mitotic divisions than did the young CFU-S, which represented an approximately 20% reduction in proliferative potential. Similar conclusions were reached by an *in vitro* analysis of aged CFU-S (Mauch *et al.*, 1982), in which long-term bone marrow cultures were established. The number of CFU-S initially generated in the aged marrow cultures was greater than in the young cultures, but over the entire culture period the total number of CFU-S generated was higher in the young. Self-renewal of CFU-S derived from aged and young animals was compared *in vivo* in a serial transplantation protocol (Ogden and Micklen, 1976) in which both aged and young bone marrows were cotransferred into a single irradiated recipient. Both marrow populations underwent a sharp decrease in the number of CFU-S between the second and third transfer, and neither appeared to have a selective advantage over the other. Thus, although the aged stem cells appear to have a slightly reduced capacity for proliferation, it would appear that the CFU-S compartment has enough reserve to produce hematopoietic cells for periods far exceeding the normal life span of the animal (Lipschitz *et al.*, 1984; Lipschitz and Udupa, 1986).

2. *Bone Marrow Stromal Elements*

The maintenance and renewal of stem cells are dependent on as-yet incompletely understood functions of the bone marrow stromal cells. The effect of aging on the stromal cells of the bone marrow has also been assessed. Aged human bone marrow stromal cell populations appear to contain a higher number of mitotically inactive cells and fewer of the dividing "progenitor" cells (Mets and Verdonk, 1981). Stromal cells derived from aged animals appear to be normal in their ability to establish monolayers in culture (Matthews and Crouse, 1981; Mauch *et al.*, 1982; Schofield *et al.*, 1986). The ability of these aged stromal layers to support the generation of CFU-S is unimpaired, and in fact both the number and rate of CFU-S production are higher in the aged cultures than in similar layers established from young animals (Matthews and Crouse, 1981; Tyan, 1982). However, young stromal cultures may "rejuvenate" aged hematopoietic stem cells after long-term culture (Mauch *et al.*, 1982).

B. Lymphoid Stem Cells

Aging appears to affect more severely the committed stem cells of the lymphoid compartment and the maturational microenvironment of the bone marrow and thymus. A number of studies have examined the ability of aged bone marrow to reconstitute immune responsiveness in irradiated young hosts, while others have attempted to assess the self-renewal capacity of lymphoid stem cells through serial transplantations. Recently, new *in vitro* techniques have been employed to study stem cell activity. Some degree of controversy still exists regarding the conclusions of these studies. In particular, the validity of the use of irradiated, bone marrow-reconstituted animals as a model system has been questioned (Ross *et al.*, 1982), and the preparation of the bone marrow cells has also been criticized (Eren *et al.*, 1988; Zharhary, 1988).

1. *B Cell Precursors*

The ability of aged bone marrow to support B cell differentiation has been examined. Early experiments utilizing bone marrow reconstitution of irradiated hosts were focused on the reconstitution of anti-SRBC plaque-forming cell responses. The conclusion drawn from these studies was that if untreated bone marrow cells were transferred into host animals, aged cells functioned as well or better than young bone marrow when assayed 1–20 months after transfer for the ability to respond to SRBCs (Harrison and Doubleday, 1975; Harrison *et al.*, 1977; Farrar *et al.*, 1974). However, in one of these studies, the bone marrow was treated prior to transfer with antilymphocyte serum, which depleted Thy-bearing cells; in animals receiving these T-depleted bone marrow cells, the aged bone marrow was markedly deficient in restoring B cell responses (Farrar *et al.*, 1974). Recent work by Zharhary (1988) would support the conclusion that aged bone marrow is not as effective as is the young in supporting B cell differentiation. This study examined the *in vitro* differentiation of B cells from bone marrow that had been depleted of sIg^+ cells. Following a period of culture in liquid media, the presence of B cells was quantitated by fluorescence with anti-Ig reagents, the number of B cell colonies able to grow in soft agar, and LPS-induced proliferation. As measured by all three of these assays, the aged bone marrow was less effective in giving rise to B cells. Because no change in the kinetics in B cell generation was noted, these deficiencies were not due to slower differentiation in the aged cultures. Duwe *et al.* (1979) also reported that the number of bone marrow B cell colonies arising from cultures of aged cells was diminished relative to young bone marrow cells, although the dextran sulfate-induced bone marrow pre-B cell

proliferative response was unaltered by aging. The interpretation of these data is difficult due to the varying protocols utilized, including using thymectomized versus intact recipients of transferred bone marrow, the length of time prior to assay after cell transfer, and the use of untreated bone marrow versus Thy- or sIg-depleted marrow. It does appear certain, however, that in the absence of mature sIg-bearing cells, differentiation of B cells from late pre-B precursors in the bone marrow is deficient in aged animals. Whether early pre-B cells are normal is still questionable, in light of the ability of aged bone marrow to give rise to functional B cells at times as late as 10 months after reconstitution.

2. *T Cell Precursors*

The number of bone marrow pre-T cells has been quantitated in two studies. In one, the ability of pre-T cells to synergize with cortisol-resistant thymocytes in a proliferative response was used to enumerate their number in aged bone marrow, which was determined to be reduced by at least 50% (Cohen and Fairchild, 1979). The other analysis examined the number of bone marrow null cells induced to express Thy-1 by exposure to ubiquitin. Fewer cells from aged bone marrow are triggered to express this marker, reflecting a possible failure in the differentiation of null cells (Twomey *et al.*, 1982) to Thy^+ T precursors. Tyan (1976, 1977) has identified an age-associated defect in bone marrow pre-T cells by focusing on the ability of bone marrow to repopulate the thymus in irradiated animals. Cellularity of the thymus and the cycling of thymic cells as determined by [^{3}H]thymidine uptake were assessed. Tyan's findings indicated that although the number of CFU-S in the bone marrow remained unchanged by aging, the ability to reconstitute the thymus was diminished. The self-renewal capacity of bone marrow-derived T cell precursors has also been assessed. In these studies, the bone marrow was serially transplanted. In the first recipient, the response of the aged cells was lower than that of the young. In subsequent transfers the activity of the aged cells equaled that of the young cells (Averill and Wolf, 1985), although it was significantly reduced over control values for both sets of recipients. However, it has been reported that the loss of functional repopulation with serial transplantation is most likely an artifact of the experimental protocol (Ross *et al.*, 1982), thus the question of the self-renewal capacity of lymphoid stem cells in the aged remains unresolved. In another system the function of transferred bone marrow in two T cell assays was assessed over the course of a year after cell transfer (Gozes *et al.*, 1982). Early after transfer the mixed lymphocyte response (MLR) and PHA-induced proliferation were equal in cultures from animals receiving aged and young bone marrow. However,

at 8 months, two of three aged recipients demonstrated reduced responses, and by 11 and 12 months both the MLR and T cell mitogenic responses were extremely reduced in cells from animals which had received aged bone marrow. Interpretation of these experiments is made more difficult by the small numbers of animals analyzed. For example, at 5 months, the response of one of two animals which had received aged bone marrow is approximately half that seen in the animals receiving young cells. In a different type of experimental protocol, Eren and co-workers (1988) assessed the ability of aged and young bone marrow cells to differentiate into functional T cells in fetal thymus *in vitro*. Aged cells did give rise to functional T cells in this system; however, this group concluded that the aged bone marrow T cell precursors were deficient relative to the young in competition analyses, where mixtures of young and aged bone marrow cells were allowed to codifferentiate in the fetal thymus. In these experiments the young cells represented a larger percentage of the differentiated cells than predicted from the initial mixture. These authors further criticize the conclusion of others regarding the ability of aged bone marrow to give rise to functional T cells, pointing to the number of mature Thy^+ cells in the bone marrow, which is higher in the aged, and which were not removed in earlier reconstitution experiments.

Gorczynski and his co-workers, in a series of articles (Gorczynski *et al.*, 1982, 1983, 1984; Gorczynski and Chang, 1984; Chang and Gorczynski, 1984), have described experiments on the differentiation of cytolytic T lymphocytes (CTLs) from bone marrow-reconstituted animals, particularly examining the precursor CTL (pCTL) repertoire of the thymus and spleen. In these experiments it was found that the repertoire of the bone marrow-derived pCTL was subtly altered with age, and that these alterations were preserved even in a young differentiative environment. However, they also found that the repertoire of the thymic CTL was similar in young and aged animals, suggesting that peripheral expansion of the repertoire occurred and that the environment of the aged animal thus influences the repertoire of the splenic CTL. These experiments further indicated that the aged thymus was able to support the differentiation of bone marrow pre-T cells into functional CTLs.

C. Thymic Microenvironment

The ability of the aged thymus to serve as the site of T cell maturation has been questioned. The human thymus involutes, beginning at puberty, progressively losing lymphoepithelial tissue (Tosi *et al.*, 1982). The number of thymocytes in the cortex diminishes as does the synthesis of thymic hormones (Weksler, 1983). T cell export in the mouse drops

sharply with age from approximately 1% of the cell mass to 0.1% at 6 months of age (Scollay *et al.*, 1980). These changes precede functional declines in the T cell compartment, and thymic involution has been suggested to be the primary cause of immunosenescence. Although thymic function is believed to greatly diminish in the aged, the number of peripheral T cells does not proportionately change. This is in contrast to the situation of adult thymectomy, in which animals undergo a gradual decline in the number of T cells (Miller and Osoba, 1967), thereby suggesting that the aged thymus must retain considerable capacity for supporting peripheral T cells. The ability of murine thymic lobes of various ages has been elegantly examined in a series of experiments by Hirokawa and co-workers (Hirokawa *et al.*, 1976; Hirokawa and Makinodan, 1975; Hirokawa, 1977; Hirokawa and Sado, 1978). Thymic lobes of varying age were transplanted into adult thymectomized, irradiated, and bone marrow-injected animals. Following a 3- to 4-month recovery period, the number of Thy^+ cells in the lymph node and spleens was quantitated, as well as the mitogenic response to lectins and alloantigen and the ability to generate an anti-SRBC response. All of these aspects were reconstituted to at least a limited degree by thymic lobes of all ages (from newborn to 33 months). However, only the newborn thymus effectively reconstituted all these parameters. The ability to support T cell repopulation of the lymph nodes is lost earliest, followed by lectin mitogenesis, the number of splenic Thy^+ cells, and anti-SRBC responsiveness, while the proliferation to allogeneic cells is preserved longest. Repeating this type of experiment using athymic mice, it was found that only newborn thymic grafts were able to support the differentiation of CTLs, mitogenic responsiveness, and anti-SRBC responses. The ability to support CTL differentiation was lost immediately after birth, while the ability to support helper T cell generation was preserved until adulthood (Hirokawa *et al.*, 1982). Thymic lobes from senescent animals were still able to support near-normal levels of Thy^+ cells which proliferated in response to PHA and Con A. T cell reactivity in aged animals was also restored to near-normal young levels by reconstitution with newborn thymic grafts and adult bone marrow (Hirokawa *et al.*, 1976). Taken together, these data indicate that the capacity of the thymus to support the complete spectrum of T cell differentiation is lost shortly following birth in the mouse. However, even at old age the thymic reticulum is capable of supporting some degree of T cell differentiation.

Several studies suggest that alterations occur within the reticuloepithelial portion of the thymus with age, making it less hospitable to repopulation by bone marrow-derived precursors. When nude mice

are cografted with thymic lobes derived from a newborn and an adult mouse, the newborn thymus is more efficiently regenerated by host precursors than is the adult thymic lobe (Loor and Hagg, 1977a,b). Parabiotic mice constructed by joining thymectomized mice to intact mice bearing the T6T6 marker were examined for the presence of T cells derived from the thymectomized partner. However, the genotype of all the mitotic T cells was of the T6T6 type (Kay, 1984). These results were interpreted as suggesting a very early reduction in stem cell to T cell traffic, secondary to an alteration in the thymus which prevents or does not induce inward migration of stem cells.

The role of thymic hormones in maintaining or generating functional peripheral T cells is not fully understood (Stutman, 1981). However, a number of studies have demonstrated that peripheral T cell function in aged animals can be improved by *in vitro* or *in vivo* exposure to thymic hormones (Weksler *et al.*, 1978; D'Agostaro *et al.*, 1980; Cowan *et al.*, 1981; Godberg *et al*, 1981; Verhaegen *et al.*, 1981; Grinblat *et al.*, 1983; Ershler *et al.*, 1984; Frasca *et al.*, 1986, 1987; Barcellini *et al.*, 1988).

D. Summary of Lymphoid Stem Cell Differentiation

The conclusion that may be drawn is that in the aged animal the differentiation of mature lymphocytes is defective, due largely to deficiencies in the maturational environment, and in part to loss of pre-B and pre-T cells in the bone marrow. This change in differentiative environment has led to two hypotheses: (1) that aged animals and humans contain larger numbers of immature cells and (2) that in the aged animal or human, larger numbers of memory cells exist. A series of papers has addressed the possibility that aged individuals accumulate immature T cells in their peripheral blood (Hallgren *et al.*, 1983; O'Leary *et al.*, 1983; Jensen *et al.*, 1986). In these studies, several enzymatic and cell-surface markers were examined. The loss of $T3^+$ cells and changes in the reduced lactate dehydrogenase chain ratio had suggested to these investigators that less differentiated cells accumulated with age. However, a further analysis of the ratio of adenosine deaminase to purine nucleoside phosphorylase demonstrated a normal ratio, not supportive of this conclusion.

The second hypothesis, that aged individuals accumulate memory cells, is supported by several lines of evidence. Several analyses of the cell surface marker Pgp, thought to be found on memory cells (Budd *et al.*, 1987a,b), found that almost all peripheral T cells are highly Pgp^+ (D. Ernst and R. Miller, personal communication). In B cells the increased frequency in the production of autoantiidiotypic antibody formation is suggestive of the accumulation of memory cells. Also, the frequency analysis of

precursors of B cells reactive with environmental antigens demonstrates that, in contrast to chemical haptens, the number of precursors is not reduced, but in fact is increased in the aged (Zharhary and Klinman, 1983, 1984, 1986a,b). Therefore, it would appear that aged individuals may accumulate B and T memory cells in their peripheral lymphoid tissues, while differentiation of naive cells from committed precursors may be diminished. The decline in the generation of mature lymphocytes most likely involves both deficiencies in the number of precursors and the differentiative environment. Impairment of the hormonal interaction between the thymus and the bone marrow may also contribute to the diminution in bone marrow pre-T cells.

V. Regulatory Changes Occurring as a Function of Age

Several processes have been described which have the effect of suppressing or down-regulating immune responses. Both antiidiotypic antibody and suppressor cells have been identified in aged animals and these immunoregulatory processes have been suggested to be responsible for the diminished immunoresponsiveness of the aged.

A. Idiotypic Regulation

Antiidiotypic antibody production increases with age and is apparently responsible, in large part, for the apparent reduction in the amount of high-affinity antibody production and the decrease in the avidity profile of antibody produced by the aged (Szewczuk and Campbell, 1980; Goidl *et al.*, 1980, 1983; Klinman, 1981). One difference seen in aged and young animals is an increased regulation of the immune response by autoantiidiotypic antibody. The presence of autoantiidiotypic antibody has been detected in a plaque-forming cell (PFC) assay in which free hapten was added. The excess free hapten displaced the autoantiidiotypic antibody from the antibody-secreting cells, reversing the inhibition of antibody secretion, thus increasing the number of PFCs detected (Schrater *et al.*, 1979; Goidl *et al.*, 1979). Using this assay it has been determined that down-regulation by antiidiotypic antibody is much higher in the aged, and the amount of antiidiotypic antibody in the serum is much higher in aged animals. Cell transfer experiments have demonstrated that the degree of hapten augmentation (HA) of the PFC response is stable upon transfer (Goidl *et al.*, 1983). Injection of aged spleen cells into irradiated young animals results in a high degree of HA-PFCs. In contrast, transfer of aged bone marrow into irradiated young recipients results in a response which has a low percentage of HA-PFCs, and therefore appears similar to the response of young adult animals.

Similar experiments in which young cells were transferred into carrier-primed aged recipients also revealed that the aged environment may exert a suppressive effect on a primary B cell response, but only when the donor and host shared a common heavy chain allotype locus (Klinman, 1981). Cotransfer of carrier-primed spleen cells and naive B cells into irradiated young hosts also demonstrated the suppressive effect of aged cells on the PFC response. This suppression could be eliminated by depletion of Thy^+ cells from the transferred cell population.

Long-lived peripheral T cells exert considerable conrol over the magnitude of the autoantiidiotypic response of mice as demonstrated by Kim *et al.* (1985). In this study, aged and young mice were bone marrow shielded, irradiated, and allowed to recover. Their response to TNP-Ficoll was then analyzed, particularly with respect to the percentage of animals mounting a significant antiidiotypic response and the magnitude of the antiidiotypic response as measured by the percentage of HA-PFCs. The results clearly indicate that repopulation from bone marrow restores the "youthful" low-antiidiotype pattern in aged mice. This pattern could be altered by splenic T cells derived from aged animals. Thus the experience of the peripheral T cells appears to determine the level of the antiidiotypic response.

The idiotype repertoire apparently changes with age (Goidl *et al.*, 1980). This is suggested by the inability of serum from young animals to effectively suppress PFC responses in aged animals, although the serum contains antiidiotypic antibody, and the greater effectiveness of serum from aged animals in suppressing the responses of animals of a similar age. This change in idiotype repertoire is apparently a function of peripheral influences and is not programmed by changes in the precursor bone marrow B cells, because aged bone marrow, when transferred to a young recipient, reconstitutes a "younglike" idiotype profile, while peripheral T cells strongly influence the antiidiotypic response.

These data support the conclusions that (1) aging results in increased antiidiotypic regulation of the antibody response and (2) the idiotype repertoire is altered with age. This change in the idiotype repertoire with age suggests that aging is accompanied by an alteration in immunoglobulin variable region gene usage.

Direct evidence for alterations in immunoglobulin gene usage has been obtained by Klinman *et al.* (1988). Examination of the response to phosphorylcholine (PC) revealed that the response of young animals is largely composed of antibodies utilizing the V_H S107 gene. In contrast, the frequency of V_H S107 usage in the PC response of aged animals is significantly lower, while the majority of the cells utilize other variable region genes. McEvoy and Goidl (1988) have reported data which further

support the hypothesis that alterations in gene usage occur with age. In their study, hybridomas were constructed from spleen cells of aged and young animals immunized with TNP-Ficoll. After cloning, the reactivity of the hybridomas (MAbs) to several self-antigens was determined. A high percentage of the hybridomas generated from aged animals were cross-reactive with self-antigens and >60% of the MAbs bore the AD8 idiotype, while none of the young monoclonal antibodies was of this idiotype. These data support the hypothesis that a programmed alteration in immunoglobulin gene usage occurs with aging.

T cell receptor gene selection may also change with age, as suggested by the findings of Gorczynski *et al.* (1983), who examined the receptor repertoire of aged CTLs and concluded that the aged lymphocytes use recognition structures distinct from those on young animals. The CTL repertoire undergoes reproducible changes with age, although the total number of CTLs is not altered. These changes are also found in irradiated recipients of aged bone marrow cells, suggesting that these changes reflect maturational alterations in the CTL precursor cells.

B. Suppressor Cells

A second regulatory system, mediated by suppressor cells, has also been hypothesized to play a significant role in the dampening of cellular and humoral immunity in the aged. The literature contains many reports and several reviews of the role of suppressor cells in aging. No consensus has been reached regarding the activity of suppressors, although several types have been identified in aged humans and experimental animals, including spontaneous and induced antigen-nonspecific and antigen-specific types (Goidl *et al.*, 1976; Makinodan *et al.*, 1976; Segre and Segre, 1976, 1977; Hallgren and Yunis, 1977; Antel *et al.*, 1978, 1983; Roder *et al.*, 1978; Duwe and Singhal, 1979; Kishimoto *et al.*, 1979, 1982; Barrett *et al.*, 1980; DeKruyff *et al.*, 1980; Schulof *et al.*, 1980; Globerson *et al.*, 1981, 1982; Delfraissy *et al.*, 1982; Gutowski and Weksler, 1982; Liu *et al.*, 1982; Cinader *et al.*, 1983; Bash and Vogel, 1984; Zharhary *et al.*, 1984; Clark *et al.*, 1985; Ellis and Demartini, 1985; Irimajiri *et al.*, 1985).

Although this large body of work has documented the presence of suppressor cells, controversy still remains regarding the role of suppressor cells in the diminished immune responsiveness of the aged animal. For example, Gottesman *et al.* (1981, 1984, 1985) have obtained data which indicated suppressor cells were unlikely to be responsible for reduced proliferative responses in most aged animals. Using a system wherein the proliferative, cytotoxic, and suppressor activities could be measured with cells from the same animal, spleen cells derived from individual

aged animals were activated by allogeneic cells, and proliferation and cytolytic activity were measured. Suppressor activity was quantitated by irradiating and transferring these cells to secondary cultures containing fresh young spleen cells and allogeneic stimulators. The suppression was antigen specific and required Thy^+ cells, and was not idiotypic. In one experiment, seven aged animals were examined. Six of seven had reduced allogeneic proliferative responses; however, the ability to generate cytolytic and suppressor cells was retained by the majority. These data suggest that effective generation of effector cells does not require a vigorous proliferative response, and that this type of suppressor cell is not responsible for the decline in proliferation. Although illustrating that proliferation is not necessary for effector cell generation, the addition of IL-2 augmented CTL and suppressor cell production in aged cultures, suggesting that the loss of lymphokine production with age influences suppressor cell generation. In agreement with this conclusion (Thoman and Weigle, 1983), the generation of T suppressor cells in a mitogen-induced system is depressed in the aged, but can be largely corrected by the addition of IL-2-containing lymphokine preparations. These results indicate that in systems which require a proliferative event, or an IL-2-sensitive step, aged individuals are deficient.

The role of suppressor cells in the regulation of aged responses has been further questioned by data generated by Doria *et al.* (1982). Azobenzenearsonate-specific T suppressor (Ts) cells were generated and were found to be significantly more active in aged spleen cell preparations than in young populations when assayed on young lymph node indicator cells. However, primed aged lymph node cells were resistant to the activity of either young or aged Ts. These data would suggest that antigen-specific Ts may have little influence on the aged response. Unfortunately, the effect of Ts on the generation of primed cells was not addressed.

In contrast to the conclusion that suppressor cells do not play a large role in immunosenescence is the conclusion drawn by Liu *et al.* (1984), from experiments in which animals were injected with anti-I-J antibody beginning in midlife until old age, at which time they were tested for the ability to mount a secondary response to avian γ-globulin. Anti-I-J treatment resulted in higher responses, a result which was interpreted to indicate that depletion of an I-J suppressor cell subset allows a greater secondary response in aged animals. Furthermore, these animals experienced a prolonged life span relative to their control (saline-injected contemporaries).

Another very clear demonstration of the suppressive effect of aged T cells on the humoral immune system was made by Zharhary (1986).

The frequency of precursor B cells responsive to DNP and NP was compared among young and aged athymic, nude mice, and was found to be identical. This is in striking contrast to conventional mice, which demonstrate a decline with age in precursor frequency to these antigens. Further investigating the role of T cells in this phenomenon, Zharhary constructed two types of chimeric mice, ones in which bone marrow-derived sIg^- pre-B cells and splenic T cells (derived from either young or aged mice) were transferred into irradiated recipients, and ones in which mature splenic B cells and T cells were transferred. Regardless of the B cell source, those animals receiving aged T cells displayed reduced B cell precursor frequencies for the test antigens. The conclusion drawn from these studies was that aged T cells can down-regulate the function of naive antigen-specific B cells in the absence of antigenic stimulation.

Thus, in the aged, a number of suppressor cell systems operate. Regulatory T cells control autoantiidiotypic antibody production and directly down-regulate certain immune responses. Non-T suppressor cells have also been found. Although the aged animal in certain circumstances displays greater suppressor cell activity, the diminution of many responses is not primarily due to suppressor cells.

VI. Mucosal Immunity

Perhaps one of the most intriguing aspects of immunosenescence is the emerging data which indicate that aging does not equally affect all tissues. Szewczuk and colleagues (Szewczuk and Campbell, 1981a,b; Szewczuk *et al.*, 1981; Wade and Szewczuk, 1984, 1988; Wade *et al.*, 1988) first described the vigorous B cell responses of aged mucosal-associated lymphocytes. In a series of experiments, the ability of splenic, peripheral lymph node and mucosal-associated lymph node lymphocytes to produce antibody of various isotypes was compared, following injection of TNP-conjugated bovine γ-globulin (TNP-BGG) or keyhole limpet hemacyanin (TNP-KLH). The magnitude of the IgM, IgG, and IgA PFC responses declined with advancing age in the spleen and peripheral lymph nodes. In contrast, however, the response of the bronchial lymph nodes was increased with age. Similarly, while the heterogeneity and the avidity profile of the anti-TNP response by spleen cells was altered by aging, with a loss in high-avidity PFCs and a restricted heterogeneity, the response of aged BLNs did not reflect these alterations but retained the young pattern.

T cell reactivity in the Peyer's patches derived from aged animals also is resistant to the deleterious effects of aging (Ernst *et al.*, 1987, 1989).

Proliferation in response to mitogens, anti-CD3 reagents, and alloantigen, IL-2 production, and expression of activation antigens have been compared in young and aged spleens and PP lymphocytes. Proliferative responses to Con A, PHA, and alloantigen were unaffected by aging in the PP lymphocytes, while splenic responses declined with advancing age. Examination of the frequency of IL-2-producing cells by limiting dilution confirmed that there was no age-associated decline in the number of IL-2-producing cells in the aged Peyer's patches. In bulk cultures, the amount of IL-2 produced by aged Peyer's patch cells remained unchanged from that produced by younger lymphocytes, while that produced by splenic lymphocytes declined with advancing age. Expression of the IL-2 receptor by Peyer's patch cells was also largely unaffected by age, while the percentage of splenic cells stimulated to IL-2 receptor expression declined with age. These data suggested that the antigen-nonspecific functions of T cells derived from the Peyer's patches retained the resistance to age-associated declines that mucosal B cells demonstrate.

The mechanisms by which mucosal lymphoid tissues escape the deleterious effects of aging are unknown. The suppressor mechanisms which down-regulate peripheral responses may not operate in the gut-associated lymphoid tissue (GALT). For example, the autoantiidiotypic antibody which dampens aged splenic responsiveness is not in evidence in the mucosal response. Szewczuk and Campbell (1981) demonstrated that the mucosal response was composed of very few hapten-augmentable plaques, and suggested that the idiotype repertoire of the mucosal-associated lymphoid tissue might be distinct from the peripheral lymphoid system. That the B cells derived from the mucosa are sensitive to antiidiotypic regulation was illustrated by the experiments in which animals were intragastrically immunized with TNP–BGG. Although the primary response of the mucosal lymph nodes was undiminshed compared to those of young animals, upon secondary challenge the responses of the spleen and mucosal lymph nodes were inhibited by the presence of antiidiotypic antibody.

Another hypothesis which has been suggested, is that the activity of suppressor T cells is greatly reduced in the GALT. There appears to be no investigation of this possiblity, but the T cells of the Peyer's patch are predominately of the Lyt-1$^+$ phenotype, with a correspondingly low percentage of Lyt-2$^+$ cells, which population is thought to contain the bulk of the suppressor population. Thus, if Ts are involved, they may either be of non-CD8$^+$ phenotype, or of increased potency.

The maturational environment of the GALT is likely to differ from

that of the peripheral lymphoid tissues, as evidenced by the data of Eldridge *et al.* (1983). The GALT of the CBA/N xid mouse were shown to contain mature, functional Lyb-5^+ B cells that were lacking in the periphery. These cells respond normally to type II T-independent antigens and SRBCs, in contrast to the splenic B cells of the CBA/N mice. These undefined differences between the maturational environment of the GALT and the peripheral lymphoid tissue have been suggested to contribute to the resistance of the GALT to the deleterious effects of aging (Wade and Szewczuk, 1984, 1988).

