ADVANCES IN IMMUNOLOGY

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Immunology

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Immunology

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ADVANCES IN IMMUNOLOGY, VOL. 44

Diversity of the Immunoglobulin Gene Superfamily

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I. Introduction

The vertebrate immune response consists of a complex set of cellular and serological reactions that provide protection from foreign and abnormal self-macromolecular structures (antigens). These responses are mediated by an equally complex array of immune recognition elements. During the past decade, many of these molecules have been characterized and have been found to share a common evolutionary precursor, the immunoglobulin homology unit (Cunningham *et al.*, 1973; Strominger *et al.*, 1980; Williams, 1984; Hood *et al.*, 1985). Recently, several molecules with no known immunological functions have also been shown to share this same precursor element (Hunkapiller and Hood, 1986; Williams, 1987). Together, the genes encoding these related molecules have been defined as the immunoglobulin gene superfamily (IgGSF) and include both multigene and single-gene representatives. These IgGSF products represent an amazingly diverse array of functions from immune receptors to cartilage formation, reflecting the versatility of the shared common structure (see Fig. 1).

We summarize here our understanding of the members of the IgGSF and speculate particularly on their evolutionary history as well as both the evolutionary and somatic mechanisms responsible for their diversity. We define here a new variation on the homology unit motif that probably more nearly represents the primordial sequence organization than either of the two previously defined units. We believe that the unique ability of the immunoglobulin homology unit to accommodate diversity (information) has made possible the evolution of the complex phenotypic traits of the IgGSF. We also suggest that the diversity potential of the informational multigene families (Hood *et al.*, 1975) of the immune receptors is generally more important evolutionarily than the individual strategies used to generate it. Thus, many different, but selectively equivalent, diversifying strategies are seen in the immune receptor gene families of the IgGSF. Finally, the genetic organization of IgGSF

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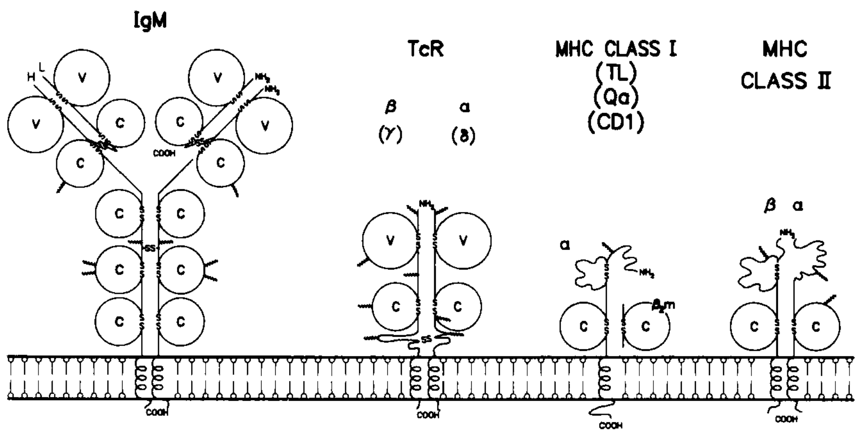
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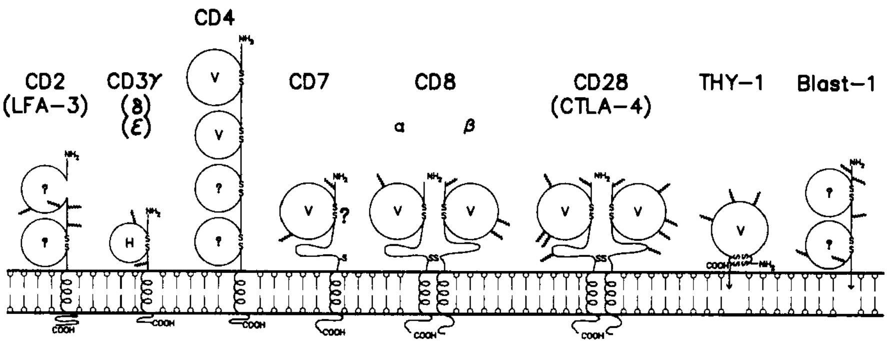
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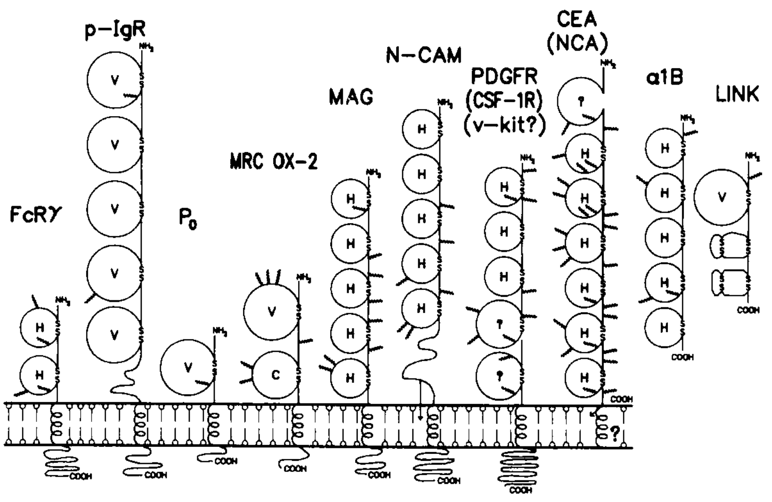
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members and gene families as well as structural and interactive properties of their proteins impart unique possibilities for their further evolutionary diversification. Recently, a review on the IgGSF has appeared elsewhere (Williams, 1987).