VII. Conclusions

The aged lymphoid population is functionally mosaic, composed of nonresponsive and responsive elements. Both the T and B cell compartments are deficient in activation to cell cycle entry, cycle transit, and subsequent differentiation. It is not possible to identify a single age-sensitive step in this sequence, but rather a series of deficiencies contribute to the decline in the competency of the aged lymphocytes to undergo normal activation. Thus very early events, such as Ca^{2+} increases, accumulation of protooncogene transcripts, expression of activation markers, and synthesis of new proteins, are deficient in the aged. However, defects have also been identified in the ability of cells to undergo repeat rounds of cell cycle, possibly due to the accumulation of DNA abnormalities, and inability to respond to lymphokines which drive differentiation. The mechanisms contributing to the accumulation of these defective lymphocytes are not known. Shifts in the proportion of various functional subsets may play a minor role. Also, suppressor cells and antiidiotypic antibody certainly play a role in down-regulating immune responsiveness in the aged, and alterations in the maturational environment, stem cell gene usage, and proliferative potential also contribute to the accumulation of nonresponsive lymphocytes. One as yet unexplained aspect of immunosenescence is the mosaic nature of the lymphocyte pool. Why do some cells retain "youthful" functional vigor, while others are nonresponsive, and is "responsiveness" transmitted to the progeny of the competent cells? The mucosal immune system provides evidence that not all lymphoid tissues are equally adversely affected by age. Thus while the response of lymphocytes derived from the peripheral lymphoid organs is diminished, the gut-associated lymphocytes from the same individual are often normally responsive. These intriguing problems await answers from further investigations of the nature of the age-associated changes in stem cells and maturational environments.

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Immune Mechanisms in Autoimmune Thyroiditis

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I. Introduction

The thyroid gland is the target in two different organ-specific autoimmune disorders with opposite clinical outcomes in man: Hashimoto's thyroiditis (HT) can result in hypothyroidism and Graves' disease (GD) can result in hyperthyroidism (Table I). These pathologic processes are diametrically opposed not only in terms of endocrine outcome but also in immunological characteristics. In this review, only studies on autoimmune thyroiditis (in man and animals) have been considered; an extensive review on Graves' disease was published by Burman and Baker (1985).

According to Burnet's clonal selection theory (1959), autoimmune thyroid diseases result from the persistence of forbidden B cell clones. In this hypothesis, the contact of antibody (Ab)-forming cells with their respective antigens (Ags) during fetal life leads to the destruction of the corresponding clones. In this way, self-reactive clones, so-called forbidden clones, would be deleted unless they originated later in life by somatic mutation of lymphocytes. Numerous experiments have failed to verify this hypothesis for autoreactive B cells (Dresser, 1978; Steele and Cunningham, 1978) as well as for autoreactive T cells; these cells are readily detected in high frequencies in normal animals (Charreire and Bach, 1975) and healthy humans (Bankhurst *et al.*, 1973; Fournier and Charreire, 1977, 1978).

By the 1970s, autoimmune reactivity was viewed as a loss of natural tolerance to self-components by T cells (Chiller *et al.*, 1971; Weigle, 1971; Allison, 1971). A self-antigen (S-Ag), such as thyroglobulin (Tg), which circulated in low concentration, was shown to induce T cell tolerance, whereas only B cells were able to develop an immune response leading to Tg autoantibody (Tg A-Ab) production. This hypothesis was reevaluated following the demonstration that naive T cells from normal animals proliferated in response to numerous S-Ags when the latter were presented in a self-Major Histocompatibility Complex (MHC) context.

In the 1980s, as previously reported for alloreactivity (Kindred and Shreffler, 1972; Rosenthal and Shevach, 1973; Katz *et al.*, 1973;

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TABLE I

ENDOCRINE AND IMMUNOLOGICAL CHARACTERISTICS OF HYPO- AND HYPERAUTOIMMUNE THYROID DISORDERS

Substance/process	Hypothyroidism	Hyperthyroidism
Thyroid hormone	Decreased	Increased
Thyrotropin (TSH)	Increased	Decreased
Antigen (Ag) responsible	Thyroglobulin (Tg)	TSH receptor
Experimental models	Spontaneous (e.g., obese chicken); induced (e.g., mice, rats)	Exclusively a human pathology
Genetic control in humans	DR3	DR5
Genetic control in mice	H-2^k (susceptible strain); H-2^b (resistant strain)	—
T cell invading the thyroid gland	CD4	CD8
Autoantibodies	Anti-Tg Anti-thyroid peroxidase (anti-TPO)	Anti-Tg (in 40–50% of the patients) Anti-TSH receptor (stimulating, blocking, thyroid growth inducers)

Zinkernagel and Doherty, 1974), Bottazzo *et al.* (1983) suggested that the initial event of autoimmune reactivity in thyroiditis was the activation of specific immunocompetent T cells by thyroid cells expressing S-Ag complexed to class II MHC antigen. The MHC Ag expression on nonlymphoid cells, such as thyroid cells, might be under the influence of factors such as interferon-γ (IFN-γ). In this hypothesis, self-Ags or viral Ags would be responsible for auto-Ag (A-Ag) mimicry (Oldstone, 1987) and would be processed in the thyroid cell much as foreign antigens are in macrophages (Unanue, 1981).

In 1986, two different hypotheses were proposed to further specify the mechanisms of self-recognition and non-self-recognition. In each of the postulates, MHC antigens are pivotal.

The "peptidic self" model (Kourilsky and Claverie, 1986; Claverie and Kourilsky, 1986; Kourilsky *et al.*, 1987) is based on the assumption that the function of MHC molecules is peptide presentation: self as well as foreign proteins would yield peptides to be presented by MHC molecules. Two sets of self-antigens would therefore coexist: the first one, called somatic self, would be represented by peptides derived from the somatic cell proteins; the second one, called immunological self, would be represented by idiopeptides, supplied by peptides from antibodies or T cell receptor (TcR) variable regions. In this model, T cells would be tolerant to somatic self, whereas they would react to immunological self; this reactivity defines the basis of a special idiotypic network (in the sense of Jerne, 1971, 1974), which would discriminate between self and nonself. Such a network requires a novel form of T cell Ag recognition. MHC molecules which are restricted to the presentation of self-peptides present the peptides in a context such that they are recognized by T cells, in the absence of a requisite MHC–TcR interaction. Reports are now accumulating in favor of direct T cell binding to MHC-bound peptides (Walden *et al.*, 1986; Werdelin, 1987). In contrast, the existence of idiopeptides functioning as fundamental regulatory elements has scant experimental support (Julius *et al.*, 1988).

The second hypothesis is based on the selection of immunodominant peptides and on unresponsiveness due to "holes in the T cell repertoire" (Schwartz, 1986; Guillet *et al.*, 1986, 1987; Berzofsky *et al.*, 1987). It takes into account the basic data demonstrating that the interaction between an Ia molecule and processed Ag is specific, that T cells recognize a preformed bimolecular complex of Ia and processed Ag (P. M. Allen *et al.*, 1984; Babbitt *et al.*, 1985, 1986; Buus *et al.*, 1986a,b) and that Ia molecules cannot by themselves distinguish between self and nonself because this discriminatory capacity is a T cell function. The authors postulated that the role of the class II molecules in T cell

activation was to hold the peptide in the form of a potentially immunogenic peptide–Ia complex. They hypothesized that class II molecules possessed only one type of peptide-binding site, composed of both chains of the Ia molecule, and that, within an Ag, the selection of an immunodominant peptide for T cell recognition rested on its ability to bind to a class II molecule, homologous to the peptide itself. This identity might therefore account for a hole in the repertoire. Briefly, the absence of self-reactive T cells in the repertoire would be due to negative selection during thymic development because of the homology between self-proteins and class II molecules.

Recent evidence casts doubt on this hypothesis. Immunization with individual synthetic peptides has revealed additional T cell determinants not seen following immunization with the native molecule. The lack of responsiveness to these determinants cannot be explained by an absence of T cells in the repertoire or a failure to bind a particular MHC molecule (Gammon *et al.*, 1987; Brett *et al.*, 1988). Furthermore, positive selection of cells bearing receptors with low affinity for self-MHC was recently demonstrated (Kappler *et al.* 1987; Kisielow *et al.*, 1988; Marrack and Kappler, 1988). This positive selection should occur during the contact between thymic epithelial cells and immature thymocytes (Kruisbeek *et al.*, 1985). Differentiation Ags borne by the immature T cells, CD4 and CD8, would also contribute to the successful triggering of thymocytes bearing low-affinity receptors for MHC (Teh *et al.*, 1988).

Over the past 30 years our thinking about self and nonself discrimination has evolved to focus on the role of class II MHC Ags on the cell surface.

Experimental autoimmune thyroiditis (EAT) was induced for the first time 33 years ago, by Rose and Witebsky (1956), while Roitt *et al.* (1956) were discovering anti-Tg A-Abs in the sera of patients with HT. Understanding the pathogenesis of the naturally occurring syndromes and the experimental autoimmune thyroid diseases has been a central theme in the experimental immunology of autoimmunity and today converges on the thyroid epithelial cells (TECs) in terms of their possible role as antigen-presenting cells (APCs) in thyroiditis. This review covers the last 10 years, taking up where Weigle's extensive review finished in 1980.

II. Thyroid Antigens Involved in Thyroiditis

One common feature of thyroiditis is the existence of autoantibodies directed against Tg and microsomal (Mic) Ags. This characteristic renders these two proteins serious candidates for the role of Ags involved in induction and/or perpetuation of thyroiditis.

A. THYROGLOBULIN (Tg)

Among the various proteins exposed on the thyroid cell membrane, why is Tg the Ag responsible for EAT? One possible explanation is its rapid degradation from the circulation, varying between 4 and 14 hours depending upon the method used (Izumi and Larsen, 1978; Ikekubo *et al.*, 1980; Taura *et al.*, 1985). This degradation process produces Tg fragments which may be iodinated and/or glycosylated. These last modifications interfere with the immunogenicity of the Tg molecule.

Tg is a homodimeric iodinated (0.3-0.5%) phosphorylated (10-12 phosphates per molecule of protein) glycoprotein (8-10% sugars) with a molecular mass of 660 kDa and an isoelectric point of 5.4. It is secreted from stores in the lumen of the thyroid follicle (Lissitzky *et al.*, 1975; Vassart *et al.*, 1975; Lissitzky, 1984; Spiro and Gorski, 1986; Consiglio *et al.*, 1987). It is supposed to reenter the follicular cell in the form of colloid droplets, where it is digested by lysosomal enzymes; then free iodiothyronines diffuse from the lysosomes and filter into the cellular space.

Topological similarity between the two subunits of the Tg molecule was demonstrated, using monoclonal anti-Tg A-Abs with different antigenic specificities and electron microscopy to visualize immune complexes. Three conformation-dependent antigenic sites were directly localized near each end of a single subunit of the Tg molecule (Kondo *et al.*, 1985).

As a precursor and support of thyroid hormone synthesis, Tg production is under the physiological control of the pituitary glycoprotein hormone, thyrotropin (TSH). From the approximately 120 tyrosine residues it contains, only 25 to 30 are available for iodination into 3-iodotyrosine and 3,5-diiodotyrosine, from which only 8 residues can couple to form the hormone residues. Enzymatic iodination of Tg showed that efficiency of coupling with iodine was determined by the initial iodine concentration and not only by the native structure of the Tg (Lamas *et al.*, 1986). *In vivo* and *in vitro* experiments (Marriq *et al.*, 1982; Dunn *et al.*, 1983; Lejeune *et al.*, 1983) have shown that on one subunit, three out of the four hormonogenic sites of Tg were localized at the carboxyl-terminal portion of the molecule most susceptible to proteolysis, especially when Tg was highly iodinated. However, recent data from Bagchi *et al.* (1985b) and from Becks *et al.* (1987) contradict this observation; an excess of iodine acutely inhibited Tg hydrolysis and thyroid hormone secretion.

The complete primary structure of Tg derived from the sequence of its complementary DNA has been reported for bovine Tg by Mercken *et al.* (1985a) and for human Tg by Malthiery and Lissitzky (1987). A

partial primary structure is available for rat Tg (Musti *et al.*, 1986). In humans, the Tg gene has been localized to chromosome 8 and regionally to band q24 (Baas *et al.*, 1985). Its transcription is under the positive control of TSH, probably mediated by cyclic AMP following TSH-receptor binding on thyrocytes (van Heuverswyn *et al.*, 1985).

Comparisons of bovine and human amino acid sequences show a great homology (81.75% on the coding region at the mRNA level). Furthermore, the first 20 amino acids which contain one hormonogenic site are identical in all the species studied. Three internal homologies, repeated 10, 3, and 5 times (Mercken *et al.*, 1985b; Malthiery *et al.*, 1989), were detected in Tg primary structures. They were localized outside the four hormonogenic domains of the Tg molecule, between amino acids 13 and 2169 in bovine Tg. More than 75% of the Tg sequence consist of repetitive structures, indicating that this large protein evolved from the serial duplication of limited numbers of building blocks. Examination of the protein data bank demonstrated a 38% homology (Koch *et al.*, 1987) between the sequence repeated 10 times in human Tg and the extracytoplasmic domain of the murine Ia-associated invariant chain Ii41 (Charron and McDevitt, 1980). This homology between a Tg repeat and Ii41 led to the hypothesis that they might perform similar functions. This supposition is reinforced by their similar intracellular pathways. The Tg repeat is transferred to lysosomes and then to the basal cell surface through a lysosomal acidic compartment; similarly, invariant chains associate transiently with MHC class II Ag α and β chains before dissociation in an acidic compartment, migration, and expression on the cell surface of only α and β chains of class II Ags (Machamer and Cresswell, 1982, 1984).

Another amino acid sequence homology was found with the enzyme acetylcholinesterase (AChE). It is localized to the carboxyl-terminal portion of the Tg molecule, which also includes three of the four hormonogenic sites (Schumacher *et al.*, 1986). These sites have 28.3% homology, extend over 544 residues, and encompass 90% of AChE. The homology of their hydrophilicity profiles (Swillens *et al.*, 1986) suggested that Tg and AChE could share common antigenic determinants and could adopt similar three-dimensional structures possibly with similar binding functions. This homology with AChE could explain the demonstration of immunoreactive Tg in neurons or eye muscle from Graves' ophthalmopathy (Konishi *et al.*, 1974; Kodama *et al.*, 1984). Furthermore, monoclonal anti-Tg A-Abs were obtained after immunization of mice with human eye muscle (Tao *et al.*, 1986). However, cross-reactivity in this system is not straightforward; Weetman *et al.* (1988) have demonstrated anti-AChE Abs in patients with Graves' ophthalmopathy and HT,

as well as in normal donors who had neither anti-Tg A-Abs nor an ocular pathology.

Structural differences localized near the thyroxine-forming sites in the Tgs from different animal species have been detected by monoclonal anti-Tg A-Abs (m anti-Tg A-Abs). The known primary sequence similarities result in different three-dimensional structures of the sites. Chan *et al.* (1986) suggested that these species-specific structures were formed because sequences from distant regions are brought close by chain folding to form the Tg hormonogenic sites.

Several factors determine the immunogenicity of the Tg molecule. Iodine content is one factor both in humans and in experimental models. In patients with a single "hot" thyroid nodule, Mariotti *et al.* (1986) reported the occurrence of A-Abs to Tg and subsequent hypothyroidism after radioactive iodine therapy. Furthermore, in patients whose HT is masked by chronic iodine deficiency, elevated serum basal Tg levels were observed (Sava *et al.*, 1986; Lima *et al.*, 1986). However, iodine therapy not only increases the Tg iodine content but also thyroid lymphocytic infiltrations (Beierwaltes, 1969; Harach *et al.*, 1985).

Similar observations have been made in experimental models. Diabetes-prone BB/W rats (Allen *et al.*, 1986a,b) develop a high incidence of spontaneous lymphocytic infiltration of the thyroid gland in addition to insulin-dependent diabetes mellitus. Administration of 0.05% iodine in the drinking water at 1 month of age significantly increases the incidence of thyroid lymphocytic infiltrations and the level of anti-Tg A-Abs in the absence of signs of hypothyroidism. However, when these BB/W rats were hemithyroidectomized before iodine supplementation, hypothyroidism was observed (E. M. Allen *et al.*, 1987). The explanation for this phenomenon remains elusive.

In another model of spontaneous autoimmune thyroiditis (SAT), feeding the Cornell C strain (CS) of chickens an iodine-deficient diet produces a weak thyroid lymphocytic infiltration without anti-Tg A-Ab production. However, birds fed 2 and 20 mg/ml of iodine showed, respectively, mild and very acute lymphocytic infiltration of their thyroid glands and produced anti-Tg A-Abs (Bagchi *et al.*, 1985a). Recently, Sundick *et al.* (1987) extracted and purified Tg from CS birds given either iodine-supplemented or iodine-deficient water. When the two Tgs with, respectively, 60 and 5 atoms of iodine per molecule were injected without adjuvant into normal birds, the highly iodinated Tg induced higher levels of anti-Tg A-Abs which reacted well with highly iodinated Tg, but poorly with weakly iodinated Tg. Comparison of the characteristics of the anti-Tg A-Abs obtained from spontaneously diseased obese strain (OS) chickens to those from chickens immunized with high-iodine-content Tg revealed

that the specificities of two kinds of anti-Tg and A-Abs were different. No cross-reactivities were detectable. This indicates that during SAT in the OS chicken pathogenic anti-Tg A-Abs are synthesized regardless of the iodine content in their diet.

The effect of iodine on Tg immunogenicity was further investigated in EAT-susceptible strains of mice, at the level of the immune T cell response. Champion *et al.* (1987b) reported that noniodinated Tg was unable to induce significant thyroid lesions *in vivo* but could trigger the production of anti-Tg A-Abs. Moreover, cloning of Tg-specific helper T cells allowed them to demonstrate that the ability of T cell clones to recognize and proliferate under Tg stimulation was correlated with a sufficient iodine content of the Tg molecule. It must be noted that these results were obtained with Tg-specific T cells unable to transfer EAT; nonetheless, EAT transfer is a property of some Tg-specific helper T cells, lines, or clones (Charreire and Michel-Béchet, 1982; Maron *et al.*, 1983; Romball and Weigle, 1987a). Does it mean, as suggested by the authors, that helper T cells involved in anti-Tg A-Ab production are distinct from those involved in EAT induction? This hypothesis would be in agreement with our experiments (Salamero *et al.*, 1987a) demonstrating that tryptic fragment(s) [TF(s)] from the Tg molecule, recognized by one monoclonal anti-Tg A-Ab, induce typical EAT with a very minimal level of A-Abs to Tg, whereas the other TFs could not. This observation suggests the existence of different Tg-specific helper T cells recognizing various Tg epitopes. However, only certain cells with particular specificities would induce and mediate EAT.

In summary, the observations describing an increase of Tg immunogenicity in relation to iodine content are convincing. However, no relationship with the induction or maintenance of the pathology has been established for either B cells producing anti-Tg A-Abs or for T cells recognizing iodinated Tg, whether the disease occurs spontaneously or is experimentally induced. Therefore, it can be hypothesized that the iodinated sequences of the Tg molecule do not include the epitope(s) recognized by immunocompetent cells responsible for thyroiditis.

Glycosylation of the Tg molecule was also reported to interfere with its immunogenicity. Tg is one of the few glycoproteins containing a glucuronic acid unit with endoglycase F (Endo F) sulfate groups (Spiro, 1977; Schneider *et al.*, 1988). The two 330-kDa subunits bear 25–30 asparagine-linked CHO chains forming biantennary (75%) or triantennary (25%) structures decorated with more sialic acid under TSH stimulation (Yamamoto *et al.*, 1984; Ronin *et al.*, 1986) and with α-galactose under basal conditions (Spiro and Bhoyroo, 1984). Taking into account that sialylation precedes Tg iodination and that, in some

human or experimental thyroiditites a defect of Tg glycosylation was concomitant with an impairment in iodination and exocytosis (Monaco and Robbins, 1973), a relationship between glycosylation and immunogenicity of the Tg molecule can be envisaged. In its favor, experiments conducted by Fenouillet *et al.* (1985, 1986) indicated that carbohydrate chains had noticeable effects on antigenic sites of Tg molecules. More precisely, when they compared the reactivity of poorly glycosylated Tg obtained from serum-free cultures of thyroid cells to that of native highly glycosylated Tg toward polyclonal anti-Tg A-Abs, they found that the poorly glycosylated Tg was fourfold less immunoreactive than was the highly glycosylated Tg. These results appear to be in conflict with recent data we obtained. In a study of the epitopes of human Tg (hTg) recognized by anti-Tg A-Abs from HT patients, hTg was first digested with trypsin and then with glycosidases, including neuraminidase, Endo-F, and Endo-H. Then their recognition by anti-Tg A-Abs was measured. As shown in Table II, 80% of the reactivity was lost when tested against the trypsinized hTg (thTg). Further treating the thTg with each of these glycosidases did not alter the reactivity with anti-Tg A-Abs, indicating that glycosylation did not mask the immunogenicity of trypsinized hTg.

Altogether, these data indicate that iodine content and glycosylation are involved in the immunogenicity of the native Tg molecule. It can be thought that degraded circulating fragments of the Tg molecule, both highly iodinated (up to 2%) (Chernoff and Rawitch, 1981) and/or glycosylated, behave as potent B cell stimulators inducing A-Ab production, similar to polyclonal B cell activators. The epitopes borne by these fragments are very rarely detected by A-Abs to Tg from patients or animals suffering from thyroiditis. It can be hypothesized that, under normal physiological conditions, the glycosylated and iodinated structures could assume a preventive role against thyroiditis by rendering the pathogenic determinants inaccessible.

TABLE II
A-Abs TO Tg FROM HT PATIENTS SIMILARLY RECOGNIZE GLYCOSYLATED OR DEGLYCOSYLATED HUMAN TRYPTIC FRAGMENTS FROM hTg

Patients' sera	Native hTg	trypsinized hTg plus treatment by: 0	Endo F	Endo H	Neuraminidase
1	430[a]	53	55	70	65
2	465	125	112	110	107
3	387	76	75	72	71

[a] Optical density at 405 nm in an ELISA.

B. Thyroid Peroxidase (TPO)

Patients with HT have a high prevalence of Abs directed against a component different from Tg and now called TPO instead of thyroid microsomal (Mic) antigen (Roitt *et al.*, 1956; Belyavin and Trotter, 1959). This Ag was detected by Khoury *et al.* (1981) on the apical cell surface of thyroid follicular cells and was on a cloned rat thyroid cell line grown *in vitro* by Chiovato *et al.* (1985). It plays a fundamental role in the biosynthesis of thyroid hormones, catalyzing both the iodination of the precursor protein Tg and the intramolecular coupling of specific iodotyrosines into thyroid hormones. Its expression under TSH regulation involves cyclic AMP production and requires mRNA formation and subsequent protein synthesis (Chiovato *et al.*, 1988). Moreover, lectin and leukoagglutinin were able to increase Mic Ag expression on the thyrocyte cell surface, an effect further augmented in the presence of IFN-γ (Iwatani *et al.*, 1987a).

TPO was characterized and recently isolated with a monoclonal Ab (MAb) in hog (Ohtaki *et al.*, 1985, 1986) and in man (Czarnocka *et al.*, 1985; Hamada *et al.*, 1985) using Western blot and immunoprecipitation techniques. TPO is a 107-kDa protein with an isoelectric point of 7.0. In 1987, the molecular cloning of the genes coding for porcine (Magnusson *et al.*, 1987) and human TPO (Kimura *et al.*, 1987; Libert *et al.*, 1987) was reported. In humans this gene, which maps to the short arm of chromosome 2, codes for a 104-kDa protein consisting of 933 amino acids. Moreover, in its extracellular part, TPO has homologies with bovine cytochrome *c* oxidase polypeptide, human C4b, and epidermal growth factor receptor gene families. These numerous homologies render TPO an A-Ag with a mosaic structure of nuclear and mitochondrial gene products.

The identity between TPO and Mic Ag was suspected when comparing their biochemical and immunological reactivities (Portmann *et al.*, 1988; Hamada *et al.*, 1987). Using sera from HT patients, significant correlations were observed between Mic Ag and TPO activities (Portmann *et al.*, 1985; Czarnocka *et al.*, 1985; Mariotti *et al.*, 1987). More precisely, TPO immunoactivity was immunoprecipitated by sera containing anti-Mic Abs and, conversely, TPO was able to absorb anti-Mic Abs. In these reactions, interference with Tg or anti-Tg Abs was never observed. The identity between TPO and Mic Ag was proved when the sequence of one Mic Ag was reported by Seto *et al.* (1987); the homology with TPO was verified at the amino acid sequence level and in the identity of the mRNA sizes.

Recently, MAbs were raised against thyroid Mic Ag (Portmann *et al.*, 1988). The identity between TPO and Mic Ag was once again confirmed with these reagents. Mic Ag, like TPO, is formed by two polypeptides

which share some common epitopes recognized by the MAbs. These common epitopes are different from those recognized by polyclonal sera. After enzyme treatment, they behave differently: the immunoreactivity of the Mic Ag is highly dependent upon a trypsin-sensitive site, important in both the recognition of the Ag by polyclonal sera and in the biological effect of the Mic Ag. More precisely, Nakajima *et al.* (1987) reported that TPO activity and the major Mic Ab-binding site(s) was (were) located on trypsin fragments which contained a disulfide bridge. It also appeared that, in a rat thyroid cell line named FRTL-5 (Ambesi-Impiombato *et al.*, 1980), some Abs to Mic or TPO did not totally overlap in their abilities to bind TPO or Mic Ag, suggesting some conformational discrepancies between these two Ags (Chiovato *et al.*, 1988). One can wonder if these discrepancies might be due to the use of a particular rat thyroid cell line. The role of Mic/TPO A-Abs in HT was postulated in thyroid destruction because of their ability to fix complement (Holborow *et al.*, 1959; Khoury *et al.*, 1981). In addition, their titers were shown to be highly correlated with the levels of cytotoxic effects of HT sera (Bogner *et al.*, 1984; Salvi *et al.*, 1988).

III. Experimental Models

Experimental models of thyroiditis have been useful in elucidating some of the pathogenic mechanisms involved in the triggering and the development of thyroid autoimmune reactivity. In contrast to spontaneous models, induced experimental models enable, and even require, the definition of immunization procedures, particularly in terms of antigenic characterization, i.e., dose or purity of Ag, nature of the adjuvant used, and injection schedule.

Several models of thyroiditis have been described. Some occur spontaneously in various species of animals; others are induced after *in vivo* treatment. In the latter case, either suitable adjuvants rendering the A-Ag immunogenic or the manipulation of the recipient's T cell subsets are used. For the diagnosis of EAT, the following criteria have to be fulfilled: (1) circulating anti-Tg A-Abs and (2) infiltration of the thyroid gland by lymphocytes. These two criteria for EAT are not equivalent; EAT can develop independently of the presence of A-Ab production. This discrepancy will be discussed below.

A. Spontaneous Autoimmune Thyroiditis

Different species of animals, such as the OS chicken, the Buffalo (BUF) and the BB/W strains of rats, and Praomys, a desert rodent, develop SAT.

The most remarkable predisposition to SAT was inherited by the OS chicken. The model was established by Cole in 1966 at a breeding center where the diseased animals represent 1% of the female birds. Chickens

are particularly convenient laboratory animals because it is possible to selectively deplete B lymphocytes by bursectomy (Wick *et al.*, 1970) and T lymphocytes by thymectomy (Tx). Moreover, the embryo develops in an accessible egg and many offspring can be derived from one pair of parents (for review see Wick *et al.*, 1985, 1986).

OS chickens, among which over 95% spontaneously develop SAT, were selected from the CS chickens on the basis of phenotypic symptoms of hereditary hypothyroidism. Phenotypically, females are smaller than normal and develop long, downy, juvenile feathers and large subcutaneous fat deposits. SAT in OS chickens occurs during the first 3 weeks after hatching and is characterized by massive lymphoid cell infiltration of the thyroid gland with consequent hypothyroidism and circulating A-Abs to Tg after 4 weeks of age. The OS chickens are hyperresponsive to several Ags (Khoury *et al.*, 1982; Aichinger *et al.*, 1985).

The OS chicken has proved a particularly useful animal model for HT for two reasons (Rose *et al.*, 1981; Wick *et al.*, 1982). First, germinal centers, detected at 6 to 8 weeks of age, replace functional thyroid tissue and result in fibrosis of the thyroid glands. Second, at the time of hatching, autologous C3 and IgG deposits forming immune complexes consisting of Tg and A-Abs to Tg transferred from the mother are present in the basal lamina of thyroid glands (Katz *et al.*, 1981, 1986; Kofler *et al.*, 1983). It was thought that the immune complexes, which included Tg or thyroid Ags, could prevent the immune system from interacting with these Ags during the tolerance induction period. This lack of tolerance induction in early embryonic life could be the cause of the appearance of antithyroid autoimmunity.

While thymocytes from OS chickens can transfer EAT (Livezey *et al.*, 1981), B cells and their products cannot do so (Polley *et al.*, 1981). However, anti-Tg A-Abs were shown to be important in the SAT of the OS chickens: they cause pathological changes and precipitate the disease when injected into OS chickens (Jaroszewski *et al.*, 1978; Neu *et al.*, 1985).