II. The Immunoglobulin Homology Unit and the IgGSF

We define a superfamily as a series of genes that share an evolutionary homology (i.e., common ancestor), but do not necessarily share function, genetic linkage, or coordinate regulation (Hood *et al.*, 1985). Members of the IgGSF have been defined by the presence of one or more regions homologous to the basic structural unit of immunoglobin (Ig) molecules, the Ig homology unit (Hill *et al.*, 1966). These units are characterized by a primary sequence about 70–110 residues in length with an essentially invariant disulfide bridge spanning 50–70 residues and several other relatively conserved residues involved in establishing a tertiary structure referred to as an antibody fold. Two basic homology unit types have been defined from crystallographic analysis of the variable (V) and constant (C) regions of Ig (see below). The tertiary structure of a V region is dominated by a series of nine antiparallel β strands, connected by variable-length loop sequences, that assume a characteristic barrel or sandwichlike structure with two β sheets, stabilized by the disulfide bridge (Fig. 2) (see Amzel and Poljak, 1979). There are four β strands in one sheet and three in the other. The extra pair of β strands is essentially stituated between the faces of the sandwich. The β strands are characterized by alternating hydrophobic and hydrophylic amino acid residues. The hydrophobic side chains are oriented toward the interior and help stabilize the interaction between the two sheets. The outpointing hydrophylic residues mediate the interchain interactions. The disulfide bridge further stabilizes this basic structure, providing compact, globular domains that are relatively proteolytically insensitive.

FIG. 1. Schematic diagrams for the members of the IgGSF. Disulfide bonds are represented by (s–s). Homology units are indicated as loops labeled V, C, or H (see text). Loops of uncertain relationship to homology units are labeled with a question mark. Different-sized loops illustrate the relative differences in length between the conserved disulfide bond of the labeled types. Membrane-spanning peptides are shown as simple helices. Glycophospholipid linkage to the membrane is represented as an arrow. Intracytoplasmic regions are drawn with wavy lines that indicate their relative lengths. Extra- and intracellular orientations are indicated by NH_2 and COOH labels on the protein chains, respectively. Possible asparagine-linked carbohydrates are shown as jagged lines extending from the protein chains. Note that these sites are not necessarily conserved between alleles or across species, but are representative of at least one known example of the labeled protein. Related IgGSF members are illustrated with a single structure, as indicated by the name labels above each structure.

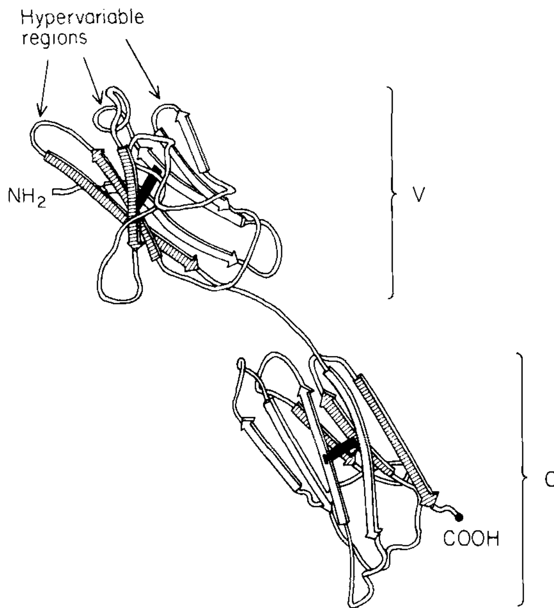

FIG. 2. Tertiary organization of V and C homology units (after Edmundson *et al.*, 1973). This illustrates the tertiary structure of an Ig light chain. The β strands and their orientation are shown as flat arrows. Opposite faces of the sandwich structure are indicated by either blank or hatched β strands. The disulfide bond is indicated with the solid bar.

The C region units lack the pair of internal β strands (Fig. 2), but otherwise assume the same general structure with a distinct, but overlapping, series of conserved residues. The lack of this extra pair of β strands decreases the distance between the two cysteine residues of C regions relative to those of V regions. The extra loop sequence connecting these two strands in V regions is critical to the formation of the antigen-binding pocket of antibodies (see below). Though there are enormous variations of primary sequences used, the basic scaffold structure of the antibody fold appears to be highly conserved within the IgGSF members. As new members of the IgGSF have been characterized, their homology units have generally been defined as either V- or C-like based on primary sequence similarities and secondary structure predictions. Many of the more recently discovered members have a primary and secondary structural motif that, although shared among themselves, does not preferentially fit either the previously defined V or C homology units. It is a more compact unit, even shorter in length between the two cysteines than most C-like units. Accordingly, we define a new class of homology unit, denoted H, to encompass these members. This third type of

homology unit has also recently been defined as C2 (constant 2) mostly due to its lack of the V-defining extra β-strand pair (Williams, 1987). Overall, however, this unit appears to have a relatively equidistant relationship to both V and C homology units. Therefore, we prefer a more generic definition that does not suggest any functional relationship to one or the other homology unit types and emphasizes the building block nature of this unit. Because both V and C units are each more closely similar to H units than to each other, the H unit probably reflects a more primordial motif (see below), suggesting that the original members of the superfamily arose in early metazoa and clearly carried out cell-surface recognition functions unrelated to vertebrate immunity. Figure 3 represents the consensus structures of each of the three homology unit types.

To discuss this or any other superfamily, criteria must be defined that establish the likelihood that any particular sequence is a homolog to the superfamily. When attempting to define evolutionary homology between any two sequences, various mathematical calculations can be made to advance arguments of statistically significant similarity (e.g., Dayhoff *et al.*, 1983). These methods rely on defining the similarity or distance score between any two protein sequences, calculated as a