Among the multiple possible causes of SAT in these animals, the roles of abnormal or increased circulating Tg, of previous viral infection, and of the influence of sex hormones have been examined. Studies devoted to detecting abnormal Tg with unique determinants in OS chickens have failed. A-Abs to Tg from OS chickens similarly recognize normal and OS Tg. In spite of these negative results, Wick *et al.* (1986) undertook new investigations using different methodologies: gel filtration, ultracentrifugation in a sucrose gradient, negative-staining electron microscopy, and the production of MAbs to normal chicken Tg. Preliminary results indicate a higher degree of heterogeneity of OS Tg.

Sanker *et al.* (1983) demonstrated that Tg levels were highly increased exclusively in the sera of OS chickens, independent of their B haplotypes or of their breeding-center origins, while CS chickens, the original parental strain of OS obese chicken, showed four to five times less circulating Tg.

SAT in OS chickens appears to be linked to (1) a general T cell hyperactivity assessed by increased T cell proliferation, interleukin 2 (IL-2) production, and IL-2 receptor expression (Schauenstein *et al.*, 1985); (2) a decrease in glucocorticoid production (Fässler *et al.*, 1986); (3) a deficiency of the neuroendocrine feedback control of the immune system in response to cytokine (Schauenstein *et al.*, 1987); and (4) specific endogenous virus ev22. Wick *et al.* (1985, 1986) have hypothesized that molecular mimicry could occur between this endogenous virus and thyroid A-Ags, leading to A-Ag-specific T cell stimulation and therefore to autoimmunity. In these studies, endogenous virus, which harbored retrovirus-related sequences, was detected only in OS chicken thyroid glands.

In BUF rats, Silverman and Rose (1971, 1975a) observed the formation of germinal centers in the thyroid glands and a low incidence of SAT in females—14% in 9- to 12-week-old animals rising to 48% in exbreeders. This level of thyroiditis can be greatly increased in both males and females by neonatal thymectomy (NTx) or simultaneous immunization with Tg (Silverman and Rose, 1974a; Penhale *et al.*, 1975, 1976) and various agents, such as subcutaneous administration of trypan blue (Reuber, 1970) or of subcarcinogenic doses of 3-methylcholanthrene (Glover *et al.*, 1968; Silverman and Rose, 1971, 1975b).

In 1987, S. B. Cohen and Weetman demonstrated that in the BUF rats hypothyroidism after NTx closely resembled human HT in terms of levels of anti-Tg A-Abs, enlargement of the thyroid gland, elevated TSH level, and severity of the disease. Later, when they studied this thyroiditis with MAbs specific to MHC Ags or lymphoid cell subpopulations, they found an increase in endothelial class II Ag expression and an infiltration of B and T cells expressing both helper and cytotoxic/suppressor phenotypes. The thyroid follicular class II Ag expression, which was detected only in the presence of T cell infiltrates, in a minority of animals, was maximal between 12 and 24 weeks post-NTx, concordant with the peak severity of the thyroiditis. This NTx-induced EAT in BUF rats is a transient disease: it was not apparent during the first 5 weeks after NTx and disappeared 34 weeks post-NTx (Cohen *et al.*, 1988).

In SAT occurring in humans, rats, OS chickens or Praomys (Solleveld *et al.*, 1982), a significant female preponderance for the thyroid lesions and hypothyroidism was observed. This higher frequency in females is

a general hallmark of autoimmune disease (Talal, 1976). One explanation proposed for this sex linkage was that testosterone affected the development of the immune system, resulting in decreased activity of autoreactive clones (Geschwind and Behan, 1982). Supporting this hypothesis, testosterone administration to the OS chickens significantly decreased thyroid infiltration by lymphocytes, while castration of the CS chickens significantly enhanced infiltration (Gause and Marsh, 1985). The mechanisms by which testosterone exerted its effect are not clear. There could be suppressive effects associated with changes in thymic development and in effector or regulatory T cell activity as suggested by the effect of testosterone treatments of OS chickens from hatching to 6 weeks of age; the frequencies of Ia^{+} cells and T and B cells in the thyroid are reduced while thymuses were slightly enlarged (Gause and Marsh, 1986).

These studies lead us to conclude that none of the parameters described above (strain, sex, level of Tg, T and B cell depletion, viral infection, etc.) can alone trigger SAT. However, we can envision synergies which would allow the disease to occur. This point will be further considered in Section IV.

B. Induction of Experimental Autoimmune Thyroiditis

Two main approaches were developed in mice or rats to induce EAT. Their fundamental goal was either to increase the circulating level and the immunogenicity of Tg by the use of suitable adjuvants or to increase the number of Tg-specific T cells. These methodologies were the direct consequence of the demonstrated role of Tg as the Ag responsible for EAT (Rose and Witebsky, 1956; ElRehewy *et al.*, 1981). This role was advanced by Rose and Witebsky in 1956. They were the first to induce thyroiditis in rabbits by subcutaneous immunization with a syngeneic thyroid gland homogenate emulsified in complete Freund's adjuvant (CFA). The disease was confirmed by the presence of A-Abs to homologous Tg determinants, infiltration of the thyroid gland by mononuclear cells, and damage to acinar cells. Moreover, after they separated (Shulman *et al.*, 1955) the cellular and soluble compartments of the thyroid gland, they showed that thyroiditis was induced by the soluble fraction where Tg represents 75–80% of the proteins (Shulman, 1971). Adjuvants were used to increase Tg immunogenicity. Some, such as CFA, an emulsion of the Tg with mineral oil and mycobacteria, allowed continuous and slow delivery of the Tg in a putatively modified conformation. Even though the complete action of CFA has never been totally elucidated, emulsified Tg in CFA is highly immunogenic (Rose *et al.*, 1971; Rose and Kong, 1983). Other adjuvants, such as lipopolysaccharide

(LPS) (Esquivel *et al.*, 1977; Okayasu and Hatakeyama, 1984), a polyclonal T cell-independent B cell activator, or muramyl dipeptide (Rose *et al.*, 1981) or poly(A:U), a stimulator of both Ab production and cell-mediated immunity (Esquivel *et al.*, 1978; Hovanessian *et al.*, 1988), have enabled investigators to study autoimmune responses induced by aqueous, unmodified syngeneic Tg. Since EAT can be induced in 50% of mice that receive only injections of syngeneic murine Tg (MTg), 20 μg for 4 days per week, for four weeks, this fully confirms that unmodified Tg can induce EAT. In this case, the need for adjuvant was bypassed by the repeated injections of Tg. More recently, Williams *et al.* (1987a) assessed two adjuvants, SGP (a synthetic copolymer of starch, acrylamide, and sodium acrylate) and Quil A (a plant saponin) for their abilities to induce EAT in mice using MTg as the Ag. They demonstrated the effective adjuvant activity of SGP, while Quil A was ineffective.

Among these various methods, the classical procedure was the subcutaneous injection of 30–100 μg of homologous or heterologous native Tg emulsified in CFA, followed 7–15 days later by a booster injection of the same amount of Tg emulsified in incomplete Freund's adjuvant (IFA). Using this protocol, the first signs of thyroiditis appear on day 4 after the first injection; severe lesions are seen between the third and fifth week, and then EAT remits; anti-Tg A-Abs are detectable at their highest levels as of the third week (Tomazic and Rose, 1977).

More recently, using immunochemical technology, we purified and characterized a small fragment (TF) obtained after trypsin digestion of porcine Tg (PTg) (Salamero *et al.*, 1987a). This 5- to 10-kDa TF reacted with one of the monoclonal anti-Tg A-Ab we had produced blocking the thyroid-specific T cell proliferative response (Salamero *et al.*, 1987b). Moreover, we found that this TF was able to induce EAT when injected into a good responder (GR) strain of mice (H-2^k), whereas control animals which received TF-deleted PTg (tPTg − TF) had no signs of thyroiditis. It must be noted that anti-Tg A-Ab levels were very low in the sera of mice suffering from EAT induced by PTg TF (Table III). Weigle (1965) and Kondo and Kondo (1984) previously reported experiments in total agreement with these findings. They demonstrated that rabbit Tg, partially digested by enzymes such as papain, pepsin, or leukocyte proteinases, could, in the absence of CFA, elicit the development of anti-Tg A-Abs and, more interestingly, of thyroiditis. It was postulated that the enzymatic digestion exposed pathogenic immunogenic determinants which are not normally accessible in the native Tg molecule.

The injection of MTg, considered an absolute requirement in the past for murine EAT induction, was proven not to be essential (Romball and Weigle, 1984). This can now be explained by the homology of the

TABLE III
ANTI-Tg A-Abs AND THYROID HISTOLOGY OF MICE IMMUNIZED WITH NATIVE OR TRYPSINIZED PTg PREPARATIONS IN CFA[a]

Mice injected with	Anti-Tg A-Abs	Histology
Crude PTg	1.582 ± 0.09[b]	Mild EAT
tPTg	0.481 ± 0.07	Very acute EAT
tPTg − TF	0.134 ± 0.02	Normal
TF	0.076 ± 0.08	Acute EAT

[a]Day 28 postimmunization. From Salamero *et al.* (1987a).
[b]Optical density at 405 nm in an ELISA.

sequences observed in Tgs purified from different species (Section II,A). Similarly, Simon *et al.* (1986) demonstrated the existence of epitopes shared by human and murine Tgs and recognized by murine Tg-specific cytotoxic T cells.

It must be noted that, in EAT, signs of hypothyroidism assessed by Tg or thyroid hormone production were rarely evaluated. However, in EAT-susceptible mice or rats immunized with Tg in CFA, Vladutiu and Kenney (1985) and Hassman *et al.* (1988) measured Tg, TSH and thyroxine levels, and radioactive iodine uptake by thyroid tissues. In all groups of mice, thyroxine levels and iodine uptake were significantly decreased from 2 to 8 weeks after immunization, reflecting the transient thyroid damage, whereas in rats, Tg and TSH levels were strongly augmented to reach 10 times the basal level at 5 weeks postimmunization and then returned to normal values. Similarly, Eishi and McCullagh (1988b) studied TSH and Tg levels in DA and PVG/c strains of rats immunized with syngeneic Tg. They found that, after immunization, PVG/c rats, which are resistant to EAT, developed high levels of circulating Tg, while TSH remained low, demonstrating that during EAT physiological thyroid function is decreased instead of disturbed.

These findings indicate (1) the existence on the Tg molecule of some epitopes responsible for EAT induction; (2) that these epitopes are included in almost linear fragment(s) of the Tg molecule; and (3) that most of the anti-Tg A-Abs detected during EAT recognize conformation epitopes. However, some linear fragments were also seen. These observations could explain the discrepancies between the levels of anti-Tg A-Abs and the existence of EAT.

The second approach used to induce EAT took advantage of the fact that T lymphocytes have two functions: as effectors in cell-mediated immunity and as helpers in Ab formation. The first experiments were those of Penhale *et al.* (1973), who showed, in rats, that depletion of

T lymphocytes by NTx followed by several low doses of irradiation led to the spontaneous development of typical EAT, without Tg injection. Furthermore, they demonstrated that EAT can be prevented by reconstitution of NTx and irradiated rats with viable lymphoid cells from syngeneic rats (Penhale *et al.*, 1976). These experiments provide the basic support for the argument that EAT is a consequence of the selective depletion of a T cell subpopulation responsible for the suppression of autoreactivity toward the thyroid components. Similarly, Wick *et al.* (1974) demonstrated that Tx of newly hatched OS chickens accelerated and aggravated the lymphoid infiltration of the thyroid gland and raised the incidence of birds with anti-Tg A-Abs. The existence of T suppressor cells specific to Tg was further investigated by Kong *et al.* (1982), who induced tolerance to autologous MTg either by injection into good responder strains of mice of exogenous MTg or by thyroid stimulation through physiological conditions, leading to the release of endogenous Tg. The endogenous Tg release was obtained by pretreatments with TSH and thyrotropin-releasing hormone before immunization with MTg and LPS (Lewis *et al.*, 1987). Hence, Kong and Lewis (1988) hypothesized three levels of EAT regulation by suppressive mechanisms: the first would be localized at the self-tolerance level, where suppressor T cells would exert clonal dominance; the second would intervene when exogenous or endogenous Tg is suddenly increased, at which time suppressor T cells have to expand rapidly in order to suppress the important immunogenic challenge; and the third would act at the level of the genetic control of EAT induction. In addition, Kotani *et al.* (1981) postulated that, along with a suppressor T cell defect, other mechanisms, such as MHC haplotype, high radiosensitivity of the thyroid gland, or NK cell activation, could also interfere.

A role for T cells as EAT inducers was demonstrated using transfer experiments. Vladutiu and Rose (1975) thymectomized, irradiated, and reconstituted mice with either syngeneic B or B and T lymphocytes before immunization with homologous thyroid extracts emulsified in CFA. Only animals reconstituted with T and B lymphocytes developed thyroid infiltrations and A-Abs to Tg, whereas nude mice were unable to develop EAT when similarly immunized. These results were further confirmed by Esquivel *et al.* (1978), who demonstrated the existence of Tg-reactive T cells in good responder strains of mice, using poly(A:U) as the Tg adjuvant. At the same time, Livezey *et al.* (1981) provided evidence for the direct involvement of thymocytes in thyroid infiltration and production of A-Abs to Tg during OS chicken SAT.

In the 1980s, the question of the helper nature of the T cell subset inducing EAT was raised by several groups (Rose *et al.*, 1981; Yeni *et al.*, 1980, 1981). In *in vitro* experiments in the presence of MTg or syngeneic TEC, the specific proliferation of lymphocytes from HCBA ($H\text{-}2^k$) strains of mice was shown to be that of the helper T cells.

In numerous experiments, the role of helper T cells in EAT was clearly proved. EAT follows injection into mice or guinea pigs of Tg in CFA or of syngeneic spleen or lymph node cells previously stimulated by Tg or syngeneic TEC (Braley-Mullen *et al.*, 1981, 1985; Charreire and Michel-Béchet, 1982; Okayasu, 1985; Simon *et al.*, 1985; Williams *et al.*, 1987b). In the transfer of EAT by TEC-sensitized spleen cells, we demonstrated that the stimulated cells transferring EAT belong to the helper T cell subset (Charreire, 1982b) and were specific to Tg borne by TEC, as evidence by detection of a secondary response exclusively with soluble Tg presented with self-MHC. The transfer into naive syngeneic recipients of Tg-specific helper T cell lines (Maron *et al.*, 1983) or clones (Romball and Weigle, 1987a) definitively demonstrated a role for T helper cells in EAT induction. In our experiments, the specificity of the T helper cell proliferative response to Tg was further assessed by its inhibition only when monoclonal anti-Tg A-Abs were added first to the stimulator syngeneic TEC (Salamero *et al.*, 1987b). Attention must be drawn to the fact that blockage of the syngeneic proliferative T cell response on TEC needed high amounts of A-Abs (250–500 μg/ml). This could be due to both the facts that monoclonal A-Abs to autologous Tg were produced against native MTg and that TEC expressed fragments of Tg resulting from the unfolding of the large Tg molecule or from its degradation necessary for the synthesis of thyroid hormones. Similar blocking of a T cell proliferative response specific to a cytochrome *c* peptide was obtained with high amounts of MAb raised against native cytochrome *c* when the peptide was presented with self-MHC (Corradin and Engers, 1984; Walden *et al.*, 1986).

Sugihara *et al.* (1988) induced typical EAT in T cell-depleted B cell mice by adoptive transfer of naive lymphoid cells pretreated with anti-Lyt-1 Ab plus complement. They also found that a subpopulation of Lyt-1 "dull" L3T4 "bright" cells from the lymphoid tissues of normal mice were the EAT inducer cells. Moreover, they observed a correlation between anti-Tg A-Ab titer and disease severity.

Knight *et al.* (1988) induced thyroiditis in susceptible strains of mice by intravenous injection of 10^5 syngeneic dendritic cells (DCs) either pulsed *in vitro* with Tg or obtained from animals immunized with Tg in CFA 2 weeks earlier. Interestingly, the histopathological and serological manifestations were not similar in these two experiments. When animals received *in vitro* Tg-pulsed DCs, the disease was in an acute phase in less than one-third of mice 2 weeks after the intravenous immunization, and A-Abs to Tg never significantly increased. In contrast, when mice received *in vivo* Tg-pulsed DCs, EAT peaked 4 weeks after the injection in more than 50% of the recipients and A-Abs to Tg were detectable

in 80% of their sera. Aside from suggesting that *in vivo* and *in vitro* Ag pulsing are different, these experiments show that EAT is a heterogeneous disease and that the DC, a "professional" antigen-presenting cell (APC), can initiate and maintain EAT with an almost physiological concentration of A-Ag.

Romball and Weigle (1987b) examined the effects of age on the induction of EAT in susceptible mice, using the classical protocol of immunization with Tg in CFA. They showed that aged mice developed thyroid lesions either comparable to or slightly less intense than those observed in young mice, but the older animals showed a drastic reduction in their abilities to produce anti-Tg A-Abs, to mount a delayed-type hypersensitivity to MTg, to initiate Tg-specific T cell proliferation, and to develop *in vitro* T helper cell function(s) in response to MTg. This decreased capacity of aged T cells to proliferate *in vitro* under Tg stimulation was comparable to the significantly decreased T cell proliferative response we previously observed, when 18-month-old spleen or lymph node lymphocytes from susceptible strains of mice were cocultured with syngeneic TECs (Charreire, 1982a). Romball and Weigle (1987b) also observed a disparity between the loss of immune reactivity of T cells with aging and the greater susceptibility to thyroiditis; the transfer of concanavalin A-activated Tg-primed young splenocytes to young and aged recipients resulted in more acute EAT in aged animals.

Simon *et al.* (1985) demonstrated that Lyt-1 T cells proliferate in response to syngeneic and xenogeneic Tgs and that a marked proliferative response was not necessarily a predictor of the disease severity but correlated with disease susceptibility. The lack of a relationship between the T cell proliferative response to Tg and disease severity can be explained by taking into account the numerous putative epitopes on the large Tg molecule able to stimulate T cells. A dose–response relationship might be certain if the EAT-inducing Tg peptide(s) was(were) known and used for EAT induction, instead of the native Tg molecule.

Comparisons of EAT developed after injection of Tg in CFA to that induced by injection of a Tg-specific T cell subset or Tg-pulsed DCs showed that a uniform feature of the disease is the lymphocytic infiltrations of the thyroid glands; detectability of anti-Tg A-Abs is a variable parameter. In EAT induced by Tg-specific T helper cells or clones, anti-Tg A-Abs are detected at very low levels and histopathological parameters of EAT are weaker than when thyroiditis was initiated by active immunization of animals with Tg in CFA. The independence of these two parameters, cell infiltration of the thyroid gland and anti-Tg A-Ab production, was further confirmed by our recent experiments. When we induced EAT in mice with TF from PTg, we obtained a typical and

severe thyroiditis in terms of lymphocytic infiltration while a weak anti-Tg A-Ab production was measured (Table III). Moreover, these experiments demonstrated that 70–80% of the anti-Tg A-Abs detected after immunization with native Tg were directed against conformational epitopes of the Tg molecule; the majority of them were not related to EAT. However, this clear dissociation between anti-Tg A-Abs and lymphocytic infiltration was recently put in doubt by two sets of experiments: in female August rats conventionally immunized with Tg in CFA (Hassman *et al.*, 1988) and in B cell mice transferred with Lyt-1 bright-depleted normal syngeneic lymphoid cells (Sugihara *et al.*, 1988), a significant relationship was found between anti-Tg A-Abs and the severity of thyroiditis assessed by lymphocytic infiltration of the thyroid glands. In the second series of experiments, it can be postulated that transferred T cells were specific to the pathogenic epitope of the Tg molecule and, therefore, only generated pathogenic A-Abs to Tg. In the first set of experiments, no clear explanation can be proposed since the methodologies are comparable to those used previously.

To summarize, while the role of suppressor cells in EAT has never been established directly and the principle supporting observation is indirect (Penhale's experiments), nonetheless Tg-specific helper T cells as EAT inducers is clearly established through transfer experiments using lines or clones.

IV. Genetic Control of Thyroiditis

The very existence of defined strains of animals at risk for SAT suggests a primary pathogenetic role for genetic control mechanisms. The ability to increase the percentages of diseased animals among SAT-susceptible strains by crosses based on the selection of phenotypic characteristics, as that made for OS chickens (Cole, 1966), further supports a genetic control of the disease.

A. Spontaneous Autoimmune Thyroiditis

Numerous species, including, rats, chickens, guinea pigs, and dogs (for review, see Weigle, 1980), develop SAT. For the majority of these animals, the MHC is not defined, and pure strains, genetic recombinants, and reagents to identify cell-surface markers are not available. However, in rats and chickens, knowledge of the MHC has been sufficiently developed (Hala, 1977; Guillemot *et al.*, 1988) to enable the study of the genetic control of SAT.

In 1974, Bacon *et al.* first reported that OS SAT was under the control of the B locus of the chicken MHC: B15/B15 was a good responder whereas B5/B5 was a poor responder (PR). This result was not verified (Bacon *et al.*, 1981; Boyd *et al.*, 1983) and a three-locus model was

proposed (Wick *et al.*, 1979; Rose *et al.*, 1980). Observations suggested that the organ-specific autoimmune disease resulted from a dysfunction of the immune system and a primary, possibly genetically determined, aberration of the target organ that made it susceptible to the autoimmune attack.

A first set of genes, dominant and independent of MHC genes, would control the immune response. The existence of this set of genes was determined from SAT studies in the progeny of two sets of (OS × CB) F_1 chickens: CB (B12/B12) normal inbred males and females crossed with OS (B15/B15) birds. Circulating anti-Tg A-Abs appeared only in chickens issued from CB mothers and were detected when the offspring were 20 weeks old, instead of after hatching, as in OS birds (Neu *et al.* 1985, 1986). The progeny from the reverse combination (male CB) was negative for circulating anti-Tg A-Abs and for thyroid dysfunction or gland damage. Because the thyroid destruction was not inherited by OS males, the authors concluded that maternal anti-Tg A-Abs in F_1 progeny from CB males transferred from the egg yolk to the embryo might prevent the F_1 chicken immune system from producing anti-Tg A-Abs by blocking or eliminating the pathological thyroid Ags. A second set of genes, controlling primary changes of the target organ, would be responsible for both the control of thyroid functions (iodine uptake, Tg and thyroid hormone synthesis, etc. and the susceptibility to the autoimmune process (viral infection and A-Ag alteration). A third set of genes that control the modulation of the autoimmune disease by the endocrine system was recently postulated (Schauenstein *et al.*, 1987). More precisely, a disturbance in immune neuroendocrine communication, found exclusively in OS chickens, was evidenced by a lack of production of circulating corticosterone in response to the immune signals of Ag challenge or lymphokine injection. Another disturbance of the immune endocrine circuit, localized at the level of sex hormone control, was evidenced by a significantly increased susceptibility to autoimmune disorders in females. On the basis of these numerous experimental data, genes controlling the immune response seemed to be dominant, whereas those responsible for thyroid gland susceptibility appeared to be recessive. Very recently, Kroemer *et al.* (1988) further analyzed the genetic control of extrathyroidal features of OS SAT using (OS × CB) F_1 and (F_1 × CB) back-crossed chickens. They found that OS T cell hyperfunctioning (Section III,A) was transmitted as an autosomal dominant trait and cosegregated in back-crossed animals whereas *in vivo* hyporesponsiveness to the corticosterone inducers was dominantly inherited and cosegregated with the OS-specific endogenous virus ev22. High levels of corticosteroid-binding globulin were recessively transmitted. None of these traits was linked to the MHC.

If one considers that the OS chicken develops SAT when most or all these sets of genes are simultaneously present and active (Wick *et al.*, 1985; Kuppers *et al.*, 1988), then multigenic control of SAT could represent a form of homeostasis. Polygenic controls should protect against autoimmune reactivity because the simultaneous dysregulation of several sets of genes is more difficult to trigger than dysregulation of a unique set. However, because none of these genes has been mapped, multigenic control of OS chicken SAT remains an appealing hypothesis.

Human HT might also be under multigenic control. Because HT has a very low incidence, the studies devoted to its genetic control are limited in size and number and are noted for discrepancies except when histopathological studies were performed. Briefly, HT patients were divided into two groups based on whether or not they had a goiter. The consensus was that patients with goitrous thyroiditis do not have HLA-A, -B, and -C haplotype disequilibrium (Farid and Bear, 1981). In contrast, patients with atrophic thyroiditis showed an increased incidence of HLA-B8 (for review, see Farid, 1981) and a strong association with HLA-DR3 (Thomsen *et al.*, 1983). HLA-DR5 was found to be associated with goitrous thyroiditis, while HLA-DR3 was slightly and nonsignificantly reduced (Farid *et al.*, 1981). However, Farid and Thompson (1986) and Farid and Balazs (1988) recently typed 52 patients with goitrous thyroiditis and noted an increase in HLA-DR4 and a nonsignificant decrease in DR5. This discrepancy between two sets of results obtained by the same group remains unexplained.

In various human autoimmune pathologies, including diabetes (Cohen-Haguenauer *et al.*, 1985; N. Cohen *et al.*, 1986). rheumatoid arthritis (Legrand *et al.*, 1984; Festenstein *et al.*, 1986), and multiple sclerosis (Marcadet *et al.*, 1985), the approaches of molecular biology through restriction fragment length polymorphism analysis or amino acid sequences of class II Ags (J. A. Todd *et al.*, 1988; Morel *et al.*, 1988) are highly efficient in elucidating relationships between autoimmune pathologies and class II genes. These methodologies have not yet been applied to HT.

B. Experimentally Induced Autoimmune Thyroiditis

Pure strains of mice, including intra-H-2 recombinants, and monoclonal anti-class-I or anti-class-II MHC Abs have enabled a precise study of the genetic control of EAT in mice. These studies emerged in the 1970s when significant differences in the immune responsiveness of different mouse strains to various Ags were reported and Ir genes were described (Levine *et al.*, 1963; Benacerraf and McDevitt, 1972; Benacerraf, 1978). Similar approaches were applied to detect a relationship between murine MHC haplotypes and the incidence of EAT.

In the earlier experiments of Vladutiu and Rose (1971a), the disease was induced using the classical protocol of Tg in CFA injection. The 35 different strains of mice, which cover 11 different *H-2* haplotypes, showed a striking correlation between EAT incidence and *H-2* haplotypes. The authors concluded that *H-2*^{s} and *H-2*^{k} haplotypes were consistently excellent responders, *H-2*^{q} strains were GR, *H-2*^{a} strains were fairly good whereas *H-2*^{b} and *H-2*^{d} were PR, and *H-2*^{v} were very poor. Moreover, using F_1 animals (*H-2*^{sd}), they demonstrated that susceptibility to EAT was a dominant trait.

In the 1980s, when the congenic strains of mice became available, genetic control experiments of susceptibility to EAT were reexamined (Beisel *et al.*, 1982a). After immunization with Tg in CFA, the pattern of *H-2* susceptibility to EAT in congenic mice was perfectly superimposable on the GR strains of inbred mice, demonstrating that the genetic control of EAT was a general phenomenon. Because the congenic strains of mice were selected from PR strains of mice to Tg, the authors observed that EAT induced in the strains carrying the GR *H-2* haplotypes (*H-2*^{k}, *H-2*^{a}, *H-2*^{s}) on the PR strain background was much less severe. This decreased disease severity mainly affected lymphocytic infiltration rather than anti-Tg A-Ab production, demonstrating that, in addition to the role played by the *H-2* gene, gene(s) located outside the *H-2* complex influence(s) EAT. Continuing this approach, intra-*H-2* recombinant strains of mice were similarly immunized to investigate which part of the *H-2* complex determined the susceptibility to EAT. They found that EAT was linked to the *I-A* subregion of the *H-2* complex (Beisel *et al.*, 1982b).

At the same time, using our *in vitro* model of murine T lymphocyte sensitization on syngeneic TECs in culture, we demonstrated that the intensity of the T cell proliferative responses studied in various inbred strains of mice as well as in congenic mice on a B10 background correlated with susceptibility to EAT induced by Tg in CFA: the higher the proliferative response, the more susceptible the mouse strain to EAT (Salamero and Charreire, 1983a). Two other similarities with the conventional EAT model were observed: the T cell proliferative response was also under the genetic control of gene(s) located outside the *H-2* complex and it was linked to genes located within the *I-A* subregion (Salamero and Charreire, 1983b). These last results were comparable to those of Tomazic *et al.* (1974), who described the principal gene controlling EAT using an *in vitro* proliferative response to MTg, the Tg-*Ir* gene, which mapped to the *K* end and/or the *I-A* subregion of the murine MHC.