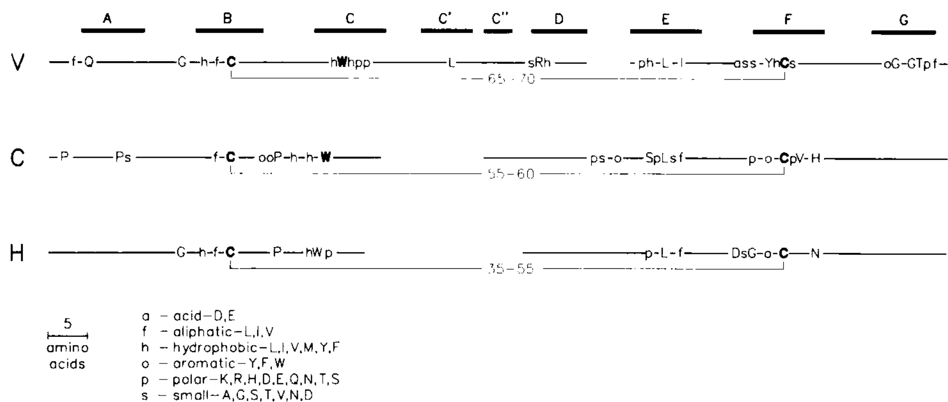

FIG. 3. Sequence motifs of each homology unit type. This figure illustrates idealized aligned examples of each homology unit type. Representative amino acid residues of each type are shown in their relative position along the polypeptides. Uppercase letters are single-letter amino acid code. Lowercase letters represent a selection of amino acids with similar functional or physical properties. The key to this code is shown in the lower left. Relative gaps are introduced into each chain to align residues conserved between types. The more nearly invariant cysteine and tryptophan residues are highlighted. The distances between the conserved cysteine residues are shown beneath each chain. The approximate locations of the β strands are illustrated with heavy bars above the sequence representations. Note the two β strands (C′ and C″) not found in the C and H domains. It is also clear that β strand D may not be found in the shorter H domain sequences.

function of the degree of sequence identity between them. A significant relationship is usually defined by a similarity score a minimum number (e.g., >2–3) of standard deviations (SD) above what would be expected from comparison of two random sequences. The expected value is generally calculated by randomizing or "shuffling" one or both sequences many times (100–150) and each time recalculating their similarity. It is the mean of these scores that forms the basis for the reference score for "unrelatedness." The greater the deviation is from this standard, the closer the relationship. Therefore, these values should reflect the evolutionary history and particular relationships within a family of related sequence, i.e., genealogy. These methods work well for relatively recently diverged sequences whose differences can be statistically viewed within a model of gradual sequence divergence at relatively constant rates. However, selective constraints against further sequence divergence between more distantly related sequences disrupt this model. For example, the conserved structure of IgGSF members is apparently mediated by a limited, but selectively conserved, set of amino acid residues at particular positions in the primary sequence, while many of the rest of the sequences are allowed to vary dramatically. There must be, then, a limit to the apparent sequence divergence between any two sequences such that they can maintain shared structural features. That the number of explicitly conserved amino acid residues need not be large is illustrated by the fact that even proteins generated by genes belonging to the same family may be less than 20% similar. However, many of the remaining residues are relatively conservative substitutions for the same position in another member. Consequently, similarity scores between proteins with variably selected residues decrease linearly with actual evolutionary distance only within a relatively restricted distance range. In other words, as evolutionary distance increases, distance scores tend to level out within a relatively narrow (and low) range, thus losing any fine distance information. Within this range (~2–6 SD) significant noise is generated by localized regions of presumably convergently similar sequences. This is because no protein sequence can be viewed as legitimately random; i.e., not all possible combinations of linear and tertiary relationships between amino acids are biologically meaningful. Hence, linear sequences that successfully define a limited number of structural motifs must convergently (independently) arise much more frequently than statistical analyses would predict from amino acid composition alone. Thus, relatedness scores between sequences that share analogous structural features will tend to overestimate their actual evolutionary relatedness. In the case of the IgGSF this is particularly true because of the dominant structural role of the β strands. The alternation of hydrophobic and hydrophylic residues certainly imposes a bias in these sequences for

the presence of certain amino acid combinations. Presumably any sequence that relies heavily on β-sheet structures will, by selective constraint, be biased toward a similar amino acid composition. Therefore, the contribution of covergent evolution cannot be ignored when considering the relationship of distantly similar sequences. Another complication that exacerbates the difficulty in distinguishing divergent from convergent relationships is the relative overrepresentation of IgGSF sequences in the data bases that new sequences are generally compared against. In the absence of truly significant matches, a data base comparison of almost any sequence will generate a list of "best" matches that includes at least some members of the IgGSF because there are so many diverse IgGSF examples in the data bases.

To identify significantly related sequences it is critical to start with reasonable sequence alignments. Alignments are the means by which residue-to-residue or base-to-base homologies are established. This means that the various penalty and weighting factors used to calculate the distances implied by the substitutions, deletions, and insertions of a particular alignment must reflect biologically and structurally "appropriate" assumptions about the likelihood of the various genetic events as well as the selective pressures for maintaining structural integrity. In this regard, it is important to take into consideration established sequence and structural homologies when evaluting the addition of a new member to the IgGSF. It is meaningless, for example, to align a new sequence to two established IgGSF members in ways that maximize its similarity to each of the two independently, but would result in a misalignment of already well-defined relationships between the two established members. Therefore, it is important that all family members fit a consensus alignment. In the IgGSF this consensus alignment must take into consideration the β-strand/loop organization and the repertoire of conserved amino acid residues. As reflected in the voluminous collection of V- and C-like sequences aligned by Kabat and Wu (1987), not only substitutions but length heterogeneities (deletions/insertions) are overwhelmingly concentrated in the loop regions of IgGSF sequences, presumably because they have less selective impact on the basic structure. Therefore, while indiscriminate use of gaps during alignment of any two IgGSF members (or potential members) may optimize the statistical score between them, it may ignore the selective constraints dictated by biological considerations.