In parallel, the influence of class I MHC antigens was suggested by the *in vitro* experiments of Tomazic *et al.* (1974), Kong *et al.* (1979) and

Maron and Cohen (1979). Using EAT induction after injection of Tg in CFA into congenic strains of B10 mice bearing differences exclusively located at the D end of the *H-2* locus, Kong *et al.* (1979) found that animals carrying D^k have the highest degree of thyroid pathology, while those bearing D^d have markedly reduced infiltrations. Similarly, Maron and Cohen (1979) found an influence of the K end locus on EAT susceptibility, using the same protocol of EAT induction in genetically defined strains of mice: $H\text{-}2^k$, $H\text{-}2^b$, and a particular $H\text{-}2^b$ mouse bearing a mutation, $H\text{-}2^{ba}$, localized at the MHC K end (Klein, 1978). In these three strains of mice, respectively, 82, 20, and 79% of the animals developed EAT, while no variations of anti-Tg A-Abs were detected. In 1980, Maron and Cohen confirmed these results by transplanting thyroid grafts bearing $H\text{-}2^{ba}$ and $H\text{-}2^b$ haplotypes into F_1 recipients ($H\text{-}2^{ba \times b}$). After classical immunization with Tg in CFA, endogenous thyroid and transplanted thyroid showed lymphocytic infiltrations in, respectively, 54 ($H\text{-}2^{ba}$), 23 ($H\text{-}2^b$), and 60% ($H\text{-}2^{ba \times b}$). Furthermore, to identify the organs in which the MHC genotype was decisive for EAT, 4 weeks before EAT induction they transplanted irradiated thymus glands carrying either $H\text{-}2^{ba}$ or the recipient MHC into nude mice bearing $H\text{-}2^{kb}$, $H\text{-}2^{db}$, and $H\text{-}2^b$ MHC. The EAT that they observed demonstrated that the syndrome's pathophysiology was controlled by specific *H-2* genotypes in both the target thyroid gland and the thymus.

At the same time, Ben-Nun *et al.* (1980) investigated the influence of MHC genes at the level of the target thyroid gland. Susceptible ($H\text{-}2^{kb}$) F_1 mice had thyroid glands originating from susceptible ($H\text{-}2^k$) and resistant ($H\text{-}2^b$) parental strains implanted under their kidney capsules, before immunization with Tg emulsified in CFA. Examination of the three thyroids showed that transplanted glands of the $H\text{-}2^k$ haplotype developed EAT, while $H\text{-}2^b$ thyroids did not. The role of mutation at the H-2K or H-2D end in altering the immune response phenotype of EAT was further confirmed in $H\text{-}2^b$, $H\text{-}2^d$, and $H\text{-}2^k$ and their respective mutants (Maron *et al.*, 1983). These authors concluded that the genetic control of susceptibility to EAT was expressed in both the immune system and in the thyroid gland. Similar observations were reported by Okayasu and Hatakeyama (1984), who induced EAT by LPS injection 6 hours after thyroid transplantation into F_1 mice; the transplanted thyroid glands provided the source of MTg.

The respective contributions of the immune system and the target organ in EAT development were recently reexamined by Eishi and McCullagh (1988a). In hosts immunologically tolerant to allografts from susceptible and resistant strains of rats, thyroid grafts were exchanged in both directions. In complete contrast to the experiments discussed

above, the severity of EAT in transplanted thyroid tissue invariably matched that of the host thyroid irrespective of the genetic susceptibility of the graft donor. These findings cast doubt on the proposal that genetically determined resistance to EAT is mediated to any extent at the level of the target organ and emphasize the substantially greater importance of the immune system in determining susceptibility to EAT. Further experiments are needed to elucidate this point since the methodologies used in the contradictory sets of experiments appear to be similar.

In all cases, the pattern of H-2 control of EAT evidenced by GR and PR strains of mice was general and was found with the multiple methods of EAT induction. As originally observed by Vladutiu and Rose (1971a), A-Abs to Tg were much less discriminating than was seen in histopathological studies; after immunization with Tg in CFA, PR strains of mice develop similar levels of anti-Tg A-Abs. This observation will be discussed later, taking into consideration the new data we obtained on the role of specific antiidiotypic Abs in EAT (Section VI).

V. Cellular Immune Responses during Thyroiditis

The thyroid gland is composed of numerous functional units, the follicles, bordered by a single layer of thyroid epithelial cells surrounding a lumen containing the colloid. Capillaries and connective tissues ordinarily comprise a small proportion of the thyroid gland. Lymphoid cells infiltrating the thyroid gland are never normal; their presence indicates a pathological status.

In HT, SAT, and EAT, the thyroid gland is diffusely infiltrated with lymphocytes, which can aggregate to form secondary follicles. Plasma cells are also present and macrophages can be seen, especially within the colloid. If the follicular architecture is disrupted, thyroiditis, as defined by the presence of anti-Tg A-Abs and the infiltration of the thyroid gland, is fulminant.

A. Lymphoid Cell Populations

When MAbs directed against human T cell subsets became available, numerous studies were conducted on peripheral blood lymphocytes (PBLs) or on lymphocytes infiltrating the thyroid glands of HT patients to determine phenotypic modifications of the T cell subsets.

a. In Humans. Compared to PBLs from normal donors, a global decrease of $CD3^+$ T cells, initially assessed by sheep rosette-forming cells and then by MAbs, was observed (Iwatani *et al.*, 1983; Fournier and Charreire, 1983; Charreire *et al.*, 1984), due mainly to a significant decrease of the inducer $CD4^+$ T cell subset. The results concerning the

$CD8^+$ subset were discordant. Canonica *et al.* (1982) and Iwatani *et al.* (1983) observed $CD8^+$ levels comparable to those of normal donors, whereas Fournier and Charreire (1983), Charreire *et al.* (1984), and Davies and Platzer (1986) showed a nonsignificant decrease, and Okita *et al.* (1981a), Raeman *et al.* (1981), Bonnyns *et al.* (1983), and Sridama *et al.* (1982) found a significant decrease. In contrast, an increase of $CD8^+$ T cell subset was recently reported by Bagnasco *et al.* (1987) and was confirmed by the use of dual-laser flow microfluorocytometry, which is a more precise technique (Chan and Walfish, 1986a,b). As regards activated T cells bearing DR Ags, their increase is uniformly described (Canonica *et al.*, 1982; Bonnyns *et al.*, 1983; Fournier and Charreire, 1983; Chan and Walfish, 1986a). This observation is in agreement with the functional tests developed by Okita *et al.* (1981b). Similarly, increased numbers of B cells bearing surface Ig (McLachlan *et al.*, 1983a; Fournier and Charreire, 1983) and of K cells (Amino *et al.*, 1982) were found.

The cell-surface markers on the lymphoid cells infiltrating the thyroid glands of HT patients have also been evaluated. An increase of B cells (Jansson *et al.*, 1983; McLachlan *et al.*, 1983b; Kontiainen *et al.*, 1987) and of activated T cells (Misaki *et al.*, 1985; Möst and Wick, 1986; Del Prete *et al.*, 1987), mainly belonging to the CD8 subset, was observed (Bene *et al.*, 1983; Margolick *et al.*, 1984; Canonica *et al.*, 1985). This presence of $CD8^+$ cells strongly favors a potential role for cytotoxic T lymphocytes (CTLs) in the thyroid damage of HT.

Recently, Möst and Wick (1986) determined the localization, the phenotype, and the distribution of the lymphoid cells on sections from HT patients' thyroids. They confirmed that a majority of cells infiltrating the thyroid tissues were T cells, approximately 10% of them expressed DR Ags, but they found that $CD4^+$ and $CD8^+$ cells were differently distributed. In the interstitium, $CD4^+$ cells, a helper/inducer phenotype, were the most abundant; whereas among the T lymphocytes *in peripolesis*, $CD8^+$ cells were much more common. This last morphological observation further strengthens the hypothesis that the $CD8^+$ cells disseminating among the thyroid follicular cells could play a role in thyroid gland destruction. A particular distribution of the $CD8^+$ cells was also reported by Misaki *et al.* (1985), who detected these infiltrating T cells between the thyroid follicles.

Moreover, T cell clones derived from cells invading HT thyroid glands expressed the CD8 phenotype and exhibited cytotoxic functions (Canonica *et al.*, 1985; Del Prete *et al.*, 1986; Noma *et al.*, 1982; Bagnasco *et al.*, 1987). It must be noted that these cytotoxic $CD8^+$ cells were not specific to Tg or other thyroid Ags, but instead they showed natural killer (NK) activity (Del Prete *et al.*, 1986) or lectin-dependent

cytotoxicity (Bagnasco *et al.*, 1987). In addition, these CD8$^+$ clones were shown to secrete IL-2 (Moretta, 1985) and IFN-γ, two lymphokines released after mitogenic or antigenic stimulation. A relationship was also demonstrated between high IFN-γ production and NK activity of T cell clones derived from HT patients' thyroid glands or PBLs. Compared to the CD8$^+$ clones obtained from the PBLs (Del Prete *et al.*, 1987; Bagnasco *et al.*, 1987), the ability of the CD8$^+$ clones from HT patients' thyroid glands to produce IFN-γ under mitogenic stimulation was significantly increased. Taking into account the capacities of these lymphokines, IL-2 and IFN-γ, to, respectively, stimulate other T or B cells and to modulate class II Ags on the target thyroid cells, the lymphokine production by CD8$^+$ T cells further argues for their effector role in the pathogenesis of HT.

Recently, Noma and Yata (1987) selected a human T cell hybridoma derived from the PBLs of a *normal* donor producing a suppressor factor specifically inhibiting anti-Tg A-Ab production. This suppressive activity, which was not MHC restricted, apart from its possible practical role in therapy, could explain the relapsing intermittent course in patients suffering from chronic HT; a failure of suppressor T cells would allow the appearance of self-reactivity.

Earlier functional studies demonstrated normal helper T cell functions, as assessed by autologous and allogeneic mixed lymphocytic responses of PBLs from HT patients (Fournier and Charreire, 1983). This result can be compared to the recent experiments of Hirose *et al.* (1988), who demonstrated that syngeneic CD4$^+$ T lymphocytes from BUF and Fisher rats can be stimulated in a restricted manner by class II-positive cloned syngeneic thyrocytes. These functional experiments indicate that, in patients or animals suffering from thyroiditis, the repertoire of T helper cells involved in thyroiditis is similar to those of their normal counterparts.

Recently, to further evaluate the existence of a functional defect in HT patients, T cell reactivity against self-components was investigated by a very sensitive method that required both limited numbers of APCs and subsequent culture of PBLs in serum-free medium (Stern and Dau, 1987). Increased organ-specific autoreactivity was detected, defined as Abs to various components such as myelin basic protein, myoglobin, and sarcolemma.

The question of the clonality of the PBLs or thyroid gland T cells in HT patients was raised. Using *Eco*RI and *Bam*HI digests of T cell DNA from patients and probes specific to T cell antigen receptor β and γ genes, i.e., T constant β and T joining γ gene probes, typical patterns of germ line polyclonal T cells were observed. This indicates a lack

of dominance of a particular T cell subset that had been rearranged and supports the hypothesis that HT is the result of a generalized defect localized at the level of the regulatory T cell circuits. However, attention must be drawn to the limited sensitivity of the technique, which detects variations only over 1% (Kaulfersch *et al.*, 1988).

Because these studies were conducted in HT patients of different ages, sex, MHC, etc., at various times after disease onset and treatment, and because most of these studies defined cell-surface markers instead of function, no clear conclusion can be established concerning the T cell dysregulation in HT patients. However, the data indicate that this dysregulation would be generalized, with possibly a dominant role of cells bearing suppressor/cytotoxic markers and expressing cytotoxic functions. This last point was further studied in experimental models.

b. In Experimental Models. Experimental models allow the study of the regulation of the immune response at the levels of both the central lymphoid organ and the target organ. The kinetics of the disease development of A-Ab production, and analysis of the respective specificities and functions of cell subsets found during thyroiditis, can be studied. The controlled experimental conditions are quite different from those found in humans and have enabled the accumulation of data concerning the initiation and the progression of thyroiditis (Section III,B).

Analyses were conducted to evaluate the B and the T cell subsets in peripheral blood and in lymphoid cells infiltrating the diseased thyroid glands of mice or rats suffering from EAT (Creemers *et al.*, 1984; Smith *et al.*, 1987). A kinetic study showed that T cells formed the main population among cells infiltrating murine diseased thyroid tissues. However, this population increased until the disease became flagrant and reached an acute phase, then declined with time and was replaced by cells without T or B cell markers. More interestingly, the early predominance of L3T4 cells was followed by a relative increase in Lyt-2^+ cells. These results, comparable to those described in the thyroid glands from HT patients, also indicate that some Lyt-2^+ T cells, specific to Tg or thyroid Ags, could be stimulated *in vivo* by the thyroid Ags or recruited by helper T cells and therefore proliferate until they initiate thyroid tissue lesions.

The role of cytotoxic T cells in EAT was first suggested by the *H-2*-restricted damage of thyroid glands reported by Creemers *et al.* (1983) and Salamero and Charreire (1985). In both studies, the protocols used were similar: after *in vivo* priming and *in vitro* stimulation with MTg or syngeneic TECs, respectively, the stimulated T cells were deposited on labeled, functional (in terms of Tg and thyroid hormone productions), syngeneic TEC targets. These cytotoxic T cells, which express Lyt-2 phenotypes, were shown to be specifically directed against

Tg epitope(s) of the TEC cultures and to be class I restricted, like classical antihapten or antivirus cytotoxic T cells. Recently, we hybridized and cloned a cytotoxic T cell, named HTC_2, which is able to lyse only syngeneic TECs, whereas allogeneic TECs or syngeneic kidney cells were never killed (Remy *et al.*, 1989). These data demonstrated the existence of class I-restricted, Tg-pathogenic, epitope-specific cytotoxic T cells.

Contrasting with TcR analysis conducted on T helper cells specific for the peptide inducing experimental autoimmune encephalomyelitis (EAE) in PL/J mice, and demonstrating a highly restricted use of V-α and V-β genes, similar studies have not yet been applied to EAT (Urban *et al.*, 1988).

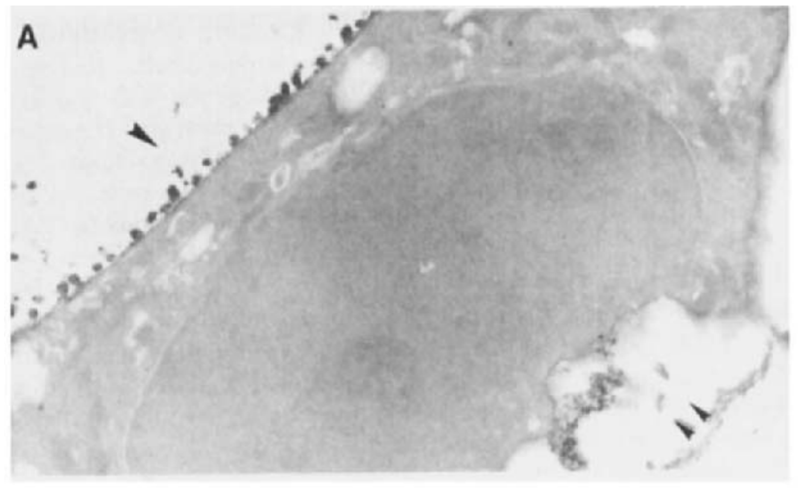
Taken together, cell-surface marker analyses and function studies of the lymphoid cell subsets infiltrating the thyroid glands of HT patients or diseased animals indicated that, at the very early phase of the disease, preexisting Tg-specific helper T cells were activated and proliferated, and were probably stimulated by exogenous and endogenous Tg increase and lymphokine production. Simultaneously, T cells expressing cytotoxic/suppressor cell-surface markers and exhibiting cytotoxic function against Tg borne by syngeneic TECs become dominant and initiate thyroid gland lesions.

B. Target Thyroid Cells

One of the major breakthroughs of the last few years was the demonstration of the expression and regulation of class II Ags on the surface of nonlymphoid cells, thyroid cells, synovial cells, or islets of Langerhans; these are the sites where autoimmunity occurs. From this discovery, new hypotheses dealing with immune regulation of autoimmunity have emerged (Bottazzo *et al.*, 1983, 1986; Volpé, 1987; Davies *et al.*, 1987; Todd *et al.*, 1986; Ada and Rose, 1988).

In 1981, we reported class II Ag expression on monolayers of murine TECs (Salamero *et al.*, 1981). The electron microscopic studies (Fig. 1) revealed that confluent monolayers expressed the ubiquitous class I Ags (K and D ends) on their apical surface, while class II Ags were detectable on the basal part of the TEC cultures; thus, the polarity of these cultured cells was reversed compared to thyroid cells *in vivo* (Mauchamp *et al.*, 1979; Chambard *et al.*, 1981; Hanafusa *et al.*, 1984). These results were reminiscent of those of Parr *et al.* (1980a,b), who described, using electron microscopic studies and immunoferritin labeling of freshly enzyme-dissociated murine thyroid cells, a minor thyroid cell population expressing class II Ags that they considered to probably represent passenger leukocytes. In our experiments, performed from day 5 to 16 of culture, there was no doubt that class II Ags were expressed on TECs as demonstrated by electron microscopy (Yeni *et al.*, 1980).

In humans, numerous investigations dealing with the existence of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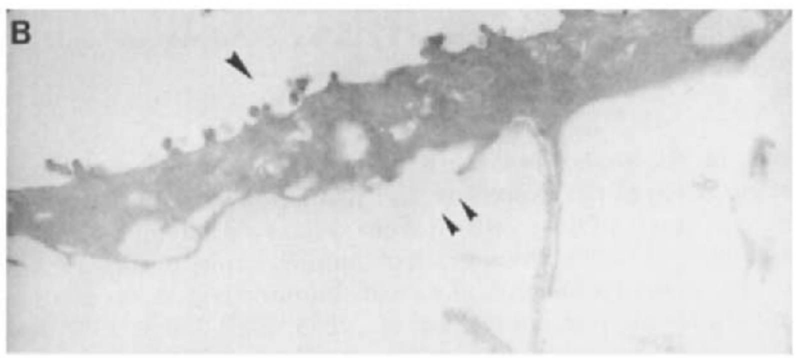
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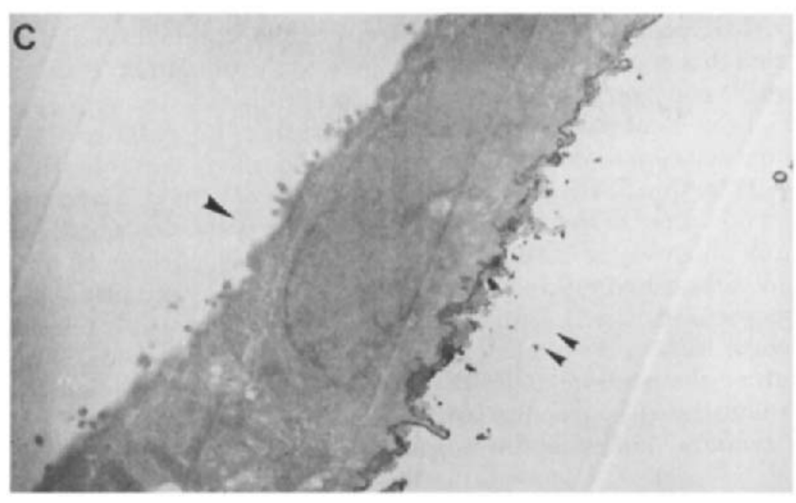
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class II Ags were undertaken on patients with various thyroid disorders. The first report, by Pujol-Borrell *et al.* (1983), examined thyroid cells from HT or GD patients cultured with phytohemagglutinin (PHA). Using indirect immunofluorescence staining, they detected class II Ags with monoclonal anti-DR Abs directed against the nonpolymorphic part of the DR Ag. DR Ag was also spontaneously expressed on the various cells found in HT and in thyrotoxic glands. In HT tissues, all remaining acini were strongly DR^+, as were most of the numerous infiltrating lymphocytes (Hanafusa *et al.*, 1983). They also demonstrated that thyroid tissues from fetuses or normal humans did not express the class II Ags. Later, Möst *et al.* (1986) found that the three distinct molecules, HLA-DR, DP, and DQ, which encode the HLA-D region, were expressed in equal quantities on Hashimoto's thyrocytes, while DR was more abundant than DP, which was more abundant than DQ, on GD patients' thyrocytes (Todd *et al.*, 1985, 1987a).

Thyroid sections from diseased murine or human thyroid glands showed that class II Ag expression was higher in the vicinity of the lymphoid cell infiltration and that its intensity was related to the stage of the disease: less class II Ag expression in early stages and more class II Ag expression in advanced stages (Hanafusa *et al.*, 1983; Aichinger *et al.*, 1985). This suggests that class II Ag expression might be a major factor in the progression and self-perpetuation of the disease. A similar relationship among class II Ag levels and lymphocytic infiltration has also been reported for astrocytes in EAE (Hirsch *et al.*, 1983; Wong *et al.*, 1984). In EAE, it was demonstrated that astrocytes bearing IFN-γ-induced class II antigens presented myelin basic protein to T cells (Fontana *et al.*, 1984) and that hyperinducibility of class II Ags on astrocytes correlated with strain-specific susceptibility to EAE (Massa *et al.*, 1987).

A characteristic of class II Ag expression on TEC is its inducibility by numerous factors, thyroid specific or not. This property was predictable as soon as Pujol-Borrell *et al.* (1983) reported class II Ag expression after the addition of PHA to human thyrocytes. Inducers of class II Ags on murine or human thyroid cells are numerous: leukoagglutinin (Iwatani *et al.*, 1986), T cell stimulators [such as PHA (Davies, 1985) and to a lesser extent concanavalin A], culture supernatants from autologous (Carel and Charreire, 1986), syngeneic (Salamero *et al.*, 1985) or allogeneic stimulated T cells (Iwatani *et al.*, 1986), IFN-γ (Bottazzo

FIG. 1. Electron microscopic studies of class I and class II MHC Ags on murine TEC cultures labeled with peroxidase-coupled antimouse IgG (7800×). (a) K end; (b) D end; (c) I region. The single arrowhead points to the apical TEC surface with numerous microvillosities; the double arrowheads point to the basal TEC surface [reproduced from *C.R. Acad. Sci. (Paris)* 1981 293, 745].

et al., 1983; Salamero *et al.*, 1985; Weetman *et al.*, 1985b; Davies, 1985; Möst and Wick, 1986), thyrotropin (Carel and Charreire, 1986; Todd *et al.*, 1987b) and its second messenger dibutyryl cyclic AMP (dbcAMP) (Todd *et al.*, 1987b), tumor necrosis factor (TNF) (Todd *et al.*, 1987c), and antibodies to TSH receptor (Wenzel *et al.*, 1986, 1987; Bodolay *et al.*, 1987). One may predict that this list is not exhaustive.

Among these factors, IFN-γ is the most potent, acting in the physiological range (Trinchieri and Perussia, 1985). In contrast, other T cell products, such as IFN-α and IFN-β (Todd *et al.*, 1987c) and IL-2 (Todd *et al.*, 1987c; Davies, 1985), have no effects on class II Ag expression on thyrocytes, as was the case for LPS, a T cell-independent polyclonal B cell activator (Iwatani *et al.*, 1986), and for cyclosporin A (CyA), which is known to block lymphokine production. More precisely, CyA inhibited lectin-mediated levels of DR Ags (Weetman *et al.*, 1985b; Piccinini *et al.*, 1987) and had no effect on DR induced through IFN-γ; these effects were detected at both the mRNA and the protein levels (Piccinini *et al.*, 1987).

Synergistic effects of TSH, dbcAMP, isobutyl methylxanthine, TNF, and Graves' immunoglobulin G (IgG) in the enhancement of IFN-γ-induced class II molecules on thyrocytes have been described. Although TNF was reported by Todd *et al.* (1987c) to act synergistically with IFN-γ in inducing class II Ag expression on human thyrocytes, a down-regulation of HLA-DR α-chain mRNA expression was reported by Londei *et al.* (1987a). If this surprising down-regulation is confirmed, it will be of special interest, because in HT, TNF is produced by intrathyroidal T lymphocytes (Londei *et al.*, 1987b) and thus could be of crucial importance in the control of the thyroid autoimmune process. Interestingly, antithyroid drugs have no effect on IFN-γ-induced class II Ag expression on human thyrocytes (Aguayo *et al.*, 1988).

Epidermal growth factor (EGF), a substance which stimulates thyroid growth in culture and suppresses TSH stimulatory processes [such as iodine efflux or Tg iodination through specific receptors expressed on TEC (Westermark *et al.*, 1983, 1985, 1986)], behaved like an inhibitory agent of class II Ag expression on thyroid cells (Todd *et al.*, 1986). These opposite effects of EGF and TSH on IFN-γ-induced class II Ag expression by thyrocytes could contribute to the suppression of the autoimmune process; down-regulatory phenomena are as important as stimulatory processes (Cohen *et al.*, 1985). Moreover, the effect of EGF depends primarily on the responder cell type; although EGF suppressed class II Ag expression on thyrocytes, it enhanced them on human monocytes (Acres *et al.*, 1985). These contrasting effects of EGF on thyrocytes and on classical antigen-presenting cells were hypothesized to have a bearing on the occurrence of organ-specific or non-organ-

specific autoimmune disease; the effect of EGF on TECs might favor the development of organ-specific autoimmunity, whereas its effect on APCs would lead to less specific activity. That a defined factor influences class II Ag expression differently depending on the target cell further increases the complexity of the regulation of autoimmune reactivity. This complexity is increased even more, if one takes into consideration that IFN-γ not only induces class II MHC Ag expression, but also affects the susceptibility of thyroid cells to cytokines, such as TNF (Taverne *et al.*, 1987), or enhances macrophage or NK cell production of interleukin 1 (IL-1), which is necessary for T cell activation (Bendtzen *et al.*, 1987).

Numerous questions were raised by the demonstration of class II Ag expression on nonlymphoid cells: Is it the result of a neosynthesis or of a passive absorption by contaminating class II-positive lymphoid cells? Are the thyroid class II Ags the same as those found on lymphoid cells? Are they able to present specifically an Ag to T cells and to induce a T cell proliferative response? Is this class II Ag expression a primary or a secondary phenomenon?

Response to the first question can be deduced from our experiments (Salamero *et al.*, 1985). We demonstrated, in a kinetic study of Ia appearance on murine TECs, that these cells were lysed in the presence of monoclonal anti-Ia^k Ab and rabbit complement, indicating that murine class II Ag expression is the result of neosynthesis and not a passive absorption onto the TEC surface. More precisely, class II antigens appeared initially with a patchy distribution around the TEC nucleus, then they migrated to the cytoplasm and, 24 hours after inductive treatment, were expressed on the cell surface. Furthermore, Möst *et al.* (1986) provided evidence for the existence of intracellular HLA-DR γ chains in Hashimoto's thyrocytes. Similarly, study of the regulation of class II antigens in human thyrocytes at the transcriptional level demonstrated the existence of mRNA coding for the HLA-DR α chain (Piccinini *et al.*, 1986, 1988) and more recently for the HLA-DP and DQ α chains (Londei *et al.*, 1987a).

The fine specificity of the thyroid class II Ags was defined with polyclonal anti-class II antibodies on murine TEC cultures (Salamero *et al.*, 1981) and, later, with monoclonal Abs directed against nonpolymorphic determinants of α and β chains of murine or human class II molecules. More recently, class II Ags were simultaneously sought on B cells and thyrocytes from the same individuals using 55 polyclonal antisera and routine lymphocyte typing procedures. A complete concordance between B cell and TEC HLA-DR expressions was found, demonstrating the same fine specificity of the class II molecules on the two cell types (Dower *et al.*, 1988).

One of the major functions of class II antigens is to present the

processed Ag to the T cell and, consequently, to initiate an immune response (P. M. Allen *et al.*, 1987). This well-established property led Bottazzo *et al.* (1983) to postulate that class II Ags on TEC could enable the TECs to act as APCs to T cells. *In vitro* studies conducted in humans (Davies, 1985; Iwatani *et al.*, 1987b) and mice (Yeni and Charreire, 1981; Charreire, 1982b) established that TECs can induce thyroid-specific syngeneic or autologous proliferative responses. Recently, Hirose *et al.* (1988) demonstrated that $CD4^+$ lymphocytes can be stimulated by cloned rat syngeneic or allogeneic thyroid cells only if the cells express class II Ags. It was also shown that human thyroid cells can present peptidic viral Ag to specific T cell clones, while they were not able to process crude viral particles (Londei *et al.*, 1984), favoring the hypothesis that TECs could present Ag to T cells only if T cells were specific to Ags normally exposed on the thyroid cell surfaces (Remy *et al.*, 1986).