Unfortunately, the evolutionary distance between the major groups of the IgGSF and the nonrandom modes of modification of its members make standard methods of statistical comparison singularly unreliable for establishing not only the broader relationships within the superfamily but also for determining whether a sequence belongs to the IgGSF at

all. For example, one attempt to calculate the relative distances between various distantly related IgGSF members using standard techniques (Williams *et al.*, 1987) determined that the Thy-1 molecule (a T cell surface antigen; see below) had a similarity score 4.2 SD above random with the fibrinogen γ chain and a score of 6.6 SD with the poly(Ig) receptor (see below). These scores represent highly significant p values of about 10^{-5} and 10^{-9}, respectively. However, the fibrinogen γ chain and the poly(Ig) receptor had a score of only 0.8 SD, exhibiting no significant relatedness. Except in unusual circumstances, a syllogism between scores should be maintained, i.e., if sequence A and sequence B are related to sequence C, then A and B should also be related. If these relationships are lacking, then one must question whether, in the example given above, any significant evolutionary relationships are reflected. Accordingly, other criteria must also be considered when attempting to establish IgGSF relationship(s). For example, secondary and tertiary protein structure conservation can be critical when considering the relationships within a family of molecules so strongly dependent on a conserved structure. However, there is no three-dimensional structural information for most IgGSF members, only calculated predictions based on less-than-perfect methods (Novotný *et al.*, 1984). Also, there are the caveats concerning the possibility that presumably unrelated sequences can convergently assume a similar tertiary structure (e.g., Richardson *et al.*, 1976). Therefore, we have taken a broadly cladistic approach to defining members of the IgGSF and their relationships, based upon a series of hopefully reasonable, objective, and subjective predefined taxa of features and assumptions, no single one of which is absolute. Primarily, a sequence must have a consistent, statistically significant degree of primary sequence identity to many, divergent representatives of the IgGSF. In addition, a sequence must also minimally maintain most of a small series of particularly conserved residues (Fig. 3) and a predominant β-strand potential throughout its sequence for us to consider it a member of the IgGSF. Also, direct or functional association with other, better defined homology units can lend subjective support to considering a more ambiguous sequence as a relic unit, even if it does not meet enough of the more objective criteria alone. Several of the larger, polydomain sequences (to be discussed later) exhibit sequence regions that can be argued to be derived evolutionarily from IgGSF sequences, given this latter assumption. Another striking general feature of IgGSF members, also useful in defining IgGSF membership, is that individual homology units of IgGSF members are encoded by discrete exons (with a few exceptions to be discussed later). Moreover, splicing between these exons always occurs between the first and second base of the boundary codon (½ rule). Other factors such as percentage identity, the ability to undergo somatic DNA rearrangements, the particular gene segments

used in the rearrangement process, the comparative exon organization of sequences, the functional roles and associations, and the chromosomal linkages can be considered to refine further the genealogical relationships. The sequences discussed in this paper all comfortably conform to this set of rules and are all generally accepted as members of the IgGSF.

III. Receptors of Immune Recognition

Any discussion of the diversity of the IgGSF must, by weight of data, be dominated by the molecules that mediate specific recognition of antigens: the Ig of B cells, the antigen-specific receptors of T cells (TcR), and the class I and class II proteins of the major histocompatibility complex (MHC). Ig can be expressed either as cell surface-bound receptors or as secreted humoral antibodies. Ig are capable of recognizing soluble or "free" antigens and play a role in both the cell surface triggering of B cell differentiation and as the effector molecules of humoral immunity. TcR, in contrast, occur only as surface-bound molecules and are able to recognize antigen only when it is presented in association with a class I or class II MHC molecule on a target cell. Hence, T cell recognition is said to be MHC restricted (see Schwartz, 1984). The T cells generally mature in the thymus and it is there that self-reactive T cells are eliminated and the processes that confer MHC restriction occur (Bevan, 1981). In general, class I molecules present antigen to cytotoxic T cells while class II molecules present antigen to regulatory or helper T cells. TcR do not appear to mediate directly the effector responses that occur upon T cell activation. Rather, they are present in a matrix of accessory molecules (CD3, CD4, CD8, etc.) that are involved in T cell activation and responses (see Townsend, 1985).

The basic Ig structure is a tetramer constructed of two identical, disulfide-linked heterodimers, each composed of one light (L) and one heavy (H) chain. Both chains are divided into an N-terminal V and a C-terminal C region. This interaction is homophylic in the case of the C-terminal C_H domains and heterophylic between the V regions and the C_L and C_{H1} regions. The V and C_L regions are single homology units while C_H regions consist of two to four related, but distinct, units tandemly linked. Pairs of similar homology units between chains associate to generate globular domains which represent the functional units of the molecules. The V and C homology units use opposite faces of the sandwich structure to interact with like units through mostly β-sheet interactions. Interchain association is often stabilized through disulfide bonds. Together, the V regions from both chains form the antigen-binding domain while the C region domains mediate various effector responses. As will be discussed later, many of the C-related functions

are initiated through further heterophylic associations of the C domains with homology units of other IgGSF members. Ig are humoral or membrane-bound depending on which exons of the C_H genes, i.e., those responsible for secretion or those responsible for membrane insertion, are included in the mature RNA by alternate pathways of RNA splicing (Early *et al.*, 1980a). The antigen-binding site of an Ig V domain is derived mostly from three or four relatively "hypervariable" (HV) loop sequences from each chain that connect the β strands of the more conserved "framework" regions (Wu and Kabat, 1970; Capra and Kehoe, 1974). The second HV region is generated by the connecting loop of the V-specific pair of β strands (Amzel and Poljak, 1979).

The predominant TcR are heterodimers, composed of an α and a β chain (see Hannum *et al.*, 1984; Meuer *et al.*, 1984). The α/β TcR have been found on all functional cytotoxic T cells and helper T cells. Together, they appear to be necessary and sufficient to confer specificity for antigen and MHC (Yagüe *et al.*, 1985; Ohashi *et al.*, 1985; Dembić *et al.*, 1986; Kuo and Hood, 1987). Each receptor is associated in the membrane with CD3, a multimeric protein complex of four or five polypeptides that appears to have a transducer role in T cell activation (see Terhorst *et al.*, 1986). Molecular analyses have shown that both the α and β chains have a single V and C homology unit, a connecting sequence of unknown function, a transmembrane, and a cytoplasmic region (Fig. 1) (see Davis, 1985; Kronenberg *et al.*, 1986). The C_α homology unit has a highly unusual structure apparently lacking an internal β strand and a highly conserved tryptophan residue important in stabilizing the overall fold. It is assumed to be evolutionarily derivative of the C unit lineage rather than that of H units, because of a gene and protein organization analogous to other immune receptor C units (see below). Although the sequence similarity between T and B cell V regions is low, the V regions of both receptors share about 15 conserved residues (Kronenberg *et al.*, 1986) that have been shown in Ig to be critical sites for determining three-dimensional structure through intra- and interchain interactions (see Amzel and Poljak, 1979). Various theoretical calculations of structure potential also suggest that the V domains of both receptors share essentially the same three-dimensional form (Goverman *et al.*, 1986; Novotný *et al.*, 1986). However, a reduced intracysteine distance in both V_β and V_α relative to Ig sequences may indicate a generally shallower (flatter) binding site (Goverman *et al.*, 1986). The total variability between the known V_β sequences examined so far is greater than that of the available Ig chains (Patten *et al.*, 1984), and this variability is more distributed throughout the sequence. However, analysis of extensive sequence data indicates that there are HV regions in V_β sequences at

positions homologous to those of Ig chains (Barth *et al.*, 1985; Behlke *et al.*, 1985; Concannon *et al.*, 1986). High background variability leads to ambiguous HV results for the V_α sequences (Arden *et al.*, 1985; Becker *et al.*, 1985; Hayday *et al.*, 1985a). The broader distribution of variability, combined with possibly flatter loop structures, may indicate that a generally larger area of the TcR is available to interact with antigen than is available in many Ig. Although Ig can also have relatively flat interactive surfaces with antigen (Amit *et al.*, 1986; Colman *et al.*, 1987; Sheriff *et al.*, 1987), such a general trend in TcR may have implications for the nature of the antigenic surfaces they recognize. Ig interact primarily with epitopes (antigenic sites) that are defined by a tertiary relationship between amino acids of a protein rather than their linear relationship along the sequence. It seems that the most flexible local structures of proteins may be generally more antigenic than the stiffer ones, as they would be able to adjust their conformation and generate a structure that better fits an Ig binding pocket (Westhof *et al.*, 1984; Fieser *et al.*, 1987). It has been suggested that an analogous flexibility of the Ig binding site could reciprocally facilitate such interactions (Colman *et al.*, 1987). The extremes seen in Ig, from the deep binding pockets of antihapten Ig to the flat surfaces of antiprotein Ig, might demonstrate a steric accommodation of the loop structures to the antigen with which they are bound more than some general binding-pocket geometry. TcR are not generally thought to interact with tertiary epitopes, but rather short linear sequences presented in a planar motif (see below). Hence, there would be less need for TcR as opposed to Ig to adjust to the variable shape of the antigen. However, it seems likely that there are no fundamental differences in the antigen-binding structures of Ig and TcR. This implies that they also share basic recognition strategies (Goverman *et al.*, 1986; Novotný *et al.*, 1986).

A second class of TcR also associated with the CD3 complex has a heterodimeric structure consisting of γ and δ chains (Brenner *et al.*, 1986; Bank *et al.*, 1986; Ioannidis *et al.*, 1987). This class of TcR is found on early thymocytes and about 1-5% of peripheral T cells (Lew *et al.*, 1986; Borst *et al.*, 1987; Moingeon *et al.*, 1987; Pardoll *et al.*, 1987; Bluestone *et al.*, 1987). The γ chain is more similar in overall sequence and organization to the TcR β chain (Saito *et al.*, 1984; Kranz *et al.*, 1985; Lefranc and Rabbits, 1985; Murre *et al.*, 1985). The δ chain is much more similar to the α chain, including sharing the deletion of the same internal β strand (Chien *et al.*, 1987a). The role of a γ/δ TcR is unknown, but, as mentioned above, neither the γ nor the δ chain is necessary to confer antigen or MHC specificity. The concordance between the structures of γ and β chains and δ and α chains does,

however, seem to imply a complementary function. The γ/δ TcR might define an important functional subset of T cells, with either or both a unique target repertoire or functional response. Some γ/δ-expressing cells can apparently exhibit both nonspecific and MHC-linked killing (Matis *et al.*, 1987).

A. ORGANIZATION, REARRANGEMENT, AND DIVERSIFICATION OF Ig AND TcR GENES

Ig chains are encoded by three unlinked gene families, the heavy chain gene family and the κ and λ light chain gene families (Fig. 4) (see Honjo, 1983). In each family, V gene formation occurs through a process of gene segment rearrangement. The V_L genes are constructed by the juxtaposition of a V gene segment and a joining (J) gene segment, while a V_H gene is generated from the joining of a V, a diversity (D), and a J gene segment. RNA processing removes the intervening sequence between the rearranged J gene segment and the C gene. TcR α and β chains are encoded similarly, with V_α genes employing V and J gene segments and V_β genes constructed from V, D, and J gene segments (Fig. 4) (see Davis, 1985; Kronenberg *et al.*, 1986). The organization and sequence of the γ gene are most similar to those of the λ light chain genes, and do not appear to employ D segments (Fig. 4) (Hayday *et al.*, 1985b). The δ gene, while more similar to the α gene, does include at least two D segments (Fig. 4) (Chien *et al.*, 1987b). More interestingly, the δ gene family is entirely located within the α locus. The C_δ gene is located 5′ of the J_α gene segments and appears to rearrange both with uniquely V_δ gene segments and with V_α gene segments.

DNA rearrangements of Ig and TcR gene segments are mediated by specific recognition sequences proximal to the gene segments in complementary orientation (Fig. 5) (see Honjo, 1983; Kronenberg *et al.*, 1986). These sequences consist of a conserved heptamer linked by 12 or 23 nonconserved nucleotides to an A/T-rich nonomer. A recognition sequence with a 12-base spacer joins to one with a 23-base spacer and vice versa (Fig. 5) (Early *et al.*, 1980a; Sakano *et al.*, 1980). Transfected TcR D_β and J_β gene segments can rearrange appropriately in B cells (Yancopoulos *et al.*, 1986), and Ig/TcR hybrid rearrangements are known (Baer *et al.*, 1986). Thus, the same or similar enzymes are used for B and T cell gene rearrangement. Tissue-specific rearrangements probably reflect the tissue-specific accessibility of the local chromatin structure of the loci.