However, new data have led to the reassessment of the ability of class II-positive TECs to act as APCs. It was demonstrated that IFN-γ-induced class II-positive murine follicular epithelial cells, from good and poor responder strains of mice or from cloned rat thyroid cells, were unable to present A-Ag or allo-Ags to Tg-sensitized lymph node cells or to auto- or alloreactive Tg-specific T cell clones or hybridomas (Ebner *et al.*, 1987). However, the same group demonstrated the capacity of a murine TEC line, used after class II Ag induction by IFN-γ, to present Ag to T cell hybridomas specific or not to Tg (Stein and Stadecker, 1987). In these last experiments, a potent Ag presentation by the class II-induced TEC line was provided by the addition of phorbol myristate acetate (PMA), a drug which gave a signal, probably stimulating APC function through an increase of protein kinase C activity (Nishizuka, 1984; Finnegan *et al.*, 1985). In the numerous examples of effective T cell stimulations by class II-positive murine or human thyroid cells previously reported, the use of PMA was never mentioned; however, in these experiments, confluent monolayers of TECs were used instead of a uniform thyroid cell line. Therefore, it cannot be excluded that contaminating cells participated in the antigenic presentation procedure. In particular, DC or B cells are very potent APCs. Dendritic cells in HT or EAT diseased thyroid glands are regularly located in close contact with the intrathyroidal lymphocytes invading the glands (Kabel *et al.*, 1987, 1988). Moreover, in EAT of August rats, the DC number was highly increased during the first week following immunization (Hassman *et al.*, 1988). Because it is well known that DCs are the most potent cells for antigenic presentation (Steinman and Cohn, 1972; Steinman, 1986), a minor contamination of the TECs by DCs could be responsible for the antigenic

presentation to T cells. Likewise, Hutchings *et al.* (1987) established that B cells could also be highly efficient APCs to Tg-specific T cell clones, with their surface Ig acting as high-affinity receptors for Ag.

Up to this point, it can be hypothesized that TECs could exclusively present thyroid Ags to specific T cells; this function would mainly influence the perpetuation of the disease, whereas highly powerful APCs, such as DCs or B cells, working with physiological concentrations of Ags, could be responsible for the triggering of the Tg- or thyroid Ag-specific T cells.

Another extensively debated question is that of class II Ag expression as a cause or a consequence of autoimmune aggression (Khoury, 1987). The data obtained with EAT-susceptible mouse strains favored the hypothesis that induction of class II Ag expression is a cause of the thyroiditis. Boudier *et al.* (1988) reported class II Ag expression on freshly dissociated murine TECs from GR strains. Similarly, the detection of HLA-DR γ chains inside the thyroid cells and of class II mRNA in normal thyroid cells indicated that nonexpression of class II Ags by epithelial cells was due to regulatory mechanism(s) located at the posttranscriptional level. It is even possible that a posttranscriptional defect preventing class II Ag expression on thyrocytes represented another protective mechanism against the onset of autoimmune reactivity.

Conversely, the *in vitro* experiments, using activated T cell supernatants as class II Ag inducers, and the *in vivo* studies, showing the preferential localization of class II Ags near mononuclear cell infiltrates, favor the hypothesis that class II Ag expression is a secondary phenomenon, a consequence of T cell activation. In our laboratory, we attempted to answer this question by injecting either one unit of murine recombinant IFN-γ (r-IFN-γ) or the saline used to dilute the r-IFN-γ into the thyroid lobes of susceptible mice. The mice were not immunized with Tg. On day 28 after r-IFN-γ inoculation, anti-Tg A-Abs were detected in 70% of the r-IFN-γ-injected mice but never in the control group. Moreover, mild lymphocytic infiltrations were observed only in r-IFN-γ-injected mice (Remy *et al.*, 1987). Similar experiments were conducted by Rayfield *et al.* (1988), who detected anti-Tg A-Abs in 12% of the r-IFN-γ-injected mice and mild lymphocytic infiltration in 37% of only r-IFN-γ-treated animals 7 weeks posttreatment. In these experiments, class II Ags on thyroid cells appear to be a cause of EAT.

As hypothesized by Bottazzo *et al.* (1983) and reinforced by the recent data on EAT induction by reovirus type I (Srinivasappa *et al.*, 1988), it can also be advanced that a virus could first cause mild focal thyroiditis

due to the triggering of an antiviral T cell immune response. During this T cell response to virus, a sequence of events could be triggered leading to autoimmunity: lymphokine production, followed by class II Ag expression by TECs and proliferation of thyroid-specific T cells. All these steps have been observed *in vitro*. Klavinski *et al.* (1988) performed experiments favoring a role for a virus in the pathogenesis of thyroid disorders. After immunization of three strains of newborn mice with lymphocytic chorionomeningitis virus, they found that the virus can persist in TECs secreting Tg and observed a decrease of thyroid hormone production. To date, no relationship between thyroiditis and viral infection has clearly been demonstrated. Furthermore, in subacute de Quervain's thyroiditis, in which the viral origin has been definitively established, a lack of class II Ags on the thyroid tissues was reported (Volpé, 1987). The hypothesis that a viral infection triggers thyroiditis begs further testing and support. In this regard, the detection of ev22 virus exclusively in OS chicken thyroid glands is promising (Wick *et al.*, 1985, 1986).

Finally, it may be that class II Ag expression is one of many triggering events in thyroiditis. Class II Ag expression would lead to the stimulation of thyroid-specific T cells, then to cytokine production and therefore to the appearance and maintenance of the disease. This question, still open and widely debated, could be answered using new experimental models, such as transgenic mice (Benoist *et al.*, 1986). Approaches similar to those developed for studying the respective roles of IFN-γ and class II genes in diabetes (Sarvetnick *et al.*, 1988) could be applied to thyroiditis (Pujol-Borrell and Bottazzo, 1988).

VI. Humoral Response(s) in Thyroiditis

One of the common features of thyroiditis is the production of A-Abs directed against defined thyroid cell membrane components (such as Tg), against microsomal Ags (Banga *et al.*, 1985, 1986) (also called TPO; Section II,B), or against new thyroid Ag(s) unrelated to Tg or TPO and recently identified by immunoblotting (Weetman *et al.*, 1987) or by molecular cloning (Rapoport *et al.*, 1986, 1987; Chan *et al.*, 1989). In addition to these thyroid Ags, other potential candidates derived from cytoskeleton components stimulate Ab production in HT patients (Rousset *et al.*, 1983). Nevertheless, in HT, SAT, and EAT, the thyroid gland is the only severely affected target organ and anti-Tg A-Abs are always detected, while anti-TPO A-Abs are found in variable percentages (25–50%) of HT sera (Ruf *et al.*, 1987; Pinchera *et al.*, 1987).

A. Autoantibodies to Tg

Anti-Tg and anti-TPO A-Abs have been the subjects of controversy, whether they were measured in sera or were produced *in vitro* by cultured

PBLs from normal individuals or HT patients. These variable observations reflected both the heterogeneity of the immune status of patients with HT and the lack of correlation between circulating anti-Tg A-Abs and the existence or severity of the disease. In normal sera, Guilbert *et al.* (1982) and Ericsson *et al.* (1985) reported that 27% of subjects possessed anti-Tg A-Abs, detected by the highly sensitive solid-phase immunosorbant radioassay. This possibly reflects subclinical thyroiditis as lymphocytic infiltrations of the thyroid glands of normal individuals are commonly detected at autopsy (Bastenie and Ermans, 1972).

The subject of *in vitro* production of anti-Tg A-Abs is also controversial. PBLs from HT patients synthesize anti-Tg A-Abs either spontaneously (Noma *et al.*, 1982) or after stimulation with pokeweed mitogen (PWM) (McLachlan *et al.*, 1983a,b; Mariotti *et al.*, 1984) or with both PWM and Cowan I (SAC), i.e., protein from *Staphylococcus aureus* (Iwatani *et al.*, 1987b). As expected, *in vitro* production was found in HT patients but not in all of them (Sekino *et al.*, 1988) and, more surprisingly, these Abs were also synthesized by normal human B cells activated by Tg and appropriate T cell signals (Logtenberg *et al.*, 1986). Moreover, although a relationship between anti-Tg A-Ab titers in patients' sera and the amounts of anti-Tg A-Abs produced *in vitro* was not demonstrable by Weetman *et al.* (1985a), it was by Petersen *et al.* (1986). This discrepancy is difficult to understand unless unusual conditions of *in vitro* anti-Tg A-Ab production were used. Similarly, Iitaka *et al.* (1988) reported *in vitro* production of anti-TPO Abs when peripheral blood B cells from normal subjects were cultured with PWM and SAC and stimulated by autologous or allogeneic $CD4^+$ cells. They also found that the helper activity of HT patients' $CD4^+$ cells was greater than that of $CD4^+$ cells from normal donors.

In these experiments, A-Ab production by normal or patients' B lymphocytes can be thought to reflect an increased helper activity for thyroid-specific Ags. However, it cannot be excluded that, in HT patients, an impaired suppressor activity is also responsible for A-Ab production.

Using the same methodology or a sensitive plaque-forming cell assay, it was demonstrated (Weetman *et al.*, 1982a; McLachlan *et al.*, 1983a) that B cells infiltrating the thyroid glands also produced anti-Tg A-Abs. Weetman *et al.* (1984) showed that the immune response in autoimmune thyroid disease was fairly well localized to the thyroid glands and to the surrounding lymphoid tissues. Furthermore, McLachlan *et al.* (1986) reported that dispase digestion of a patient's thyroid tissue, followed by separation of thyroid follicular cells, yielded a lymphocyte population with a high T:B cell ratio and a significantly increased ability to produce anti-Tg or anti-Mic A-Abs compared to PBLs from the same patient. It was hypothesized that the B lymphocytes, which were closely associated

with thyroid follicular cells and were released after enzyme digestion of thyroid tissues, could make a major contribution to anti-Tg A-Ab synthesis. Recently, Baker *et al.* (1988) reported the production of antithyroid A-Abs restricted to the thyroid gland of a patient whose antithyroid A-Ab serology was negative. Moreover, several MAbs were produced from lymphocytes extracted from this patient's thyroid gland, including an IgM κ with specificity restricted to human Tg. Two other associations among monoclonal intrathyroidal anti-Tg A-Abs and HT have been reported (Matsubayashi *et al.*, 1986; Timsit *et al.*, 1988). These observations argue that anti-Tg A-Abs associated with the disease are mainly synthesized by the lymphocytes infiltrating the thyroid glands. However, this hypothesis of the diseased thyroid gland as the center of anti-Tg A-Ab production must be reevaluated, taking into account the reports by Farrant *et al.* (1986a,b), who used autologous DCs from PBLs to present almost physiological doses of Tg. By this method. they stimulated peripheral blood B cells from HT patients, previously selected for their high levels of circulating anti-Tg A-Abs. Then they measured anti-Tg A-Abs spontaneously produced or generated after the addition of Tg or B cell growth factor. They found that anti-Tg A-Ab synthesis correlated in some patients with circulating anti-Tg A-Ab levels, but this relationship did not exist in every HT patient with elevated anti-Tg A-Abs. This discrepancy can be understood if one considers that each B cell produces various amounts of anti-Tg A-Abs, probably directed against different epitopes of the vast Tg molecule; the *in vitro* culture system can differently and fortuitously select which B cell will grow or which helper T cell will cooperate with these B cells independent of both the amount and the specificity of the anti-Tg A-Abs they produce.

To investigate the function of B cells from HT patients in an analytical way, Weetman *et al.* (1985a) studied *in vitro* anti-Tg A-Ab production using a combination of lymphokines and other stimuli that act at various stages of B cell differentiation. Broad heterogeneity of anti-Tg A-Ab production was found among HT patients, possibly reflecting differences in activation and in migration of circulating B cells.

In humans, the isotypes of circulating or *in vitro*-produced anti-Tg A-Abs were studied and a restricted heterogeneity was found; most belong to the IgG_1 subclass (60–70%), then to the IgG_2 (20–25%), IgG_3 (7-10%), and IgG_4 (3–5%) (Davies *et al.*, 1986; Forouhi *et al.*, 1987) subclasses. This hierarchy was also observed with Epstein–Barr virus-immortalized B cell lines derived from HT patients (Walker *et al.*, 1983; Thompson *et al.*, 1983). The great proportion of IgG_1 suggested that complement-fixing A-Abs could play a role in the destruction of the thyroid gland and therefore in thyroid failure (Parkes *et al.*, 1984).

The same questions were asked of experimental models. Surprisingly, in rats with overt thyroiditis, anti-Tg A-Ab synthesis in a plaque-forming cell assay was demonstrated with bone marrow lymphocytes but not with thyroid gland lymphocytes (Weetman *et al.*, 1982b). These data are in contrast to those discussed herein for HT patients. Moreover, in experimental models, the role of anti-Tg A-Abs was never clearly established and a correlation between anti-Tg A-Ab levels and pathology was rarely reported. Vladutiu and Rose (1971b) transferred EAT into mice by injecting immune sera from diseased animals into normal mice. Jaroszewski *et al.* (1978) demonstrated that repeated inoculations of pooled OS sera, containing anti-Tg A-Abs, into newly hatched OS chickens having an MHC associated with mild thyroiditis resulted in more extensive lymphoid infiltration of their thyroid glands. Thus, anti-Tg A-Abs would be potentiators of thyroiditis. More recently, Polley *et al.* (1981) and Okayasu (1985) reported that immune sera from animals with EAT failed to transfer EAT into syngeneic recipients.

The specificity of anti-Tg A-Abs was investigated; polyclonal A-Abs raised in animals immunized with hTg in CFA recognized multiple antigenic determinants on the hTg molecule, approximately 40 different epitopes; however, anti-Tg A-Abs from HT patients recognized a more restricted number of determinants, only two to four as assessed by the restricted spectrotypes of their sera, but with different patterns (Nye *et al.*, 1980; Roitt *et al.*, 1981b). Recently, Delves and Roitt (1988) examined the spectrotypes and the idiotypes of anti-Tg A-Abs during the course of HT. For idiotypic studies, they used polyclonal rabbit antiidiotype Abs raised against human anti-Tg A-Abs from HT patients. At several-year intervals during the evolution of HT, they found that the spectrotype as well as the idiotype of anti-Tg A-Abs were not modified once they had been established.

These results partially disagreed with those of Stott *et al.* (1988), who studied at various times the spectrotypes of A-Abs to Tg in August and PVG/c rats, in which EAT was induced either by immunization with autologous Tg in CFA or after NTx and sublethal irradiation, respectively. Surprisingly, the two serum spectrotypes were different. In August rats, the anti-Tg A-Ab response was oligo- or polyclonal, with dominant spectrotypes and considerable variations with time, resembling most cases of HT, whereas in PVG/c rats, the spectrotypes of immune sera were highly restricted and stable with time, reflecting those found in few HT patients and suggesting that the majority of the anti-Tg A-Abs were produced by a small number of B cell clones. These opposing data obtained in EAT in two rat strains further attest that thyroiditis is a heterogeneous disease in its manifestations and pathogenesis. The two different EAT

induction protocols selected different helper T cells recognizing various epitopes of the Tg molecule. Hence, when rats were immunized with Tg in CFA, a polyclonal spectrotype was observed, bearing witness to T cell stimulation by the multiple potential epitopes of the Tg molecule, whereas, when EAT resulted from NTx and irradiation, an oligoclonal profile was obtained, attesting to a reduced number of T cells recognizing some epitopes of the Tg molecule.

Determination of the Tg epitope(s) recognized by anti-Tg A-Abs, the subject of numerous investigations, was precisely undertaken when the cell fusion technology was successfully developed and MAbs were produced (Köhler and Milstein, 1976). The synthesis of MAbs directed against hTg (Ruf *et al.*, 1983; Kurata *et al.*, 1984; Kohno *et al.*, 1985a; Chan *et al.*, 1987; De Baets *et al.*, 1987; Shimojo *et al.*, 1988) or against MTg (Kotani *et al.*, 1985; Salamero *et al.*, 1987b; Champion *et al.*, 1987b) allowed the mapping of immunogenic epitopes of the human and murine Tg molecules. The results were similar to those obtained with polyclonal A-Abs (Kohno *et al.*, 1985b; Petersen *et al.*, 1986; Shimojo *et al.*, 1986). Poly- or monoclonal reagents detected interspecies cross-reactivities by competitive inhibition of binding of defined A-Abs to Tgs obtained from different species, but stronger reactivities were regularly found when MAbs and Tg were provided by the same species. Champion *et al.* (1987a), De Assis-Paiva *et al.* (1988) demonstrated that cross-reactive epitopes detected on Tgs from distinct species were highly conserved throughout most of the mammalian class and mainly were seen by specific T cells; conserved immunogenic epitopes on the Tgs from different species were expected, taking into account the amino acid similarities of human, bovine, and rat Tg sequences (Section II,A).

The reactivity of anti-Tg A-Abs was also evaluated versus heat-denatured or chemically denatured Tg or fragments obtained after CNBr (Marriq *et al.*, 1982), trypsin (Salamero *et al.*, 1987a), or protease V8 treatment (Shimojo *et al.*, 1988). It was found that anti-Tg A-Abs reacted with the largest fragments but that their ability to bind to small fragments was decreased, especially when the disulfide bonds of Tg were reduced by dithiothreitol or 2-mercaptoethanol treatment. Heat denaturing of Tg partially decreased reactivity while antigenicity following sodium dodecyl sulfate was conserved.

The specificity of anti-Tg A-Abs is usually measured by the binding of polyclonal or monoclonal anti-Tg A-Abs to the native Tg molecule. However, MAbs can also be characterized with functional studies, such as T cell proliferation on TECs, preincubated or not with monoclonal anti-Tg A-Abs (Salamero *et al.*, 1987b). This property is a consequence of the T cell's ability to recognize Tg fragments, while B cells need a

native or a minimally modified Tg molecule. In our experiments, a first set of murine anti-Tg A-Abs was defined by their ability to block the T cell proliferative response normally induced by syngeneic TECs; T cells recognize an epitope borne by a tryptic fragment of the Tg molecule and also exposed on murine TECs. These studies also defined a second set of anti-Tg A-Abs, with distinct specificities noncompetitive with the first set.

The question then arose as to whether the Tg produced by HT patients expressed antigenic determinants exclusively recognized by anti-Tg A-Abs from the patients and thus corresponding to a particular B cell repertoire. In hTg, the number of immunogenic regions evaluated with 10 murine anti-Tg A-Abs was limited to six. Among them, five regions were not recognized by anti-Tg A-Abs from patients (Ruf *et al.*, 1983). The immunogenic regions on the Tg molecule were located at the two extremities, including tyrosines used for iodination of the Tg, and in the middle of the large Tg molecule. However, MAbs raised against hTg epitopes bearing iodination sites showed no cross-reactivity with patients' anti-Tg A-Abs (Kurata *et al.*, 1984; de Baets *et al.*, 1987). A study conducted by Ruf *et al.* (1985) in humans concluded that a unique anti-Tg A-Ab repertoire exists which is detectable both in the normal state and in HT. This result can be compared to those of De Carvalho *et al.* (1982) and Sanker *et al.* (1983), who studied the specificity of the naturally occurring anti-Tg A-Abs from OS chickens to OS, CS, and normal chicken Tgs and found no differences related to the source of Tg they used. Kaulfersch *et al.* (1988) tested *Eco*RI digests of DNA from peripheral non-T cells or from lymphocytes infiltrating the HT patients' thyroid glands with an Ig J_H probe and found no significant rearrangement of the Ig J_H gene. It must be recalled that this method only detects rearranged genes expressed in at least 1% of the total B cell population. This limit may exceed clonal representation if one considers the numerous distinct specificities borne by each B cell due to the nonselection of this subpopulation.

Study of the idiotype borne by anti-Tg A-Abs enables further characterization. The existence of antigenic determinants on Abs was initially described by Slater *et al.* (1955). Oudin and Michel (1963) confirmed their existence and raised antibodies against them in rabbits. They termed these determinants idiotypes (id). Zanetti *et al.* (1983a) demonstrated the existence, in the majority of the BUF rat sera tested, of a cross-reactive idiotype (CRI) borne by 20–50% of A-Abs to Tg. They found that this CRI was borne by anti-Tg A-Abs from both young and old rats with thyroiditis regardless of whether they were spontaneously generated or induced by immunization. Moreover, it was also shared

by anti-Tg A-Abs from other species including humans. This CRI, named id 62, exhibited regulatory functions and was expressed on each murine monoclonal anti-Tg A-Ab, even if MAbs were generated in different fusions and recognized apparently distinct epitopes on rat Tg. Furthermore, it mapped within or close to the Ag-binding site (Zanetti *et al.* 1983b). The existence of this regulatory CRI led the authors to conclude that the immune response to phylogenetically conserved epitopes on Ags of internal origin, such as Tg, is idiotypically restricted. They exist very early in ontogeny, since they were detected in the sera of germ-free nonimmunized newborn animals, which demonstrates that certain V regions borne by one A-Ab from an adult were expressed in the neonatal repertoire; their germ line origin was therefore highly probable (Glotz and Zanetti, 1986). More surprisingly and still unexplained, regulatory id 62 has been detected independently on both the isolated light (L) and heavy (H) chains of the related monoclonal anti-Tg A-Ab and on the native A-Ab. It was detected with specific Abs raised against purified H62 or L62 chains. In addition, specific anti-H62 and anti-L62 chain antisera were able to function as the original antiidiotype A-Ab raised against monoclonal anti-Tg A-Ab bearing id 62 (Zanetti *et al.*, 1985; Zanetti and Rogers, 1987). The authors hypothesized that the simultaneous presence of the same idiotype on both H and L chains of a given A-Ab may confer regulatory properties within the idiotypic network, as postulated by Paul and Bona (1982) for a limited set of idiotypes with pivotal function(s) in autologous regulation. It must, however, be noted that the detection of CRI on monoclonal anti-Tg A-Abs was not a general phenomenon; Ruf *et al.* (1986) reported the existence of individual idiotypes on monoclonal anti-Tg A-Abs, when tested in a competitive binding assay with rabbit antiidiotype Abs.

Male *et al.* (1985) proposed several hypotheses to explain the existence of recurrent idiotypes: (1) a genetic predisposition of a given strain expressing genes producing defined idiotypes; (2) the existence of constraints on one A-Ab which binds to a particular epitope and therefore induces the recurrence of an A-Ab site-associated idiotope; and (3) the presence of idiotope-specific T cells which select expression of particular sets of idiotype-positive B cells. Among these hypotheses, propositions (1) and (3) are experimentally supported, while (2) appears less evident. Does constraint mean that a certain Ig structure, such as the demonstration of id 62 on both H and L Ig chains, would be required for this phenomenon?

Variable regions from some anti-Tg A-Abs were also analyzed using molecular biology techniques; Ig heavy chain variable region (V_H) genes encoding nine Tg-specific MAbs from murine hybridomas showed that

three V_H families are primarily utilized: V_H J558, the largest family, and V_H 7183 and V_H QPC52, the families most proximal to the Ig joining region heavy chain genes (Monestier *et al.*, 1986). These MAbs express CRI shared by MAbs with different specificities found in other autoimmune pathologies; this expression is independent of the MHC and Ig heavy chain H constant region (C_H) genes as well as the V_H gene utilized.

Attention must be drawn to the fact that most of the anti-Tg A-Abs studied in humans or experimental models were probably directed against conformational epitopes with no proved relationship to pathogenicity. If similar studies were to be conducted with anti-Tg A-Abs related to HT, SAT, or EAT, new insights into thyroiditis and into autoimmune reactivity in general would surely be obtained.

B. Antiidiotype Autoantibodies (anti-id A-Abs)

In his idiotypic network theory, Jerne (1974) proposed that, in addition to the immune system regulation by interactions among the Ags and their specific Abs, interactions among Abs and, more precisely, among idiotypes borne by Abs could also occur. Since this theory was advanced, there has been increasing evidence that interactions among idiotypes and antiidiotypes were crucial for the regulation of the immune response to self-antigens (Colvin and Olson, 1985; Male, 1986).

In EAT, a role for anti-id A-Abs was suggested by Nakamura and Weigle (1969), who transferred EAT into healthy rabbits by injecting sera containing anti-Tg A-Abs from donors soon after Tg immunization, whereas antisera containing anti-Tg A-Abs taken after the lesions had fully developed were not effective.

Spontaneously occurring anti-id A-Abs to anti-Tg A-Abs were found in the BUF rat (Zanetti *et al.*, 1983a) as well as in 10% of HT patients (Sikorska, 1986); moreover, a monoclonal anti-id A-Ab to anti-Tg A-Ab was detected in the serum of one patient with multiple myeloma (Zouali *et al.*, 1984).

Antiidiotype A-Abs to human anti-Tg A-Abs have also been raised in rabbits (Roitt *et al.*, 1981a; Matsuyama *et al.*, 1983; Delves and Roitt, 1984; Ruf *et al.*, 1986). Some only reacted with the homologous idiotype and showed no cross-reactivity with the anti-Tg A-Abs from other patients, whereas others recognized a CRI shared by different patients' A-Abs.

Zanetti and Bigazzi (1981) first described the production of antiidiotype sera to anti-Tg A-Abs in rabbits immunized with the spontaneously developed anti-Tg A-Abs from several BUF rats. They showed that antiidiotype A-Abs partially blocked the Tg–anti-Tg interaction *in vitro* and significantly decreased the level of anti-Tg A-Abs when injected into

sublethally irradiated BUF rats suffering from SAT. Moreover, the number of rat spleen cells bearing the idiotype was also decreased. Unfortunately, the thyroids from antiidiotype-treated BUF rats were not subjected to histopathological analysis. In humans, comparable data were reported by Kojima *et al.* (1986), who demonstrated that human anti-Tg A-Abs produced by Epstein–Barr virus-infected B lymphocytes from an HT patient could be suppressed by private as well as by cross-reactive anti-id A-Abs to human IgG.

Zanetti *et al.* (1986, 1988) studied the effect in rats of the time interval between exposure to rabbit anti-id 62 and subsequent Tg challenge. When the interval was 2 weeks, anti-id 62 A-Abs suppressed the anti-Tg A-Ab response in approximately 70% of the animals; on the other hand, when this interval was extended to 7–14 weeks, marked increases in the levels of anti-Tg A-Abs were detected. These experiments suggest that the immune status of the recipient animals is a critical determinant for the manipulation of autoimmune disease with anti-id A-Abs.

Male *et al.* (1983) also produced in rabbits antiidiotype A-Abs to three murine monoclonal anti-Tg A-Abs; among the idiotypes recognized, one was associated with the Ag-combining site, although the other two were not. Two of these idiotypes were detected in the sera of mice with EAT, but they constitute a small percentage (1%) of the anti-Tg A-Abs present. However, although one was common and, moreover, detectable in normal mice, a second one was found in a few mice with EAT but never in normal animals. Furthermore, when the two different antiidiotype A-Abs were injected into various strains of mice, which were then challenged with a subimmunogenic dose of Tg, only one idiotype (D8) increased, and only in a few strains of mice. In this case, id D8 represents up to 30% of the anti-Tg A-Ab responses. Spectrotyping of mouse sera containing induced idiotypes associated with A-Abs to Tg showed that the overlap between anti-Tg A-Abs and id D8 was greater in CBA mice, the strain from which the D8 clone was derived, implying that CBA mice normally have cells which can be induced to produce id D8-positive anti-Tg A-Abs. These experiments also indicatd that id D8 could act as a regulatory idiotype, since it is associated with anti-Tg A-Abs in CBA mice and it recurs in all EAT mice, as previously reported for id 62 on anti-Tg A-Abs from BUF rats (Section VI,A). Moreover, its low level of expression led the authors to hypothesize that it is weakly expressed or regulated on B cells. Lastly, because stimulation by anti-id D8 also induced a considerable increase of id D8-negative anti-Tg A-Abs, Male *et al.* (1985) envisioned that Tg-specific helper T cells bearing a structure similar to D8 were also stimulated. In this respect, it can be supposed that id D8-positive T helper cells, specifically activated by previous exposure

to anti-id D8 A-Ab, would produce cytokines activating T helper cells independently of their specificity. This nonspecific activation would occur during the first 14 days prior to Tg immunization. At this time, id D8-positive T helper cells would remain a minority compared to the unlimited T helper cells, already activated by cytokines from id D8-positive cells. Then Tg immunization would mainly amplify id D8-negative T helper cells. This possible pathway could explain the considerable increase of anti-Tg A-Abs that were id D8 negative. Furthermore, another pathway could also be postulated, taking into account data assuming that B cells, id D8 positive, would use their Ig to capture Ag (anti-id D8 A-Ab) and subsequently process and present it to T cells in an MHC-restricted fashion, as do conventional APCs (Hutchings *et al.*, 1987; Lanzavecchia, 1988); more interestingly, the efficiency of specific B cell Ag presentation to T cells can be obtained with the Ag concentration 10^3-10^4 times lower than needed for conventional macrophages. These two mechanisms probably coincide.