B. MECHANISMS OF SOMATIC DIVERSIFICATION

The diversity of both Ig and TcR must complement the range of potential antigenic determinants. An amazing array of mechanisms is employed

FIG. 4. Organization of the mouse Ig and TcR gene families. Open boxes are C-region genes and filled boxes are V-region gene segments. Vertical lines illustrate D and J gene segments. Numbers between C-region genes in the heavy chain family are distances in kilobases. Double slashes without numbers indicate unknown distances. Human gene families are organized similarly. The δ gene family is shown as an insert to the α gene family.

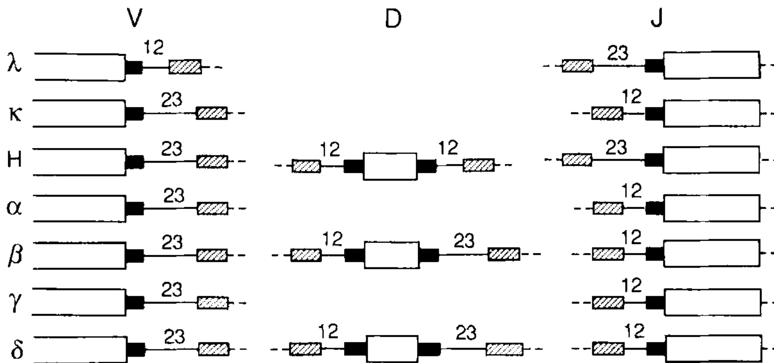

FIG. 5. Organization of V gene rearrangement signal sequences. The rearrangement signals for V, D, and J gene segments are shown in the relative orientation in which they are found in each gene family. The conserved heptamer is shown as a filled box and the nonomer is an open box. The distance in bases between each heptamer and nonomer is indicated.

to provide it. The basis of mammalian receptor diversity is the germline repertoire of gene segments and the consequent combinatorial possibilities that arise at both the DNA and protein levels during differentiation of both B and T cells (Table I) (see Honjo, 1983; Kronenberg *et al.*, 1986). At the DNA level there is a combinatorial rearrangement of gene segments within a family (e.g., any V_κ can join to any J_κ). At the protein level a combinatorial association may occur between the heterodimers (e.g., any α may associate with any β). Particular immune receptor loci may also have additional "nonstandard" combinatorial possibilities. The D_β and D_δ gene segments are flanked by asymmetric rearrangement signals, suggesting the possibility of D–D and V-J joining according to the 12–23 joining rule (Fig. 5). Possible examples of both events have been described for the β chain (Yoshikai *et al.*, 1984; Concannon *et al.*, 1986), but their frequency is unknown. However, the occurrence of D_δ–D_δ joinings is well established (Chien *et al.*, 1987b). The V_H gene segments have been shown to rearrange into already complete V_H genes at an internal sequence near the 3′ end of the V_H gene segment that mimics the 5′ rearrangement heptamer of D gene segments (Kleinfield *et al.*, 1986). This results in a combinatorial potential between any V_H gene segment and the very 3′ sequence of any other downstream V_H gene segment. Interestingly, all four families of TcR V gene segments conserve the same cryptic rearrangement signal sequence (data not shown) and, accordingly, have the potential for similar secondary rearrangements.

Two other diversifying mechanisms directly linked to the rearrangement process operate to extend the basic combinatorial diversity of both

TABLE I

DIVERSITY POTENTIAL OF IMMUNE RECEPTOR GENES

Gene	Heavy	κ	α	β	γ	δ
V gene segments	500	200	100	30	7	15
(subfamilies)	(8)	(5)	(16)	(17)	(5)	(8)
D gene segments	15	–	–	2	–	2
J gene segments	4	4	>50	12	2	2
D segments in three reading frames	+	Na	Na	++	Na	+
N region sequence	++	–	+	++	++	++
Junctional diversity	+++	+	+++	+++	++	+++
Somatic hypermutation	+	+	–	–	–	?
Nonstandard rearrangements	+	–	–	+	–	++
Combinatorial joining	$V \times D \times J$	$V \times J$	$V \times J$ 100×50	$V \times D \times J$ $(30 \times 3 \times 12)$ $+ (30 \times 3 \times 6)$	$V \times J$ 7×2	$V \times D \times J$ $(15 \times 2 \times 2)$ $+ (15 \times 2)$
Total	3×10^4	800	5000	1620	14	90
	$\times$		$\times$		$\times$	
Combinatorial association	2.4×10^7		8.1×10^6		1.3×10^3	

B and T cell sequences. First, the actual site of joining can vary across a range of several coding nucleotides proximal to the heptamer in both segments (Fig. 6) (see Tonegawa, 1983). This results in the generation of length heterogeneities and, hence, significant diversification of the protein products. However, this flexibility also results in many V and J gene segments joined out of reading frame with respect to each other and, hence, "nonproductive" V genes. Each rearrangement appears to have only the randomly expected one-in-three chance of joining the segments in frame (Alt *et al.*, 1984; Lewis *et al.*, 1985). This possibility suggests that joining efficiency in both B and T cells has been sacrificed for the diversity contributed by a flexible joining process. It has been suggested that secondary V rearrangements into the cryptic rearrangement signal may "rescue" some fraction of these nonproductive V genes (Kleinfield *et al.*, 1986).