Whereas this attractive hypothesis can neatly explain the experimental data reported above, it must be recalled that it postulates that the same idiotype (D8) could be borne by both T and B cells and, consequently, these cells would be able to recognize the same antigen specific for this idiotype; precedent for this possibility is exceptional. Nonetheless, dual recognition has been described for a few non-self-Ags (Cosenza *et al.*, 1977; Eichmann, 1978). From a fundamental point of view, the homologous sequences among genes coding for α or β chains of the TcR and for L and H chains from the Igs and the similarity among the gene products, demonstrating that they belong to the same superfamily (Kraig *et al.*, 1983; Yanagi *et al.*, 1984), would further favor the hypothesis of a given epitope being recognized by both T and B cells. Our group has generated preliminary experimental data which completely fit with this hypothesis of a given idiotype present on both T and B cells expressing a defined specificity for a Tg epitope. This point will be discussed later (Section VII).

Recently, we produced in CBA mice monoclonal anti-id A-Abs by immunization with a murine monoclonal anti-Tg A-Ab in CFA which blocked the proliferative T cell response on syngeneic TECs (Roubaty *et al.*, 1989). Antiidiotype A-Abs were selected by binding to the $F(ab')_2$ of the immunizing anti-Tg A-Ab. Among them, one totally competed with a pathogenic tryptic fragment (TF) from the PTg molecule for binding to monoclonal anti-Tg A-Ab, while another was not able to do so. They were named Ab2-β and Ab2-α (Jerne *et al.*, 1982), respectively. We then evaluated the levels of antiidiotype A-Abs in CBA mice before immunization and on day 28 postimmunization with Tg. For that purpose, these

sera were tested against (1) polyclonal anti-Tg A-Abs, (2) monoclonal anti-Tg A-Ab blocking the T cell proliferative response (Ab2-β response), and (3) monoclonal anti-Tg A-Ab, which did not interfere with the proliferative response. We found that only the Ab2-β A-Abs were increased in sera from immunized mice, while they were at low levels in mouse sera tested before Tg immunization. When total Ab2 A-Abs in sera from controls or immunized animals were tested against the same three anti-Tg A-Abs just described, they were detected at similar levels independently of the immunization procedure. These A-Abs, mostly non-Ab2-β, probably represent Ab2 A-Abs directed against CRI borne by almost all mouse IgG (Table IV).

Much more interestingly, these experiments demonstrated the variation of exclusively Ab2-β A-Abs related to thyroid pathology of mice; these antiidiotype A-Abs are significantly decreased in protected controls and significantly increased in diseased animals. Up to now, variations of antiidiotype A-Abs related to thyroid pathology were never reported. We hypothesized that we detected EAT-related Ab2 antiidiotype A-Abs because we studied a restricted anti-Tg A-Ab population, specific for a pathogenic TF of the PTg molecule (Bédin *et al.*, 1989). In theory, their serological evaluation would allow EAT diagnosis.

Taken together, these data support the existence of antiidiotype clones in animal or human repertoires and consequently brought experimental support to Jerne's hypothesis of a relationship between idiotypic regulation and autoimmune reactivity. Moreover, some antiidiotype A-Abs to anti-Tg A-Abs modulate (positively or negatively) the anti-Tg A-Ab levels when injected into animals after Tg challenge, depending upon the recipient's immune status or the idiotype that they recognize. These various parameters further increase the complexity of future therapies through antiidiotype A-Abs to anti-Tg A-Abs.

VII. Prevention and Treatment of Thyroiditis

Different therapies, either thyroid-specific or not, have been developed for susceptible strains of rats and mice to prevent or cure thyroiditis. The specific methods of EAT prevention included injection before immunization with thyroid extracts of soluble Tg, Tg-specific T cell lines or clones, or antiidiotype A-Abs specific to anti-Tg A-Abs. Nonspecific methods were also developed. They usually interfere with the immunological parameters involved in EAT and were mainly aimed at enhancing the recipient's suppressor activities or blocking effector functions.

TABLE IV

LEVELS OF ANTIIDIOTYPE A-Abs TO VARIOUS ANTI-Tg A-Abs (A-Abl) IN PREIMMUNE AND IMMUNE SERA FROM CBA MICE

Nature of the coated $F(ab')_2$ from anti-Tg A-Abs	Before immunization[a] (Day 0)	After immunization (Day 28)	*t* test
Polyclonal A-Abl	1112 ± 102[b]	1128 ± 127	NS[c]
Pathogenic epitope-related monoclonal A-Abl	430 ± 56	1456 ± 215	$p < 0.0001$
Nonpathogenic epitope-related monoclonal A-Abl	1227 ± 97	1310 ± 147	NS

[a] Mean ± SEM of seven to eight sera per group of mice.
[b] Optical density at 492 nm in an ELISA.
[c] Not significant.

In this respect, immunosuppressive agents (Borel *et al.*, 1976; Shevach, 1985) such as CyA were used in obese chicken SAT (Wick *et al.*, 1982) and in rat and mouse EAT (McGregor *et al.*, 1983; Hassman *et al.*, 1985; Vladutiu, 1983). Beneficial effects were observed on the degrees of thyroid damage in EAT but inconstant effects were noted in OS chicken SAT. In these birds, CyA improved SAT only when it was applied after hatching and aggravated the disease when given to OS embryos; anti-Tg A-Abs were never affected in either case.

Because CyA is known to induce adverse side effects and because potentiation with another immunomodulatory molecule (1,25-dihydroxy vitamin D_3) [$1,25(OH)_2D_3$] was reported in *in vitro* studies of the T cell proliferative response and IL-2 production by $CD4^+$ lymphocytes from rheumatoid arthritis patients (Gepner *et al.*, 1989), we also sought an *in vivo* synergistic effect in mouse EAT. For that purpose, a 3-week treatment regimen with suboptimal doses of CyA or $1,25(OH)_2D_3$ alone or together was initiated along with priming by Tg. Although thyroid infiltrations were never significantly affected by each drug alone, mice receiving simultaneously suboptimal doses of both drugs exhibited a signficantly lower incidence of thyroid pathology and developed milder disease.

Because of their *in vitro* immunosuppressive effects, the preventive capacities of antithyroid drugs such as methimazole were also evaluated in murine (Davies *et al.*, 1984) or BB/W rat thyroiditis (Allen *et al.*, 1986b) (Wall *et al.*, 1976; McGregor *et al.*, 1980; Weiss and Davies, 1981, 1982). In these experiments, both the splenic immune response and the degree of thyroiditis after heterologous Tg immunization were significantly reduced, while quantitative differences in the circulating and intrathyroidal anti-Tg A-Abs were not detected.

It was also reported that PHA, a T cell mitogen, prevented the development of murine EAT after LPS injections, whereas no effect was noted in EAT conventionally induced with Tg in CFA (Esquivel *et al.*, 1982).

Since genes of the *I-A* subregion of the *H-2* MHC complex have a major influence on EAT (Beisel *et al.*, 1982a; Salamero and Charreire, 1983b), monoclonal anti-I-A Abs of the relevant MHC specificity were given to susceptible mice before and after the antigenic challenge with Tg in CFA (Vladutiu and Steinman, 1987). When monoclonal anti-I-A Abs were given before immunization, the development of thyroid lesions and anti-Tg A-Abs was totally prevented; however, when they were given after the antigenic challenge, the severity of EAT and the titers of anti-Tg A-Abs were decreased but not completely suppressed.

Finally, because females are much more susceptible than males due to the sex linkage of autoimmune reactivity, testosterone was given to

PVG/c rats with chronic thyroiditis. This was reported to be beneficial (Ahmed *et al.*, 1986). This improvement was exclusively in the severity and the incidence of the lesions; the anti-Tg A-Ab levels remained unaltered.

Among the specific methods, the first indicative experiments of EAT prevention were reported by Jankovic and Flax (1963), who found that soluble thyroid extract given before or after injection of thyroid extract in CFA delayed the onset of thyroiditis, but did not reduce the incidence or severity of the disease. Similarly, Silverman and Rose (1974b) and Whitmore and Irvine (1977) showed that thyroid extract injections suppressed the development of SAT in some strains of rats. Later, Braley-Mullen *et al.* (1978, 1980) demonstrated that EAT suppression could be induced in the guinea pig (GP) by pretreatment of animals with either GPTg emulsified in IFA or with GPTg coupled to syngeneic spleen cells. In these cases of EAT suppression by GPTg, the suppressive effect was transferrable by lymphoid cells but never by sera from pretreated animals. Moreover, in these two last models of EAT suppression, the anti-Tg A-Abs titers were never significantly modified.

EAT in susceptible strains of mice was also prevented by intravenous injection of 200 μg of soluble Tg on days 3 and 10 prior to immunization with MTg and LPS (Kong *et al.*, 1982). This suppression was transferred by spleen cells from tolerant animals and mediated by T cells. The relationships among circulating MTg concentrations and the extent of prevention were studied. For that purpose, protection against EAT in H-2^k strains of mice was induced through augmentation of MTg either by exogenous intravenous injection or by physiological stimulation of the thyroid gland by TSH or thyrotropin-releasing hormone before immunization (Lewis *et al.*, 1987). It was shown that an exogenous or endogenous increase of the MTg levels blocked the development of EAT. The authors hypothesized that the MTg increase could be directly responsible for the subsequent activation of suppressor T cells leading to reinforced prevention of disease induction.

More recently, the phenotype of the cells involved in the induction of EAT suppression was reexamined by Parish *et al.* (1988a,b). While they confirmed Kong's data (1989) showing that suppression induced by soluble Tg was an active phenomenon due to T cells, their findings conflicted with the hypothesis that $CD8^+$ suppressor T cells could be activated in the recipients and they suggested that $CD4^+$ suppressor T cells or $CD4^+$ suppressor-inducer cells were involved. In their experiments, pretreatment of animals with anti-L3T4 or anti-Lyt-1 prior to tolerance induction prevented EAT suppression, whereas treatment with anti-Lyt-2 was ineffective. Interestingly, they were unable to break

this tolerance by immunizing with cross-reactive xenogeneic Tg. Similarly, Stull *et al.* (1988) showed that EAT in susceptible strains of mice could be prevented by injecting animals with Tg-sensitized spleen cells depleted of $L3T4^+$ T cells.

Tg-specific T cell lines or clones were also used to prevent EAT. The first protocols designed by Maron *et al.* (1983) were reminiscent of those used in adjuvant arthritis (Holoshitz *et al.*, 1983) or in experimental autoimmune encephalomyelitis (EAE) (Ben-Nun and Cohen, 1982). In these experiments, they selected autoimmune effector T cell lines from rats that spontaneously recovered from EAE and then became refractory to attempts to induce a second EAE; these cell lines induced EAE when injected into naive recipients. They suggested that potential autoimmune effector T lymphocytes could develop and persist without necessarily causing disease. Using the same rationale, they obtained total prevention of EAT in susceptible strains of mice when animals were injected 3 weeks prior to EAT induction with 10^6 attenuated cells of a Tg-specific T cell line. Again, the anti-Tg A-Ab levels in vaccinated mice were not modified by this preventive therapy and thus the lack of a relationship between EAT and anti-Tg A-Abs levels was observed once more. In these experiments, a positive regulatory role for either antiidiotype A-Abs directed against the TcR or for suppressor cells was also envisaged.

Recently, in other experimentally induced autoimmune diseases, including EAE (Sun *et al.*, 1988; Lider *et al.*, 1988; Ellerman *et al.*, 1988), type II collagen arthritis (Kakimoto *et al.*, 1988; Arita *et al.*, 1987), uveoretinitis (de Kozak *et al.*, 1987), or myasthenia gravis (Agius and Richman, 1986), protection against these pathologies was obtained using either antigen-specific T cell lines or clones or specific antiidiotype Abs.

We have also investigated this therapeutic approach using a cloned cytotoxic T cell hybridoma (HTC_2) specific for a pathogenic epitope(s) of the PTg molecule, class I restricted, that we had selected (Section V,A,b). For this purpose, 10^6 mitomycin-inactivated HTC_2 cells were injected into normal syngeneic recipients 21 days before immunization with Tg in CFA. As shown in Table V, a 76% reduction of lymphocyte infiltration of the thyroid glands of animals inoculated with only HTC_2 was observed. Analyses of anti-Tg A-Abs and Ab2-β anti-id A-Abs responses were conducted both on day 0 (3 weeks after HTC_2 injection and before Tg immunization) and on day 28 postimmunization, when EAT is in the acute phase. As expected, anti-Tg A-Abs were at high levels in mice on day 28 post-Tg challenge, independently of the preventive treatment. More surprisingly, they were also detected on day 0 before Tg challenge, exclusively in mice which had received HTC_2 cells. Therefore, we called these Ab1-like Abs "Ab3," because the mice had

TABLE V
PREVENTION OF EAT BY Tg-SPECIFIC T CELL HYBRIDOMA VACCINATION

T cell clone inoculated	Tg challenge	No. of mice	EAT			
			Incidence	Mean grade/corresponding no. of animals	Infiltration index ± SEM	Relative EAT (%)
None	None	5	0/5	Acute/0 Severe/0 Mild/0 Negative/5	0.40 ± 0.04	—
None	+	8	8/8	Acute/7 Severe/1 Mild/0 Negative/0	4.20 ± 0.40	100
BW 5147	+	8	8/8	Acute/3 Severe/3 Mild/2 Negative/0	3.10 ± 0.30	76
HTC_2	+	8	3/8	Acute/0 Severe/0 Mild/3 Negative/5	1.00 ± 0.23	24

never previously encountered Tg except for endogenous Tg, which was present in each mouse at similar low levels (approximately 10 ng/ml). We hypothesized that Ab3 could be generated *in vivo* from antiidiotype A-Abs. More precisely, we postulated that T cells bearing specific receptors for the A-Ag prevented EAT through two possible mechanisms. The first one supposed the generation of anti-TcR A-Abs structurally similar to the antiidiotype A-Abs (Ab2-β), which would represent the internal image of pathogenic Tg epitope(s) and would produce specific Ab1-like A-Abs or Ab3. These A-Abs would in turn block the specific pathogenic epitopes at the time of immunization and therefore the specific helper T cell response inducing EAT. Consequently, they would protect against EAT. Another explanation would envision that injected cytotoxic HTC_2 cells specifically migrated to the thyroid gland and destroyed it, thus liberating syngeneic MTg. In this latter alternative, the anti-Tg A-Abs that we called Ab3 would become conventional anti-Tg A-Abs, which would further stimulate *in vivo* antiidiotype A-Ab production. Experiments are now in progress to elucidate the nature and the origin of anti-Tg A-Abs. It can be thought that each hypothesis is not exclusive and cooperativity among at least these two mechanisms occurs.

Variations of antiidiotype A-Abs (Ab2-β) were also observed: they were significantly decreased in protected mice and significantly increased in diseased animals. As mentioned above (Section VI,B), we hypothesized that we detected EAT-related Ab2-β antiidiotype A-Abs because we studied a restricted anti-Tg A-Ab population specific for a pathogenic TF of the Tg molecule. To further investigate the potential role of these specific antiidiotype A-Abs in EAT prevention, 7 days prior to immunization with Tg in CFA, we injected mice with two monoclonal antiidiotype A-Abs, Ab2-α and Ab2-β, both specific to one monoclonal A-Ab to Tg recognizing the pathogenic epitope of the Tg molecule. This protocol was designed to induce in recipient mice injected with Ab2-β exclusively anti-Tg A-Abs specific for the pathogenic epitope of Tg during the 7 days before Tg challenge. Therefore, protection would be generated by blocking the pathogenic Tg epitopes at the time of immunization. As anticipated from our previous data, only mice receiving Ab2-β were significantly protected against EAT, while those injected with Ab2-α behaved as controls (Fig. 2). This last series of experiments even further established that a physiological idiotypic network could represent a regulatory mechanism for EAT. In these experiments, Ab2-β directed against monoclonal anti-Tg A-Abs played a pivotal role because they bind to the B cell producing the Ab1 specific to the pathogenic Tg epitope, as well as to the T cells specific to this epitope and, furthermore, they represent the internal image of the nominal antigen.

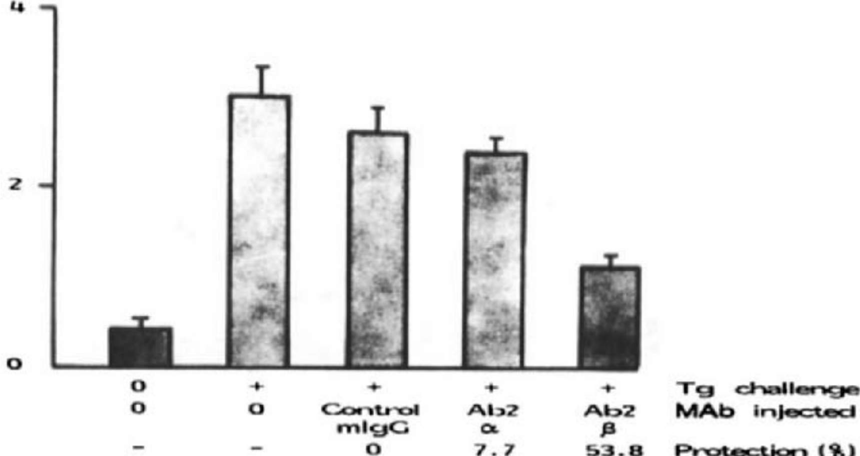

FIG. 2. Thyroid infiltration index. Monoclonal antiidiotype A-Abs to Tg protect against EAT. On day 7 before immunization with PTg in CFA, CBA mice (five per group) were injected ip with 40 μg of MAb. On day 28 postimmunization, animals were killed and their thyroids were collected for histopathological studies.

EAT regulation through a physiological idiotypic network bridging T and B lymphocytes, specific to defined pathogenic TFs from the PTg molecule inducing EAT, now seems possible (Fig. 3). Attention must be drawn to the fact that it requires the simultaneous recognition of a crucial epitope by both T and B cells. Such a hypothesis was reminiscent of Male *et al.*'s experiments (1985) (Section VI,B). It is generally accepted that T cells recognize linear peptides, whereas B cells preferentially recognize conformational structures. This dogma, further emphasized by the difficulties encountered in developing antipeptide antibodies, has been fully demonstrated for Tg as for other Ags. However, one can wonder whether a linear peptide simultaneously "seen" with class I or class II MHC Ags does not exhibit some conformational structures. Experimental data presented in this review demonstrated that a 5- to 10-kDa TF of the PTg molecule could be affinity purified by monoclonal anti-Tg A-Abs and *in vitro* could stimulate T helper cells capable of transferring EAT. Again, this 5- to 10-kDa protein may contain some recognizable conformational site(s). However, the simultaneous recognition of a given pathogenic epitope by both specific T and specific B cells has become more plausible along with the appearance, during the past few years, of data concerning TcR and Ig structures. TcR-variable α and β chains are homologous to Ig-variable L and H chains, respectively, with an average of 25–30% amino acid identity. Moreover, residues

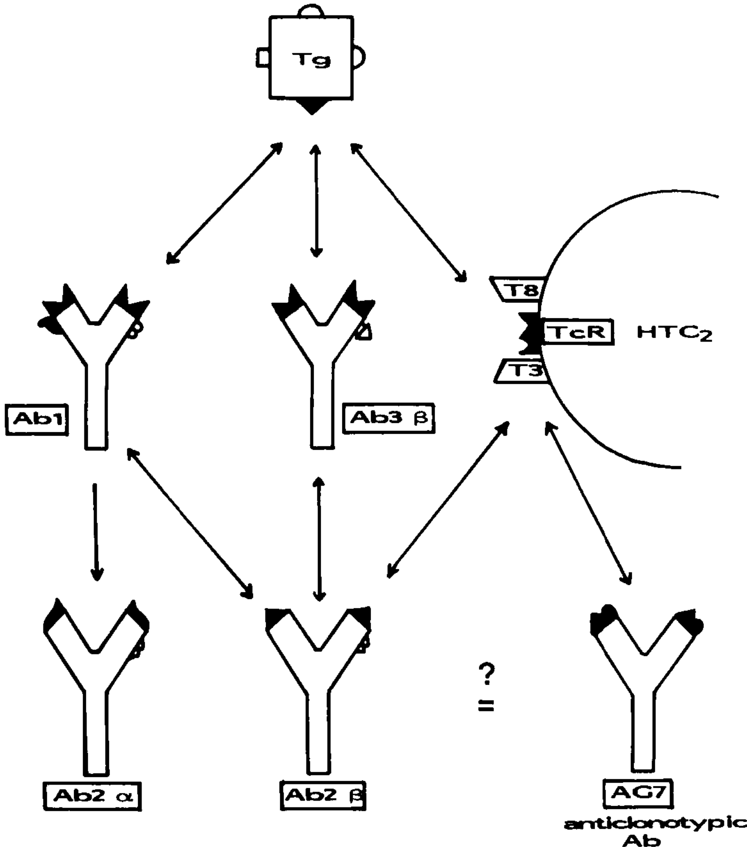

FIG. 3. Hypothetical regulation of EAT through a physiological idiotypic network, bridging T and B lymphocytes, both specific to the pathogenic epitope(s) of the Tg molecule (▼).

corresponding to important structures such as cysteines, aromatic residues (Novotny *et al.*, 1986), and amino acids forming salt bridges are conserved (Bougueleret and Claverie, 1987), and secondary structure predictions also suggest a similar folding pattern; TcRs are thus expected to have a spatial organization close to that of the Fab moiety of Ig (Claverie *et al.*, 1989). Thus their interaction with a same peptide or protein can be envisioned, for self-Ag, for example. This makes for an extremely attractive hypothesis. Nonetheless, we have not lost sight of numerous studies demonstrating that a difference of only one amino acid among two similar sequences leads to a total loss of Ag recognition by T cells.

VIII. Concluding Remarks: Toward a New Conception of Autoimmune Reactivity

During the process of reviewing the past decade's literature on the immune mechanisms of thyroiditis, it has become apparent that our understanding of the disease is at a turning point. The more our knowledge of thyroiditis accrued, the more complex and intricate seemed its cause. The reasons for this discrepancy are the multiplicity and the complexity of the immune mechanisms involved.

In addition to models of spontaneous thyroiditis, numerous methods were used to induce EAT in animals. All these schemes offer some pathogenetic insight. Similarly, the genetic control of thyroiditis was demonstrated to be multigenic. Moreover, multiple cells produce multiple A-Abs and multiple cytokines modulate the disease. In the forthcoming years, studies devoted to a better understanding of the mechanisms controlling the interplay between cells, autoantibodies, and cytokines promise better comprehension of thyroiditis, autoimmune reactivity, and, to a larger extent, immune reactivity.

Molecular biology technology, which allowed the determination of the amino acid sequences of Tg, thyroid peroxidase, and yet unidentified thyroid Ags, must be further exploited and crystallographic studies are needed to determine the secondary and tertiary structures of the various thyroid Ags. The field of immunogenetics promises answers to how a given MHC gene regulates or facilitates thyroiditis and how it cooperates with genes coding for Tg, A-Abs, cytokines, or other products. The powerful transgenic mouse model appears to be one of the most appropriate tools to answer these questions.

Cell biology studies have to be further pursued to elucidate such fundamental relationships as the existence of a receptor for Tg on thyroid cells, the nature of the mechanisms regulating the secretion of very small amounts of Tg into the blood, and Tg processing by thyroid cells, macrophages, or B cells.

Basic immunological puzzles approaching solution in other systems will surely be clarified in HT in the near future. These include the clonality of T cells transferring EAT, that of B cells producing anti-Tg A-Abs, the antigen presentation to T cells, the potential APC function of TECs, and the definition of the immunodominant minimal amino acid sequence of Tg-inducing thyroiditis.

During the past few years, considerable efforts have been made to characterize the structures of Ig genes and their regulation. To date, however, few data have enhanced our knowledge of anti-Tg A-Abs. Perhaps the lack of induction of EAT by anti-Tg A-Abs and the absence of a

correlation between A-Abs and thyroiditis are responsible for this gap. Our very recent data on murine EAT, demonstrating a relationship between the pathology and a defined subpopulation of specific anti-Tg and antiidiotype A-Abs belonging to the Ab2-β population, renew interest in this field of research. Such studies can answer several questions: How many V_H genes are used for A-Ab production during thyroiditis? What is their expression frequency in the pathological state compared to normal controls? Do they belong to V_H gene families common to other V_H genes coding for other A-Abs?

Because the presence of anti-Tg A-Abs is critical for the autoimmune origin of thyroiditis, the lack of correlation between the levels of anti-Tg A-Abs and the severity of thyroiditis evaluated by lymphocytic infiltration is surprising. Perhaps the large Tg molecule presents a vast continuum of determinants, each of which may trigger A-Ab production to a given Tg epitope. Fortunately, most of these A-Abs are not directed against epitopes involved in the pathogenesis of thyroiditis. Therefore, the lack of correlation between anti-Tg A-Abs and pathology could be explained. This enigma will surely be solved in the future, probably resulting in the diagnosis of thyroiditis based on the detection of anti-Tg A-Abs directed against crucial epitopes of the Tg molecule.

Our hypothetical view, given the present state of knowledge and complexity of immune mechanisms in thyroiditis, is summarized in Fig. 3 and hopefully will offer a framework for our future investigations.

The detection of A-Abs to Tg provided the first indication of the autoimmune origin of thyroiditis, and today no doubt exists as to the role of T cell-mediated immunity in this disease. Nevertheless, the nature of the relationships among T and B cell populations remains unclear. Prevention of EAT, which follows preinjection of Tg-specific T cell clones, leading to significant variation in the specific antiidiotype A-Abs produced, demonstrates the existence of a physiological idiotypic network bridging T and B cells in EAT. However, this network must be further investigated in order to design predictable therapies for thyroiditis.

The basic question raised by the existence of this specific idiotype network and its potential to regulate EAT concerns the relationship between the TcRs carried by Tg-specific T cells and the Igs borne by specific B cells. In this network, a given epitope of the Tg molecule must be recognized by both the specific B and specific T cells. Up to now, such a "natural" coincidence has proved to be exceptional and only a few reports of experimental induction have been published. However, in the past few years, the homology of sequences and residues corresponding to important structures has been demonstrated among α and β chains of the TcR and the L and H chains of the Igs, revealing that these

products belong to the same superfamily and thus could present similar secondary conformations. Homology between T and B cell-surface products occurs, favoring similar antigenic recognition by these cells, particularly for self-Ag. It can be envisioned that thyroiditis and, to a broader extent, autoimmune reactivity would only occur when a defined A-Ag is recognized by both specific T and specific B cells. This simultaneous recognition would strongly dysregulate the homeostatic idiotype network present in any normal individual or animal. To further clarify this relationship, the precise definition of a pathogenic A-Ag and the production of anticlonotypic A-Abs to TcRs and of antiidiotype A-Abs to A-Abs specific to pathogenic Tg epitope(s) have to be obtained.

Finally, it should be recalled that thyroiditis is a relatively chronic disease with multiple parameters varying with time. How can a specific idiotype network control the regulation of initiation and progression of thyroiditis? The answer to this question may provide the insight to design an optimal intervention to prevent or cure the disease. Consequently, studies defining the presence of different cells involved, their nature, their specificity, their sensitivity to factors, the products released, etc., throughout the course of disease are high priorities.

To conclude, we hypothesize that recognition of a defined A-Ag by both T and B cells would result in a breakdown of a silent self–anti-self-idiotype network normally present. Its dysregulation could possibly occur through a sudden increase of various components, such as A-Ag, or specific B or specific T cells and their products. This mechanism is highly probable if organisms bear an MHC haplotype of susceptibility to thyroiditis and if the elusive suppressor cells are transiently deficient. Therefore, the initiation and the self-perpetuation of autoimmune reactivity would be possible. As a corollary, it can also be envisioned that the same pathways inducing anti-Tg A-Abs could be exploited to prevent or treat the disease. Stimulation of the right cell, at the right time, could lead to predictable controlled management of thyroiditis.