A consequence of joining flexibility is that the D_H and D_β gene segments can be used in all three reading frames, essentially multiplying the size of each D segment family by three (Goverman *et al.*, 1985; Kaartinen and Makela, 1985). However, D_H gene segments appear to be more restricted in the reading frames they employ than are D_β gene segments. This distinction cannot be accounted for by termination codons in the unused frames. It is unclear, then, whether this distinction is a consequence of specific differences in the rearrangement process, of selection at the protein level, or simply of a sampling bias (most characterized TcR sequences have been isolated without regard to specificity, unlike

```
                VH1                        DFL16.1                                  JH1

           Y  Y  C  A  R  D  A         Y  Y  Y  G  S  S  Y        Y  W  Y  F  D  V  W  G  A  G  T  T  V  T  V  S  S
Germline   TATTACTGTGCAAGAGATGCA     TTTATTACTACGGTAGTAGCTAC     CTACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAG
S107       ------------------          --------------               ---------------------------------------
                                                    T
M603       ------------------          -------------C               ---------------------------------------
                     T                 D        N        F G
M167       ---------A----------        GG---------A-------TTTG           ----------------------------------
                                       N                  N
```

FIG. 6. Somatic diversification of Ig gene elements. Shown are the 3′ sequences of three related heavy chain V genes that each use the same V, D, and J gene segments (see Kim *et al.*, 1981, for sequence references). This figure is a simple illustration of how somatic diversification mechanisms can generate different proteins even if the same germ line information is used. Each rearranged V gene is listed beneath the representative germ line elements from which they were constructed. Dots indicate base-to-base similarity of each V gene to the germ line sequences. Translation of the germ line sequences are shown directly above in single-letter amino acid code. Differences in translation between the germ line and rearranged sequences is also shown above the respective V gene. Junctional variation in the exact joining point between elements length can be seen. Non-germ-line N-region sequences at the joining sites are labeled. Random nucleotide differences within the body of an element are the result of somatic hypermutation.

the Ig sequences). As regards the first possibility, it should be noted that the heptamer sequences of TcR gene segments are not as conserved as those of Ig (data not shown). Rearrangement efficiency can apparently be influenced in B cells by differences in the heptamer sequence (Goodhardt *et al.*, 1987).

A second process adds random nucleotides between gene segments during joining (Fig. 6) (Alt and Baltimore, 1982; Kurosawa and Tonegawa, 1982). These "N regions" can range from 1 to greater than 10 nucleotides and are found in V_H (see Tonegawa, 1983) and in all four characterized T-cell-expressed chains (see Kronenberg *et al.*, 1986; Quartermous *et al.*, 1986; Chien *et al.*, 1987a). Human and mouse V_L sequences do not appear to include N regions, but rabbit V_κ chains may (Heidmann and Rougeon, 1983). Interestingly, mouse κ constructs transfected into rearranging B cells can include the N sequence upon rearrangement (Lewis *et al.*, 1985). This certainly implies that light chain sequences can be productive substrates for the enzyme(s) mediating N-region diversification. It is possible that the lack of recognized N regions in mouse and human V genes reflects a biased sample set, rather than biological constraints. All known Ig N sequences have a high G/C content. However, this bias is considerably reduced in mouse and human V_β N regions (data not shown). Potential V_α N sequences do not appear to be G/C rich either, but the lack of germ line data and the consequent difficulty in rigorously defining the germ line contribution to V_β and V_α junctional regions make the calculations less than certain. The V_δ N sequences, on the other hand, have a clear G/C bias. A G/C bias reduces the possibility of generating stop codons as well as several amino acid codons, such as those for aromatic residues. It is possible that clonal selection favoring or not favoring G/C-rich codons may explain this discrepancy among chains, rather than specific differences in the diversifying processes. Selection at both the DNA and protein level may play a role in this bias (Milner *et al.*, 1986). It has been suggested that the N sequence is the result of the operation of deoxynucleotidal transferase (TdT) on the free ends of DNA exposed during V gene rearrangement (Alt and Baltimore, 1982). TdT is known to add a random sequence with a G/C bias to a free end of DNA (Kunkel *et al.*, 1986), such as might be expected to exist during rearrangement. A correlation has been demonstrated between the extent of N-region sequence and the expressed levels of TdT (Desiderio *et al.*, 1984).

Both junctional and N-region diversification alter the third HV loop of Ig and TcR V regions and can have a profound effect on the repertoire of antigen specificities (e.g., Auffray *et al.*, 1981; Azuma *et al.*, 1984; Darsley and Rees, 1985; Hedrick *et al.*, 1988). In fact, these mechanisms result in the greatest variability of each of these chains being concentrated

in their third HV regions, with the possible exception of V_L chains (Barth *et al.*, 1985). This variability can be so great that essentially no recognizable germ line D gene segment sequence remains (Fig. 7). The role of the D gene segment may be more as a substrate for the diversifying mechanisms than as a contributor of specific amino acid sequence information.

Ig genes undergo a third type of somatic variation not directly linked to rearrangement. This process, denoted somatic hypermutation (Fig. 6), distributes point mutations throughout both V_H and V_L genes and flanking sequences, resulting in as much as a 3% nucleotide change from the germ line sequence (Kim *et al.*, 1981). The rate of hypermutation may approach 1×10^{-3}/base/cell generation (e.g., Wabl *et al.*, 1985; Sablitzky *et al.*, 1985), which is three to four orders of magnitude greater than expected in most cell types (Kimura, 1983). Hypermutation may contribute less than 5% of the total sequence diversity of the expressed V_H chain repertoire of the mouse (Gojobori and Nei, 1986). However, as many as 90% of B cells may express V_H genes that have undergone somatic hypermutation. These mutations may be clustered and are localized to about 1 kb of sequence centered about the rearranged V gene (Kim *et al.*, 1981; Gearhart and Bogenhagen, 1983). Although it may occur in pre-B cells without surface Ig expression (Wabl *et al.*, 1985), hypermutation appears to be an ongoing process (Rudikoff *et al.*, 1984; Clarke *et al.*, 1985). Antigen selection appears to drive the preferential expansion of mutated clones with increased affinity, contributing to the affinity maturation observed in the development of a humoral immune response (Gearhart *et al.*, 1981; Clarke *et al.*, 1985; Sablitzky *et al.*, 1985). Somatic mutation can also generate new specificities (Diamond and Scharff, 1984; Giusti *et al.*, 1987). However, somatic hypermutation probably more often has impact on affinity maturation rather than on increasing the overall repertoire of antigen specificities. The V_α and V_β and apparently V_γ genes do not frequently, if at all, undergo hypermutation (Barth *et al.*, 1985; Ikuta *et al.*, 1985; Behlke *et al.*, 1985; Davis, 1985). Information on the V_δ genes is not yet available. It is possible that T cells do not diversify in this manner in order to avoid the potential for generating autoreactive specificities outside the selective environment of the thymus (Barth *et al.*, 1985; Honjo and Habu, 1985; Eisen, 1986). Consequently, it may be that TcR affinities for antigen will be on average lower than those of Ig. The possibility of generating autoreactive B cells may pose a smaller risk because of the regulatory safeguards provided by T cells.