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Index

A

Acetylcholinesterase, autoimmune thyroiditis and, 268
Actin
  immunosenescence and, 224
  leukocyte integrins and, 151, 169
Acute lymphoblastic leukemia, 45
Acute myelomonocytic leukemia, 160
Adenylate cyclase, immunosenescence and, 226
Alleles
  complement receptor 1 and, 188, 189, 191
  complement receptor 2 and, 202
Allergic thyroiditis, murine lupus models and, 79
Alloantigens, immunosenescence and, 221, 245, 252
Allogeneic cells, immunosenescence and, 250
Allografts, autoimmune thyroiditis and, 286
Allotype
  complement receptor 1 and, 185, 187-190, 195, 196
  immunosenescence and, 248
Alum/*Borderella pertussis* (ABP), 137
Amino acids
  autoimmune thyroiditis and, 317
    antigens, 268, 272
    genetic control, 284
    humoral responses, 302
    prevention, 315, 316
  complement receptor 1 and, 184, 186, 187, 194
  complement receptor 2 and, 202, 205, 210
  leukocyte integrins and, 154-156, 159, 174, 176
  maps of immunoglobulin-like loci and, 2
    structure, 7, 8, 10, 12, 13, 15, 21, 25
    T cell receptor, 35, 38, 41
  murine lupus models and, 69
Anti-bromelaine-treated red blood cells, 72, 74
Antibodies
  autoimmune thyroiditis and, 263, 317-319
    antigens, 272, 273
    cellular immune responses, 287, 289, 290, 293-295
    experimental models, 276-278, 281, 282
    genetic control, 283, 285-287
    humoral responses, 298-307, 309
    prevention, 308, 310, 312, 314, 315
  complement receptor 1 and, 193, 196, 200, 201
  complement receptor 2 and, 206
  helper T cell cytokines and
    cross-regulation of differentiation, 131, 132
    differential induction, 127, 129, 130
    functions, 116, 118-120
    immune responses, 133, 137, 138
    precursors of differentiation states, 124
  immunosenescence and, 221, 253
    lymphocyte activation, 222
    mucosal immunity, 252
    regulatory changes, 247, 248, 250, 251
    stem cells, 246
  leukocyte integrins and, 164
  murine lupus models and, 99
    Ig germline, 65, 66, 72, 74, 76, 77
    lupus strains, 64
    T cell antigen receptor, 79, 88, 90, 98
Antibody-dependent cell-mediated toxicity, 114, 122
Antigen-presenting cells
  autoimmune thyroiditis and, 266, 317
    cellular immune responses, 289, 294-297
    experimental models, 281
  helper T cell cytokines and, 112, 128-130
Antigens
  autoimmune thyroiditis and, 263, 265-273, 317-319
    cellular immune responses, 288, 289, 291, 293-295, 297, 298
    experimental models, 273, 276, 281
    genetic control, 284, 285
    humoral responses, 298, 299, 302-305, 307
    prevention, 310, 312, 314-316
  complement receptor 1 and, 200

complement receptor 2 and, 205, 207-209, 211
helper T cell cytokines and, 111
cross-regulation of differentiation, 130-132
differential induction, 128-130
functions, 114, 115, 117-122
immune responses, 138
precursors of differentiation states, 122-127
secretion patterns, 112, 113
immunosenescence and, 253
lymphocyte activation, 222-224, 228, 231, 235
lymphocyte subsets, 237
mucosal immunity, 253
regulatory changes, 249-251
stem cells, 247
leukocyte integrins and, 149, 153, 161-163, 169
maps of immunoglobulin-like loci and, 1, 7, 29, 31, 32, 34
murine lupus models and, 65, 99
Ig germline, 72, 74, 76-78
T cell antigen receptor, 81, 90, 91
Antiidiotypic antibodies
autoimmune thyroiditis and, 318
genetic control, 287
humoral responses, 301, 304-309
prevention, 308, 312, 314
immunosenescence and, 247, 248, 252, 253
Arthritis
autoimmune thyroiditis and, 284, 310, 312
murine lupus models and, 61, 75, 79, 90
Astrocytes, autoimmune thyroiditis and, 293
Autoantibodies
autoimmune thyroiditis and, 298, 317
helper T cell cytokines and, 120
murine lupus models and, 99
Ig germline, 65-67, 71-77
lupus strains, 63, 64
Autoimmune thyroiditis, 263-266, 317-319
antigens, 266
thyroglobulin, 267-271
thyroid peroxidase, 272, 273
cellular immune responses, 287
lymphoid cells, 287-291
target thyroid cells, 291-298
experimental models, 273
induction, 276-282
SAT, 273-276
genetic control, 282
experimental induction, 284-287
SAT, 282-284
humoral responses, 298
antiidiotype autoantibodies, 305-309
autoantibodies, 298-306
prevention, 308, 310-316
Autosomes, murine lupus models and, 63, 94

B

B cell antigen receptor, murine lupus models and, 61, 65
B cells
autoimmune thyroiditis and, 263, 318
antigens, 270, 271
cellular immune responses, 288, 290, 295, 297
experimental models, 275-277, 280, 282
humoral responses, 299-304, 306, 307
prevention, 314, 315
complement receptor 1 and, 183, 193, 200, 201
complement receptor 2 and, 203-211, 213
helper T cell cytokines and
cross-regulation of differentiation, 131
differential induction, 128-130
functions, 114, 116-122
immune responses, 133, 136
precursors of differentiation states, 125, 126
immunosenescence and, 253
lymphocyte activation, 231-234, 236
mucosal immunity, 251, 253
regulatory changes, 248, 251
stem cells, 240, 242, 243, 246, 247
leukocyte integrins and, 151, 154, 166, 171
maps of immunoglobulin-like loci and, 1
murine lupus models and, 64-66, 76, 98
Bacteria
complement receptor 2 and, 205
helper T cell cytokines and, 133
leukocyte integrins and, 151, 161
maps of immunoglobulin-like loci and, 1
Bone marrow
autoimmune thyroiditis and, 301
complement receptor 1 and, 203, 204
helper T cell cytokines and, 115, 117
immunosenescence and
regulatory changes, 248, 249, 251
stem cells, 240-247
leukocyte integrins and, 160, 166
murine lupus models and, 64, 80, 81

Bronchial lymph nodes, immunosenescence and, 236, 237, 251
Brucella abortis, helper T cell cytokines and, 127, 137

C

C3-binding protein
  complement receptor 1 and, 185, 189, 195, 196
  complement receptor 2 and, 202
C4-binding protein
  complement receptor 1 and, 184, 185, 189, 195, 197
  complement receptor 2 and, 202
c-*myc*, immunosenescence and, 223, 227, 228
Cachexia, helper T cell cytokines and, 114
Calcium
  complement receptor 1 and, 199
  complement receptor 2 and, 207, 208
  immunosenescence and, 222–226, 229, 235, 253
  leukocyte integrins and, 156, 157, 159
Cancer, maps of immunoglobulin-like loci and, 1
Carbohydrate
  autoimmune thyroiditis and, 271
  complement receptor 2 and, 204
  leukocyte integrins and, 173, 174
Cartilage matrix protein, leukocyte integrins and, 158
cDNA
  complement receptor 1 and, 187, 188, 191, 193, 194, 199
  complement receptor 2 and, 211
  helper T cell cytokines and, 112, 115, 116
  leukocyte integrins and, 154–158, 160, 174, 176
  maps of immunoglobulin-like loci and, 34, 43
  murine lupus models and, 83, 87, 95
Cell cycle, immunosenescence and, 221–223, 227, 234, 235, 253
Centromeres, maps of immunoglobulin-like loci and, 26, 30, 33, 44
Chemotaxis
  complement receptor 1 and, 192, 199
  leukocyte integrins and, 151, 160, 161, 163, 164, 166, 167
Chromatids
  maps of immunoglobulin-like loci and, 3
  murine lupus models and, 83
Chromatin, immunosenescence and, 234
Chromosomes
  autoimmune thyroiditis and, 268, 272
  complement receptor 1 and, 190
  complement receptor 2 and, 202
  leukocyte integrins and, 159, 160, 170
  maps of immunoglobulin-like loci and, 2, 3, 5–7
    MHC, 28, 31, 33
    structure, 7, 9–13, 18, 19, 21, 23, 25–27
    T cell receptor, 36, 39, 42, 43, 45
  murine lupus models and, 63, 64
Chronic myelogenous leukemia, leukocyte integrins and, 160
Clathrin-coated pits, complement receptor 1 and, 199
Clones
  autoimmune thyroiditis and, 263, 317, 318
    antigens, 270, 272
    cellular immune responses, 288, 289, 296
    experimental models, 276, 279, 281, 282
    humoral responses, 298, 301, 306, 308
    prevention, 312, 313
  complement receptor 1 and, 188, 199
  complement receptor 2 and, 201, 202, 211
  helper T cell cytokines and, 111, 115, 116, 118, 121, 122
    cross-regulation of differentiation, 130, 131
    differential induction, 130
    functions, 114, 117, 119, 120
    human subsets, 139
    immune responses, 136–138
    precursors of differentiation states, 123
    secretion patterns, 112, 113
  immunosenescence and, 237, 249
  leukocyte integrins and, 156, 164, 171, 172
  maps of immunoglobulin-like loci and, 3–5
    MHC, 29, 32, 33
    structure, 11, 13, 15, 17, 18, 21, 23–27
    T cell receptor, 36, 37, 45
  murine lupus models and, 99, 100
    Ig germline, 66, 67, 71, 76–78
    lupus strains, 63
    T cell antigen receptor, 79, 82, 83, 88, 90, 91, 93, 95–97
Clonotypes, murine lupus models and, 78, 99
Colchicine, immunosenescence and, 224
Collagen
  helper T cell cytokines and, 128
  leukocyte integrins and, 153, 159
  murine lupus models and, 79, 90

Collagen arthritis, autoimmune thyroiditis and, 312
Colony-stimulating factors, 116
Complement receptor 1, 183, 208, 209
allotypes, 187–190
function
endocytosis, 198–200
erythrocytes, 198
immunoregulation, 200, 201
ligand binding sites, 193–196
regulation, 196, 197
structure, 183–187
tissue distribution, 190–193
Complement receptor 2, 183
function, 205
EBV tropism, 210–213
immunoregulation, 205–210
structure, 201–203
tissue distribution, 203–205
Complement receptor 3, 209, 210
Complement-receptor type-3, leukocyte integrins and, 150, 163, 164
Complementarity-determining regions
maps of immunoglobulin-like loci and, 7
murine lupus models and, 72, 78
Complete Freund's adjuvant, autoimmune thyroiditis and
experimental models, 276–282
genetic control, 286, 287
humoral responses, 301, 302, 307
prevention, 309–312, 314
Concanavalin A
autoimmune thyroiditis and, 281
immunosenescence and
lymphocyte activation, 223, 225, 226, 228–230
mucosal immunity, 252
stem cells, 245
murine lupus models and, 98
Cosmids, maps of immunoglobulin-like loci and, 4, 5
MHC, 29, 32, 33
structure, 17, 18, 27
T cell receptor, 36, 37
Cross-reactive idiotype, autoimmune thyroiditis and, 303–305, 308
Cyclic AMP
autoimmune thyroiditis and, 268, 272
immunosenescence and, 223, 226, 227, 232
Cyclic GMP, immunosenescence and, 223
Cyclic nucleotides, immunosenescence and, 226, 227, 232
Cyclosporin, autoimmune thyroiditis and, 294
Cysteine
autoimmune thyroiditis and, 316
complement receptor 1 and, 184, 194
complement receptor 2 and, 202
leukocyte integrins and, 154, 155
maps of immunoglobulin-like loci and, 2, 13
Cytokine synthesis inhibitory factor, 131, 133, 135
Cytokines
autoimmune thyroiditis and, 275, 298, 307, 317
complement receptor 1 and, 192, 199
helper T cell, *see* Helper T cell cytokines
leukocyte integrins and, 170, 172, 176
Cytoplasm
autoimmune thyroiditis and, 295
complement receptor 1 and, 184, 188–190
complement receptor 2 and, 202, 209
leukocyte integrins and, 154–156, 166, 171
Cytoskeleton
autoimmune thyroiditis and, 298
complement receptor 1 and, 186, 198
complement receptor 2 and, 210, 213
immunosenescence and, 224, 235
leukocyte integrins and, 163, 169, 171, 176
Cytotoxic T lymphocytes
autoimmune thyroiditis and, 288
helper T cell cytokines and, 111
cross-regulation of differentiation, 132
functions, 114–117
immune responses, 133, 135
precursors of differentiation states, 125
secretion patterns, 112, 113
immunosenescence and, 244, 245, 249, 250
leukocyte integrins and, 150
inflammation, 161, 163, 164
ligand molecules, 172

D

Decay accelerating factor, complement receptor 1 and, 189, 197
Delayed-type hypersensitivity
helper T cell cytokines and
cross-regulation of differentiation, 131
differential induction, 127–130
functions, 117
human subsets, 139
immune responses, 133, 137, 138
precursors of differentiation states, 125

immunosenescence and, 221, 237
leukocyte integrins and, 162
Dendritic cells
autoimmune thyroiditis and, 280, 281, 296, 297, 300
complement receptor 1 and, 190
complement receptor 2 and, 209
helper T cell cytokines and, 128
Diabetes
autoimmune thyroiditis and, 269, 284, 298
murine lupus models and, 79
Diacylglycerol, immunosenescence and, 223, 224
Dibutyrl cyclic AMP, autoimmune thyroiditis and, 294
Differentiation
autoimmune thyroiditis and, 266
complement receptor 2 and, 204, 206
helper T cell cytokines and
cross-regulation, 130–133
human subsets, 139
immune responses, 137
induction, 127–130
precursors, 122–127
immunosenescence and, 221, 253
lymphocyte activation, 231, 232
stem cells, 240, 242–247
DNA
autoimmune thyroiditis and, 267, 303
complement receptor 1 and, 188
complement receptor 2 and, 206
immunosenescence and, 230, 234, 253
leukocyte integrins and, 160, 174, 175
maps of immunoglobulin-like loci and, 3–6, 46
structure, 11–13, 15, 18, 25, 27
T cell receptor, 36, 37, 39, 41–43, 45
murine lupus models and
Ig germline, 66, 69, 71, 72, 76–78
T cell antigen receptor, 83, 84, 87
Down's syndrome, leukocyte integrins and, 160
Drosophila, leukocyte integrins and, 153

E

Electron microscopy
autoimmune thyroiditis and, 267, 274, 291
complement receptor 1 and, 184
leukocyte integrins and, 167
Electrophoresis
complement receptor 1 and, 189, 190, 192
immunosenescence and, 230
leukocyte integrins and, 154, 157
maps of immunoglobulin-like loci and, 5
MHC, 28, 30, 33
structure, 15, 18, 27
T cell receptor, 36, 37, 44
Endocytosis
complement receptor 1 and, 193, 198–200
complement receptor 2 and, 210
Endoglycosidase F, autoimmune thyroiditis and, 270, 271
Endoglycosidase H
autoimmune thyroiditis and, 271
leukocyte integrins and, 154, 174
Endothelial cells, leukocyte integrins and
inflammation, 162, 164, 165, 168
ligand molecules, 170–172
Endothelium, autoimmune thyroiditis and, 275
Enkephalin, helper T cell cytokines and, 116
Eosinophilia, helper T cell cytokines and, 133, 137
Eosinophils, complement receptor 1 and, 190, 191
Epidermal growth factor
autoimmune thyroiditis and, 272, 294, 295
complement receptor 1 and, 187
leukocyte integrins and, 159
Epithelium
autoimmune thyroiditis and, 266, 287, 296, 297
complement receptor 2 and, 204, 210, 213
leukocyte integrins and, 164, 172
Epitopes
autoimmune thyroiditis and, 318, 319
antigens, 270, 271, 273
cellular immune responses, 291
experimental models, 278, 282
humoral responses, 302–305, 307
prevention, 312, 314, 315
complement receptor 1 and, 196
complement receptor 2 and, 206, 207
helper T cell cytokines and, 124
leukocyte integrins and, 168, 173
murine lupus models and, 74, 76
Epstein–Barr virus
autoimmune thyroiditis and, 300, 306
complement receptor 1 and, 192
complement receptor 2 and, 204, 205, 208, 210–213
leukocyte integrins and, 173

Erythrocytes
complement receptor 1 and, 190, 191, 194, 196, 198, 199
complement receptor 2 and, 203, 206, 210
leukocyte integrins and, 163
Escherichia coli
leukocyte integrins and, 163
maps of immunoglobulin-like loci and, 5
Exocytosis, autoimmune thyroiditis and, 271
Experimental allergic encephalomyelitis, 79
Experimental autoimmune encephalomyelitis, 291, 293, 312
Experimental autoimmune thyroiditis, 266, 317, 318
antigens, 267, 270
cellular immune responses, 287, 290, 296–298
genetic control, 284–287
humoral responses, 301, 302, 306, 308
induction, 276–282
models, 273–275
prevention, 308, 310–315
Extracellular matrix, leukocyte integrins and, 149, 153
ligand molecules, 171
structure, 156–159

F

Factor H, complement receptor 1 and, 189, 197
Fc receptors, complement receptor 1 and, 198
Ferritin, complement receptor 1 and, 200
Fibrinopeptide B, helper T cell cytokines and, 128
Fibroblasts
leukocyte integrins and, 162, 171, 172
maps of immunoglobulin-like loci and, 6
Fibronectin, leukocyte integrins and, 149, 153
inflammation, 168, 169
leukocyte adhesion deficiency disease, 176
structure, 157, 158
Fluorescence
complement receptor 2 and, 204, 207, 211
immunosenescence and, 229, 231, 232, 242
Framework, maps of immunoglobulin-like loci and, 7
Freund's complete adjuvant, helper T cell cytokines and, 137

G

Generalized lymphoproliferative disease, murine lupus models and, 63, 64, 94–97
Glycoprotein
autoimmune thyroiditis and, 267, 270
complement receptor 2 and, 203, 205, 210
leukocyte integrins and, 149, 151, 153
ligand molecules, 171, 172
structure, 157, 159
maps of immunoglobulin-like loci and, 34
β-Glycoprotein, complement receptor 1 and, 184, 185
Glycosylation
autoimmune thyroiditis and, 267, 271
complement receptor 1 and, 192
complement receptor 2 and, 202, 204, 205
leukocyte integrins and, 154, 170, 174
Goitrous thyroiditis, 284
Grafts, autoimmune thyroiditis and, 286, 287
Granulocyte–macrophage colony-stimulating factor, 112, 115, 117, 122, 133
Granulocytes
helper T cell cytokines and, 115, 117
leukocyte integrins and, 151, 161–163, 166
Graves' disease, autoimmune thyroiditis and, 263
Graves' ophthalmopathy, autoimmune thyroiditis and, 268
Gut associated lymphoid tissue, immunosenescence and, 252, 253

H

Hairy-cell leukemia, leukocyte integrins and, 151
Haplotypes
autoimmune thyroiditis and, 275, 279, 284–286, 319
maps of immunoglobulin-like loci and, 32
murine lupus models and, 99
Ig germline, 66, 67, 69, 71, 72
T cell antigen receptor, 84–86, 88, 90
Hapten
helper T cell cytokines and, 120, 121
immunosenescence and, 247
Hapten augmentation, immunosenescence and, 247, 248, 252
Hashimito's thyroiditis, 263, 266, 317
antigens, 268, 269, 271–275

cellular immune responses, 287-291, 293
genetic control, 284
humoral responses, 298-301, 303, 305, 306
Helper T cell cytokines, 111
cross-regulation of differentiation, 130-133
differential induction, 127-130
functions, 114-117
DTH, 117
helper, 117-120
human clones, 121, 122
isotype regulation, 120, 121
macrophage activation, 122
human subsets, 138, 139
immune responses, 133-138
precursors of differentiation states
antigen expression, 123-125
bulk cultures, 122, 123
clones, 123
heterogeneity, 125, 126
phenotypes, 126, 127
secretion patterns, 111-114
Hematopoietic cells, leukocyte integrins and, 160, 166
Hematopoietic stem cells
immunosenescence and, 240-247
murine lupus models and, 64, 65
Hemopoietic cells, helper T cell cytokines and, 115-117
Heparin, leukocyte integrins and, 159
Hepatitis virus, helper T cell cytokines and, 129
Hepatocytes
helper T cell cytokines and, 116
leukocyte integrins and, 162
Heterogeneity
autoimmune thyroiditis and, 274, 281, 300
helper T cell cytokines and, 125, 126
leukocyte integrins and, 151, 154, 174, 175
High performance liquid chromatography, complement receptor 1 and, 187
Histoplasma capsulatum, leukocyte integrins and, 163
Hormones
autoimmune thyroiditis and
antigens, 267, 272
cellular immune responses, 298
experimental models, 274, 278, 280
genetic control, 283
immunosenescence and, 244, 246, 247
murine lupus models and, 63
Housekeeping, immunosenescence and, 234
Hybridization
complement receptor 1 and, 188-190
helper T cell cytokines and, 112
leukocyte integrins and, 159, 160, 173
maps of immunoglobulin-like loci and, 2-4
MHC, 30, 33
structure, 10, 12, 13, 15, 17, 18, 21, 25, 27
murine lupus models and, 74, 83, 85, 87
Hybridomas
autoimmune thyroiditis and, 289, 296, 304, 312
helper T cell cytokines and, 116
immunosenescence and, 249
murine lupus models and, 72, 74, 78
21-Hydroxylase, maps of immunoglobulin-like loci and, 28, 32, 33
Hypergammaglobulinemia, murine lupus models and, 63, 64
Hypothyroidism, 263, 269, 274, 275, 278

I

ICAM-1, *see* Intercellular adhesion molecule-1
Idiotype, autoimmune thyroiditis and, 318, 319
humoral responses, 302, 304-308
prevention, 315
Idiotypic regulation, immunosenescence and, 247-249
Immunofluorescence
autoimmune thyroiditis and, 293
complement receptor 1 and, 193
complement receptor 2 and, 204, 210
leukocyte integrins and, 158, 171
murine lupus models and, 88, 95
Immunoglobulin germline, murine lupus models and, 65, 66
autoantibodies, 71-76
inducing agent, 78
murine lupus, 66-71
somatic mutation, 76, 77
T cell antigen receptor, 98
Immunoglobulin-like loci, maps of, *see* Maps of immunoglobulin-like loci
Immunoglobulins
autoimmune thyroiditis and, 318
cellular immune responses, 288, 297
experimental models, 274

humoral responses, 300, 303–308
prevention, 315, 316
complement receptor 1 and, 193, 198, 200
complement receptor 2 and, 203, 205, 207, 208, 211
helper T cell cytokines and
cross-regulation of differentiation, 132
differential induction, 127, 128, 130
functions, 114, 116, 118–122
human subsets, 139
immune responses, 133, 135, 137, 138
precursors of differentiation states, 126
secretion patterns, 113
immunosenescence and
lymphocyte activation, 223, 224, 231, 232, 234, 236
lymphocyte subsets, 237, 239
mucosal immunity, 251
regulatory changes, 248, 249, 251
stem cells, 240, 242, 243
leukocyte integrins and, 149, 150, 168, 171
Immunoregulation
complement receptor 1 and, 200, 201
complement receptor 2 and, 205–210
immunosenescence and, 247
Immunosenescence, 221, 253
lymphocyte activation, 221, 222, 234–236
cell cycle, 222, 223
chromatin, 234
early events, 223–227
housekeeping, 234
protein, 227–230
secondary signal, 230–234
lymphocyte subsets, 240
human, 238, 239
murine, 236–238
marker density, 239
mucosal immunity, 251–253
regulatory changes, 247
idiotypic, 247–249
suppressor cells, 249–251
stem cells, 246, 247
lymphoid, 242–244
pluripotent, 240, 241
thymus, 244–246
Incomplete Freund's adjuvant, autoimmune thyroiditis and, 277
Inducing agents, murine lupus models and, 78
Inflammation
complement receptor 2 and, 205
helper T cell cytokines and, 115–117
leukocyte integrins and, 149–151, 160, 161, 177
animal models, 165
expression, 166–169
function, 161–165
ligand molecules, 170
monoclonal antibodies, 165, 166
Insulin
autoimmune thyroiditis and, 269
murine lupus models and, 79
Integral membrane protein, leukocyte integrins and, 154, 156
Integrins, leukocyte, *see* Leukocyte integrins
Intercellular adhesion molecule-1 (ICAM-1), leukocyte integrins and, 149, 150, 176, 177
inflammation, 163, 166, 167
ligand molecules, 169–172
structure, 158
Interferon
autoimmune thyroiditis and, 265
antigens, 272
cellular immune responses, 289, 293–298
helper T cell cytokines and, 112
cross-regulation, 131
functions, 114, 117, 120–122
immune responses, 133, 137, 138
precursors of differentiation states, 122, 123, 125
immunosenescence and, 231, 232, 237
leukocyte integrins and, 168, 170
Interleukin-1
autoimmune thyroiditis and, 295
helper T cell cytokines and, 130
immunosenescence and, 228
leukocyte integrins and, 170
Interleukin-2
autoimmune thyroiditis and, 275, 289, 310
complement receptor 1 and, 185
helper T cell cytokines and, 112
cross-regulation of differentiation, 130, 131
functions, 114, 120–122
immune responses, 136, 138
precursors of differentiation states, 122–126
immunosenescence and
lymphocyte activation, 222, 226, 228–230, 232
lymphocyte subsets, 237, 238

mucosal immunity, 252
regulatory changes, 250
Interleukin-3, helper T cell cytokines and, 116, 117
Interleukin-4
helper T cell cytokines and, 113
cross-regulation of differentiation, 130, 131
functions, 114–116, 119–122
immune responses, 133, 136–138
precursors of differentiation states, 122–125
immunosenescence and, 231, 232, 234, 237, 238
Interleukin-5
helper T cell cytokines and, 113
functions, 116, 119, 120
immune responses, 133, 136, 137
precursors of differentiation states, 122, 123, 125
immunosenescence and, 231, 232, 237, 238
Interleukin-6
helper T cell cytokines and, 116, 133
immunosenescence and, 231
Intraepithelial lymphocytes, immunosenescence and, 237
Iodine, autoimmune thyroiditis and
antigens, 267, 269–272
cellular immune responses, 294
experimental models, 278
humoral responses, 303
Ionomycin, immunosenescence and, 228
Ionotypic regulation, murine lupus models and, 65
Ischemia, leukocyte integrins and, 165, 166, 177

K

Keratinocytes, helper T cell cytokines and, 115

L

Lamina propia lymphocytes, immunosenescence and, 237
Laminin
complement receptor 1 and, 199
leukocyte integrins and, 153
Large granular lymphocytes, leukocyte integrins and, 151
Lectin
autoimmune thyroiditis and, 294
immunosenescence and, 221, 223, 245
Leishmania
complement receptor 1 and, 200
leukocyte integrins and, 163
Leishmania major, helper T cell cytokines and, 128, 129, 137, 138
Leukemia, maps of immunoglobulin-like loci and, 7
Leukocyte adhesion deficiency disease, 151, 173–176
inflammation, 161, 162, 165, 168
ligand molecules, 170
Leukocyte integrins, 149–153
chromosomal localization, 159, 160
future research, 176, 177
inflammation, 160, 161
adhesion deficiency, 161
animal models, 165
expression, 166–169
function, 161–164
functional redundancy, 164, 165
monoclonal antibodies, 165, 166
leukocyte adhesion deficiency disease, 173–176
ligand molecules, 169–173
structure, 153, 154
biosynthesis, 154
ligand binding, 158, 159
primary, 154–158
Leukocytes, helper T cell cytokines and, 117
LFA-1, leukocyte integrins and, 149–153
inflammation, 160–164, 166–169
leukocyte adhesion deficiency disease, 173, 174
ligand molecules, 169–172
structure, 153, 154, 156, 158
Ligands
complement receptor 1 and, 183, 192–196, 199, 200
complement receptor 2 and, 205, 211
immunosenescence and, 224
leukocyte integrins and, 150, 153, 169–173, 176, 177
inflammation, 163, 167
structure, 156, 158, 159
murine lupus models and, 94

Lipids
complement receptor 2 and, 210
helper T cell cytokines and, 114
leukocyte integrins and, 170
Lipopolysaccharide
autoimmune thyroiditis and
cellular immune responses, 294
experimental models, 276, 277, 279
genetic control, 286
prevention, 310, 311
complement receptor 2 and, 205
helper T cell cytokines and, 116, 119, 121
immunosenescence and, 223, 234, 242
leukocyte integrins and, 171
murine lupus models and, 63, 72
Liver
complement receptor 1 and, 198
complement receptor 2 and, 204
Long homologous repeat
complement receptor 1 and, 185, 187–189, 191, 194–196
complement receptor 2 and, 203
Lymph nodes
autoimmune thyroiditis and, 280, 296
complement receptor 2 and, 204, 209
helper T cell cytokines and, 129, 137, 138
immunosenescence and, 236, 245, 250, 251
murine lupus models and, 95, 96, 98
Lymphadenopathy, murine lupus models and, 63
Lymphoblastoid cells
complement receptor 1 and, 194
complement receptor 2 and, 201–204, 207, 209, 210, 213
leukocyte integrins and, 172
Lymphoblasts, leukocyte integrins and, 162
Lymphocytes
autoimmune thyroiditis and, 263, 318
antigens, 269
cellular immune responses, 287–289, 293, 294, 296–298
experimental models, 273, 274, 276, 278, 279, 281, 282
genetic control, 285, 286
humoral responses, 299–301, 303, 306
prevention, 310, 312, 315
complement receptor 1 and, 190, 191, 193, 198–201
complement receptor 2 and, 203, 205, 207, 209, 210, 213
helper T cell cytokines and, 123, 124
immunosenescence and, 221, 253
activation, 221–234
mucosal immunity, 251, 252
regulatory changes, 249
stem cells, 240, 246, 247
subsets, 236–240
leukocyte integrins and, 149, 151
inflammation, 162–164, 169
leukocyte adhesion deficiency disease, 173
ligand molecules, 170–172
maps of immunoglobulin-like loci and, 1
Lymphoid cells
autoimmune thyroiditis and
cellular immune responses, 287–291, 293, 295
experimental models, 274, 275, 279, 280, 282
immunosenescence and, 242–244, 246, 247
leukocyte integrins and, 162, 168, 171
Lymphoid hyperplasia, murine lupus models and, 63
Lymphoid tissues, immunosenescence and, 236, 252, 253
Lymphokines
autoimmune thyroiditis and, 283, 289, 291, 294, 298
immunosenescence and, 253
lymphocyte activation, 230–236
lymphocyte subsets, 237
regulatory changes, 250
Lymphoproliferation gene, murine lupus models and, 63, 94–98
Lymphotoxin
helper T cell cytokines and, 112
functions, 114, 115, 117, 122
immune responses, 133
maps of immunoglobulin-like loci and, 30, 33, 34
Lysosomes
autoimmune thyroiditis and, 267, 268
complement receptor 1 and, 200

M

Mac-1, leukocyte integrins and, 149–153, 176
chromosomal localization, 160
inflammation, 163, 164, 166–168
ligand molecules, 173
structure, 153, 154, 156, 158, 159
Macrophage inflammatory protein, 115

Macrophages
  autoimmune thyroiditis and, 265, 287, 307
  complement receptor 1 and, 190, 192, 200
  helper T cell cytokines and
    differential induction, 128–130
    functions, 114–117, 122
    immune responses, 133
  immunosenescence and, 228
  leukocyte integrins and, 151, 154, 163, 164, 167
Magnesium, leukocyte integrins and, 150, 156, 157, 163
Major histocompatibility complex
  autoimmune thyroiditis and, 263, 265, 266, 317, 319
    antigens, 268
    cellular immune responses, 289, 290, 295
    experimental models, 275, 279, 280
    genetic control, 282–286
    humoral responses, 299, 301, 305, 307
    prevention, 310, 315
  complement receptor 1 and, 189
  helper T cell cytokines and, 112, 117, 118
  immunosenescence and, 239
  maps of immunoglobulin-like loci and, 1, 28–34, 46
    structure, 19
    T cell receptor, 34
  murine lupus models and, 65, 79, 81, 82, 85, 86, 90, 91, 94
Mannose
  complement receptor 2 and, 202, 204
  leukocyte integrins and, 154
Maps of immunoglobulin-like loci, 1–3
  chromosomal locations, 6, 7
  mechanisms, 45–47
  methods, 3–6
  MHC, 28
    human HLA, 30–34
    mouse *H-2*, 28–30
  structure, 7
    human heavy chain, 24–28
    human κ chain, 15–19
    human λ chain, 10–12
    mouse heavy chain, 19–24
    mouse κ chain, 12–15
    mouse λ chain, 8–10
  T cell receptor, 34, 35
    α-chain, 41–45
    β-chain, 35–37
    γ-chain, 38–41
    δ-chain, 42, 43, 45
Marker density, immunosenescence and, 237, 239
Mast cells, helper T cell cytokines and, 113, 114, 116, 117
Mean fluorescence intensity, immunosenescence and, 222
Membrane cofactor protein, complement receptor 1 and, 189, 197
Memory cells, immunosenescence and, 246, 247
Microfilaments, complement receptor 1 and, 199
β_2-Microglobulin, maps of immunoglobulin-like loci and, 2
Microsomal antigens, autoimmune thyroiditis and, 266, 273, 298, 299
Microtubules, immunosenescence and, 224
Mitochondria, autoimmune thyroiditis and, 272
Mitogen
  autoimmune thyroiditis and, 289, 310
  complement receptor 2 and, 206, 208
  helper T cell cytokines and, 122
  immunosenescence and, 221
    lymphocyte activation, 223–225, 228, 234
    mucosal immunity, 252
    regulatory changes, 250
    stem cells, 244, 245
  leukocyte integrins and, 173
Mitosis, immunosenescence and, 241, 246
Mixed lymphocyte response, immunosenescence and, 243, 244
Molecular genetics of murine lupus models, *see* Murine lupus models
Monoclonal antibodies
  autoimmune thyroiditis and
    antigens, 267, 269, 272, 273
    cellular immune responses, 287, 295
    experimental models, 274, 275, 277, 280
    humoral responses, 300, 302–306, 308
    prevention, 310, 314
  complement receptor 1 and, 196, 200, 201
  complement receptor 2 and, 203, 204, 206, 211
  helper T cell cytokines and, 112, 119, 123–125
  immunosenescence and, 249
  leukocyte integrins and, 150, 151, 177
    inflammation, 162–166, 168

leukocyte adhesion deficiency disease, 173
ligand molecules, 170, 172, 173
structure, 153
murine lupus models and, 95
Monocytes
autoimmune thyroiditis and, 294
complement receptor 1 and, 190, 199–201
complement receptor 2 and, 207
immunosenescence and, 228
leukocyte integrins and, 161, 163–167
mRNA
autoimmune thyroiditis and, 273, 294, 295, 297
complement receptor 1 and, 184, 193
complement receptor 2 and, 202
helper T cell cytokines and, 112, 115, 138
immunosenescence and, 222, 228, 229
leukocyte integrins and, 174, 175
maps of immunoglobulin-like loci and, 3, 12, 32
murine lupus models and, 72, 86–88, 90, 91, 96
Mucosal immunity, immunosenescence and, 221, 251–253
Murine lupus models, 61, 62, 99, 100
Ig germline, 65, 66
autoantibodies, 71–76
inducing agent, 78
murine lupus, 66–71
somatic mutation, 76, 77
lupus strains, 61–65
T cell antigen receptor, 78, 79
autoimmune strains, 83–86
expression, 86–99
gene diversity, 79–83
Mutation
autoimmune thyroiditis and, 263, 286
complement receptor 1 and, 193, 194, 196
complement receptor 2 and, 211
immunosenescence and, 222, 234
leukocyte integrins and, 160, 171, 172, 174–176
maps of immunoglobulin-like loci and, 3, 12
murine lupus models and, 99
Ig germline, 65, 66, 69, 76–78
lupus strains, 63, 64
T cell antigen receptor, 83, 94, 98
Myasthenia gravis, murine lupus models and, 79
Myelin-associated glycoprotein, 171, 172
Myelin basic protein
autoimmune thyroiditis and, 289
murine lupus models and, 79
Myeloblasts, leukocyte integrins and, 166
Myeloid cells
helper T cell cytokines and, 114, 115, 117
leukocyte integrins and, 150, 163, 165

N

Natural killer cells
autoimmune thyroiditis and, 279, 288, 289, 295
helper T cell cytokines and, 114, 116
leukocyte integrins and, 161, 163, 169
Neonatal thymectomy, autoimmune thyroiditis and, 275, 301, 302
Nephritis, murine lupus models and, 64
Neural cell adhesion molecule, 171
Neutrophil-activating factor, 115
Neutrophils
complement receptor 1 and, 190–193, 198–200
leukocyte integrins and, 151, 177
inflammation, 160, 161, 163–169
ligand molecules, 173
structure, 154
Nippostrongylus brasiliensis, helper T cell cytokines and, 137
Nucleic acid, murine lupus models and, 76
Nucleotides
complement receptor 2 and, 201
immunosenescence and, 223, 226, 227
maps of immunoglobulin-like loci and MHC, 31, 32
structure, 13, 15, 17, 20, 21, 25, 27, 28
T cell receptor, 36, 39
murine lupus models and, 67

O

Obese strain chickens, autoimmune thyroiditis and
antigens, 269, 270
cellular immune responses, 298
experimental models, 273–276, 279
genetic control, 282–284
humoral responses, 301, 303
prevention, 310

Oligosaccharides
complement receptor 1 and, 187, 192
complement receptor 2 and, 202, 204
leukocyte integrins and, 154
Oncogenes
complement receptor 1 and, 187
complement receptor 2 and, 210

P

p150,95, leukocyte integrins and, 149–153
chromosomal localization, 160
inflammation, 164, 167
ligand molecules, 173
structure, 153, 156, 158, 159
Parasites, helper T cell cytokines and, 137–139
Peripheral blood lymphocytes
autoimmune thyroiditis and, 287, 289, 299, 300
immunosenescence and
lymphocyte activation, 224, 226–230
lymphocyte subsets, 238, 239
stem cells, 239, 240
Peyer's patches, immunosenescence and, 236, 237, 251, 252
Phagocytosis
complement receptor 1 and, 183, 186, 198, 199
leukocyte integrins and, 151, 161, 163, 168
Phenotype
autoimmune thyroiditis and
cellular immune responses, 287, 288, 290
experimental models, 274, 275
genetic control, 282
prevention, 311
helper T cell cytokines and, 111, 123, 126–128, 139
immunosenescence and, 252
leukocyte integrins and, 174–176
murine lupus models and, 64, 82, 94
Phorbol esters
complement receptor 1 and, 199
complement receptor 2 and, 208
leukocyte integrins and, 162, 163, 165–169
Phorbol myristate acetate
autoimmune thyroiditis and, 296
immunosenescence and, 224, 226, 228, 230
leukocyte integrins and, 168
Phosphatidyl inositol, leukocyte integrins and, 171
Phospholipase C
immunosenescence and, 223
leukocyte integrins and, 171
Phospholipids, immunosenescence and, 223
Phosphorylation
autoimmune thyroiditis and, 267
complement receptor 1 and, 187, 199
complement receptor 2 and, 202, 208, 209
immunosenescence and, 222
leukocyte integrins and, 155, 169
Phosphorylcholine, immunosenescence and, 248
Physical maps of immunoglobulin-like loci, *see* Maps of immunoglobulin-like loci
Phytohemagglutinin, autoimmune thyroiditis and, 293, 310
Plaque-forming cells
autoimmune thyroiditis and, 299, 301
immunosenescence and, 247, 248, 251
Plasma membrane
complement receptor 1 and, 191, 192, 197, 199
complement receptor 2 and, 204, 209
immunosenescence and, 224, 226
Plasmacytomas, helper T cell cytokines and, 116
Plasmalemma, immunosenescence and, 235
Plasmids, maps of immunoglobulin-like loci and, 4
Pokeweed mitogen, complement receptor 1 and, 200, 201
Polyadenylation, complement receptor 1 and, 184
Polymerase chain reaction, leukocyte integrins and, 175, 176
Polypeptides
autoimmune thyroiditis and, 272
complement receptor 1 and, 184
complement receptor 2 and, 204
helper T cell cytokines and, 124
immunosenescence and, 229, 230
leukocyte integrins and, 153, 156
maps of immunoglobulin-like loci and, 2, 46
structure, 7, 12, 20
T cell receptor, 35, 41–43
Position-specific antigens, leukocyte integrins and, 153
Praomys, autoimmune thyroiditis and, 273, 275

Preproenkephalin, helper T cell cytokines and, 112, 116
Protein
autoimmune thyroiditis and, 265, 266, 315, 316
antigens, 268, 272
cellular immune responses, 294
experimental models, 276
complement receptor 1 and, 185, 186, 190, 194, 197, 199
complement receptor 2 and, 201–203
helper T cell cytokines and, 115, 116, 123
immunosenescence and, 222, 223, 227–230, 234
leukocyte integrins and
inflammation, 164, 165, 169
leukocyte adhesion deficiency disease, 174, 175
ligand molecules, 171
structure, 153, 154, 158, 159
maps of immunoglobulin-like loci and
MHC, 28–30, 32
structure, 8, 10, 12, 13, 15, 17, 19
T cell receptor, 45
murine lupus models and, 69
Protein kinase C
autoimmune thyroiditis and, 296
complement receptor 1 and, 187, 199
complement receptor 2 and, 202, 208, 211
immunosenescence and, 222–227, 230, 235
leukocyte integrins and, 169

R

Raji cells, complement receptor 2 and, 201–203, 206, 207, 209, 210
Recombination
maps of immunoglobulin-like loci and, 3, 4, 47
structure, 19–21, 23
murine lupus models and, 87
Regulation of complement activation
complement receptor 1 and, 189
complement receptor 2 and, 202
Reperfusion, leukocyte integrins and, 165, 166, 177
Replication, maps of immunoglobulin-like loci and, 4
Restriction enzymes, maps of immunoglobulin-like loci and, 3, 4
Restriction fragment length polymorphism
autoimmune thyroiditis and, 284
complement receptor 1 and, 191
leukocyte integrins and, 160
maps of immunoglobulin-like loci and, 23
murine lupus models and, 66, 67
Retrovirus, autoimmune thyroiditis and, 275
RGD sequence, leukocyte integrins and, 153
ligand molecules, 171–173
structure, 158, 159
Rheumatoid factor, murine lupus models and, 72, 75, 77, 78
RNA
immunosenescence and, 227
leukocyte integrins and, 174–176
maps of immunoglobulin-like loci and, 8, 13, 25
murine lupus models and
Ig germline, 74
T cell antigen receptor, 86–88, 90–92, 95
RNase
complement receptor 2 and, 202
murine lupus models and, 86–88, 91

S

Schizosaccharomyces pombe, maps of immunoglobulin-like loci and, 5
Secretion, helper T cell cytokines and, 111–114, 127, 136
Self-antigens
autoimmune thyroiditis and, 263, 265, 305, 307, 319
murine lupus models and, 65
Sex-limited protein, 29
Short consensus repeats
complement receptor 1 and, 184–186, 188–190, 194, 195
complement receptor 2 and, 202, 203, 211
Spectrotypes, autoimmune thyroiditis and, 301, 304, 306
Spleen
autoimmune thyroiditis and, 280, 281, 306, 310–312
complement receptor 2 and, 204–206
helper T cell cytokines and, 122, 123, 128, 129, 137, 138
immunosenescence and
lymphocyte activation, 226, 228, 232
lymphocyte subsets, 237, 238

marker density, 239
mucosal immunity, 251, 253
regulatory changes, 247-251
stem cells, 240, 241, 244, 245
murine lupus models and, 64, 91, 92, 98
Spontaneous autoimmune thyroiditis
antigens, 269, 270
cellular immune responses, 287
experimental models, 273-276, 279
genetic control, 282-284
humoral responses, 298, 305, 306
prevention, 310, 311
Staphylococcus aureus, autoimmune thyroiditis and, 299
Stromal cells, immunosenescence and, 241
Sugar, immunosenescence and, 234
Suppressor cells, immunosenescence and, 249-251
Systemic lupus, 196, 200
Systemic lupus erythematosus, murine lupus models and, 65, 71

T

T cell antigen receptor, murine lupus models and, 61, 65, 78, 79
autoimmune strains, 83-86
expression, 86-99
gene diversity, 79-83
T cell cytokines, *see* Helper T cell cytokines
T cell growth factor, 112, 114
T cell receptor
autoimmune thyroiditis and, 265, 318, 319
cellular immune responses, 291
humoral responses, 307
prevention, 312, 314-316
maps of immunoglobulin-like loci and, 1, 5, 34-45
chromosomal locations, 6
mechanisms, 46, 47
T cells
autoimmune thyroiditis and, 263, 265, 266, 317-319
antigens, 270
cellular immune responses, 287-291, 293-298
experimental models, 273, 275-282
genetic control, 283
humoral responses, 299, 302-304, 306-308
prevention, 308, 310-312, 314-316
complement receptor 1 and, 183, 200, 201
complement receptor 2 and, 203, 205-207, 213
immunosenescence and, 221
lymphocyte activation, 221, 222, 224-232, 234, 236
lymphocyte subsets, 236-239
regulatory changes, 250-252
stem cells, 240, 243-247
leukocyte integrins and, 154, 169, 172
maps of immunoglobulin-like loci and, 1, 28, 29, 31
murine lupus models and, 64, 66, 67, 99, 100
T helper cells, leukocyte integrins and, 163
T suppressor cells, immunosenescence and, 251, 252
Talin, leukocyte integrins and, 169
Target thyroid cells, 291-298
Testosterone, autoimmune thyroiditis and, 276, 310
Thymectomy, helper T cell cytokines and, 125
Thymidine, immunosenescence and, 231-234, 243
Thymocytes
autoimmune thyroiditis and, 274, 279
helper T cell cytokines and, 116
murine lupus models and, 82, 94-100
Thymus
autoimmune thyroiditis and, 276, 286
immunosenescence and, 243-247
Thyrocytes, autoimmune thyroiditis and, 293-295, 297
Thyroglobulin, 263, 317-319
antigens, 266-272
cellular immune responses, 287, 288, 291, 297, 298
experimental models, 274-278, 281-283, 285, 287
humoral responses, 298, 299, 302-307, 309
prevention, 308, 310-312, 314, 315
Thyroid peroxidase, 272, 273, 298, 317
Thyroiditis, *see* Autoimmune thyroiditis
Thyrotropin
antigens, 267-269, 272
experimental models, 275, 278, 279, 294
prevention, 311
Thyrotropin-releasing hormone, 279, 311
Thyroxine, 269, 278
Tissue plasminogen activator, 166
Tolerance induction, murine lupus models and, 65

Transcription
  autoimmune thyroiditis and, 268, 295
  immunosenescence and, 223, 228
  maps of immunoglobulin-like loci and
    MHC, 30, 34
    structure, 18, 19, 21, 27
    T cell receptor, 43, 44
  murine lupus models and, 80, 95
Transferrin, immunosenescence and, 222, 227
Translocation
  immunosenescence and, 224, 225, 235
  leukocyte integrins and, 160
  maps of immunoglobulin-like loci and, 3, 26, 32, 45
Transplantation, murine lupus models and, 64
Trypsin, autoimmune thyroiditis and, 271, 273, 277, 278, 302
TSH, *see* Thyrotropin
Tumor
  leukocyte integrins and, 150
  maps of immunoglobulin-like loci and, 8
Tumor necrosis factor
  autoimmune thyroiditis and, 294, 295
  complement receptor 1 and, 192
  helper T cell cytokines and, 112, 114, 115
  leukocyte integrins and, 170, 172
  maps of immunoglobulin-like loci and, 30, 33
Tunicamycin
  complement receptor 1 and, 187
  complement receptor 2 and, 201
Tyrosine kinase, complement receptor 1 and, 202

V

Virus
  autoimmune thyroiditis and
    cellular immune responses, 297, 298
    experimental models, 274–276
    genetic control, 283
  complement receptor 2 and, 210
  helper T cell cytokines and, 127, 129, 133
  maps of immunoglobulin-like loci and, 1
Vitronectin, leukocyte integrins and, 153, 157
VLA antigens, leukocyte integrins and, 153, 157
von Willebrand factor, leukocyte integrins and, 158, 159

Y

Yeast artificial chromosome, maps of immunoglobulin-like loci and, 4, 5

CONTENTS OF RECENT VOLUMES

Volume 35

The Generation of Diversity in Phosphorylcholine-Binding Antibodies
ROGER M. PERLMUTTER, STEPHEN T. CREWS, RICHARD DOUGLAS, GREG SORENSEN, NELSON JOHNSON, NADINE NIVERA, PATRICIA J. GEARHART, AND LEROY HOOD

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JOHN ROGERS AND RANDOLPH WALL

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ROBERT J. NORTH

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DAVID R. JACOBY, LARS B. OLDING, AND MICHAEL B. A. OLDSTONE

The Influence of Histamine on Immune and Inflammatory Responses
DENNIS J. BEER, STEVEN M. MATLOFF, AND ROSS E. ROCKLIN

INDEX

Volume 36

Antibodies of Predetermined Specificity in Biology and Medicine
RICHARD ALAN LERNER

A Molecular Analysis of the Cytolytic Lymphocyte Response
STEVEN J. BURAKOFF, OFRA WEINBERGER, ALAN M. KRENSKY, AND CAROL S. REISS

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BARTON F. HAYNES

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ANDREW W. WADE AND MYRON R. SZEWCZUK

A Major Role of the Macrophage in Quantitative Genetic Regulation of Immunoresponsiveness and Antiinfectious Immunity
GUIDO BIOZZI, DENISE MOUTON, CLAUDE STIFFEL, AND YOLANDE BOUTHILLIER

INDEX

Volume 37

Structure, Function, and Genetics of Human Class II Molecules
ROBERT C. GILES AND J. DONALD CAPRA

The Complexity of Virus–Cell Interactions in Abelson Virus Infection of Lymphoid and Other Hematopoietic Cells
CHERYL A. WHITLOCK AND OWEN N. WITTE

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Membrane Complement Receptors Specific for Bound Fragments of C3
GORDON D. ROSS AND M. EDWARD MEDOFF

Murine Models of Systemic Lupus Erythematosus
ARGYRIOS N. THEOFILOPOULOS AND FRANK J. DIXON

INDEX

Volume 38

The Antigen-Specific, Major Histocompatibility Complex-Restricted Receptor on T Cells
PHILIPPA MARRACK AND JOHN KAPPLER

Immune Response (*Ir*) Genes of the Murine Major Histocompatibility Complex
RONALD H. SCHWARTZ

The Molecular Genetics of Components of Complement
R. D. CAMPBELL, M. C. CARROLL, AND R. R. PORTER

Molecular Genetics of Human B Cell Neoplasia
CARLO M. CROCE AND PETER C. NOWELL

Human Lymphocyte Hybridomas and Monoclonal Antibodies
DENNIS A. CARSON AND BRUCE D. FREIMARK

Maternally Transmitted Antigen
JOHN R. RODGERS, ROGER SMITH III, MARILYN M. HUSTON, AND ROBERT R. RICH

Phagocytosis of Particulate Activators of the Alternative Complement Pathway: Effects of Fibronectin
JOYCE K. CZOP

INDEX

Volume 39

Immunological Regulation of Hematopoietic/Lymphoid Stem Cell Differentiation by Interleukin 3
JAMES N. IHLE AND YACOB WEINSTEIN

Antigen Presentation by B Cells and Its Significance in T–B Interactions
ROBERT W. CHESNUT AND HOWARD M. GREY

Ligand–Receptor Dynamics and Signal Amplification in the Neutrophil
LARRY A. SKLAR

Arachidonic Acid Metabolism by the 5-Lipoxygenase Pathway, and the Effects of Alternative Dietary Fatty Acids
TAK H. LEE AND K. FRANK AUSTEN

The Eosinophilic Leukocyte: Structure and Function
GERALD J. GLEICH AND CHERYL R. ADOLPHSON

Idiotypic Interactions in the Treatment of Human Diseases
RAIF S. GEHA

Neuroimmunology
DONALD G. PAYAN, JOSEPH P. MCGILLIS, AND EDWARD J. GOETZL

INDEX

Volume 40

Regulation of Human B Lymphocyte Activation, Proliferation, and Differentiation
DIANE F. JELINEK AND PETER E. LIPSKY

Biological Activities Residing in the Fc Region of Immunoglobulin
EDWARD L. MORGAN AND WILLIAM O. WEIGLE

Immunoglobulin-Specific Suppressor T Cells
RICHARD G. LYNCH

Immunoglobulin A (IgA): Molecular and Cellular Interactions Involved in IgA Biosynthesis and Immune Response
JIRI MESTECKY AND JERRY R. MCGHEE

The Arrangement of Immunoglobulin and T Cell Receptor Genes in Human Lymphoproliferative Disorders
THOMAS A. WALDMANN

Human Tumors Antigens
RALPH A. REISFELD AND DAVID A. CHERESH

Human Marrow Transplantation: An Immunological Perspective
PAUL J. MARTIN, JOHN A. HANSEN, RAINER STORB, AND E. DONNALL THOMAS

INDEX

Volume 41

Cell Surface Molecules and Early Events Involved in Human T Lymphocyte Activation
ARTHUR WEISS AND JOHN B. IMBODEN

Function and Specificity of T Cell Subsets in the Mouse
JONATHAN SPRENT AND SUSAN R. WEBB

Determinants on Major Histocompatibility Complex Class I Molecules Recognized by Cytotoxic T Lymphocytes
JAMES FORMAN

Experimental Models for Understanding B Lymphocyte Formation
PAUL W. KINCADE

Cellular and Humoral Mechanisms of Cytotoxicity: Structural and Functional Analogies
JOHN DING-E YOUNG AND ZANVIL A. COHN

Biology and Genetics of Hybrid Resistance
MICHAEL BENNETT

INDEX

Volume 42

The Clonotype Repertoire of B Cell Subpopulations
NORMAN R. KLINMAN AND PHYLLIS-JEAN LINTON

The Molecular Genetics of the Arsonate Idiotypic System of A/J Mice
GARY RATHBUN, INAKI SANZ, KATHERYN MEEK, PHILIP TUCKER, AND J. DONALD CAPRA

The Interleukin 2 Receptor
KENDALL A. SMITH

Characterization of Functional Surface Structures on Human Natural Killer Cells
JEROME RITZ, REINHOLD E. SCHMIDT, JEAN MICHON, THIERRY HERCEND, AND STUART F. SCHLOSSMAN

The Common Mediator of Shock, Cachexia, and Tumor Necrosis
B. BEUTLER AND A. CERAMI

Myasthenia Gravis
JON LINDSTROM, DIANE SHELTON, AND YOSHITAKA FUJII

Alterations of the Immune System in Ulcerative Colitis and Crohn's Disease
RICHARD P. MACDERMOTT AND WILLIAM F. STENSON

INDEX

Volume 43

The Chemistry and Mechanism of Antibody Binding to Protein Antigens
ELIZABETH D. GETZOFF, JOHN A. TAINER, RICHARD A. LERNER, AND H. MARIO GEYSEN

Structure of Antibody–Antigen Complexes: Implications for Immune Recognition
P. M. COLMAN

The $\gamma\delta$ T Cell Receptor
MICHAEL B. BRENNER, JACK L. STROMINGER, AND MICHAEL S. KRANGEL

Specificity of the T Cell Receptor for Antigen
STEPHEN M. HEDRICK

Transcriptional Controlling Elements in the Immunoglobulin and T Cell Receptor Loci
KATHRYN CALAME AND SUZANNE EATON

Molecular Aspects of Receptors and Binding Factors for IgE
HENRY METZGER

INDEX

Volume 44

Diversity of the Immunoglobulin Gene Superfamily
TIM HUNKAPILLER AND LEROY HOOD

Genetically Engineered Antibody Molecules
SHERIE L. MORRISON AND VERNON T. OI

Antinuclear Antibodies: Diagnostic Markers for Autoimmune Diseases and Probes for Cell Biology
ENG M. TAN

Interleukin-1 and Its Biologically Related Cytokines
CHARLES A. DINARELLO

Molecular and Cellular Events of T Cell Development
B. J. FOWLKES AND DREW M. PARDOLL

Molecular Biology and Function of CD4 and CD8
JANE R. PARNES

Lymphocyte Homing
TED A. YEDNOCK AND STEVEN D. ROSEN

INDEX

Volume 45

Cellular Interactions in the Humoral Immune Response
ELLEN S. VITETTA, RAFAEL FERNANDEZ-BOTRAN, CHRISTOPHER D. MYERS, AND VIRGINIA M. SANDERS

MHC–Antigen Interactions: What Does the T Cell Receptor See?
PHILIPPE KOURILSKY AND JEAN-MICHEL CLAVERIE

Synthetic T and B Cell Recognition Sites: Implications for Vaccine Development
DAVID R. MILICH

Rationale for the Development of an Engineered Sporozoite Malaria Vaccine
VICTOR NUSSENZWEIG AND RUTH S. NUSSENZWEIG

Virus-Induced Immunosuppression: Infections with Measles Virus and Human Immunodeficiency Virus
MICHAEL B. MCCHESNEY AND MICHAEL B. A. OLDSTONE

The Regulators of Complement Activation (RCA) Gene Cluster
DENNIS HOURCADE, V. MICHAEL HOLERS, AND JOHN P. ATKINSON

Origin and Significance of Autoreactive T Cells
MAURICE ZAUDERER

INDEX

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