C_H class switching is a further rearrangement process found in Ig genes (see Honjo, 1983). Initially, a rearranged V_H gene is immediately upstream of the first C_H gene, C_μ (Fig. 4) (Shimizu *et al.*, 1982), to which it splices to create a complete mRNA. During class switching,

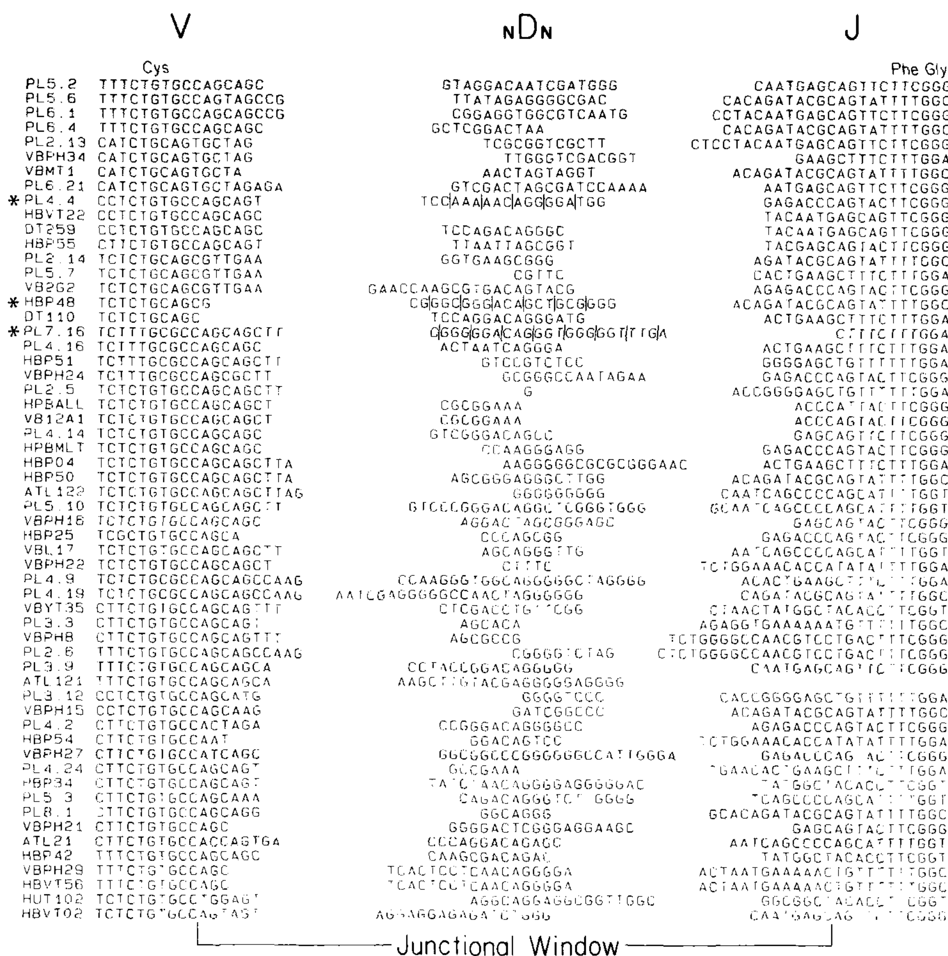

FIG. 7. Representative variation possible within a junctional window. The sequences in this figure are the 3′ sequences of a collection of human β-chain V genes (see Wilson *et al.*, 1988, for a key to the sequences). The V, D, and J gene segment sequences are labeled. The N-region sequence is included with the D sequences. An attempt was made to align the junctional sequences to the core D gene segments. However, so little of the germ line information remains in many of the regions that the alignment is often arbitrary. Likewise, the boundary between the V and junctional region is difficult to indicate precisely because of the lack of much germ line V gene segment information. The conserved Cys of the V region and the conserved Phe-Gly of the J segment are indicated for reference. Note the enormous variability of both length and coding potential between the 5′ most conserved J sequences and the 3′ most conserved V gene segment sequences even though only two D gene segments are used. Also, the (N)D(N) sequence can be translated in all three reading frames. The three sequences highlighted with an asterisk have the reading frame of their junctional sequence indicated by vertical bars separating codons. All three possible reading frames are represented.

the same V_H gene is juxtaposed 5′ to one of the other C_H genes further downstream within the C_H gene cluster, with the concomitant loss of the intervening DNA. The result is not diversification of the antigen recognition repertoire, but rather the expression of this diversity within the various functional contexts provided by the different C genes. This process occurs within repetitious "switch" sequences 5′ of most C_H genes. Like hypermutation, switching is often associated with the maturation of an immune response (Gearhart *et al.*, 1981).

C. Strategies of Diversification

There are obvious similarities among the gene families of the immune receptors (Fig. 4). However, each family and each receptor has a unique combination of strategies for organization and diversification, both species and chain specific. The λ L chain family is illustrative in this regard. The λ L chains are used in only 2–3% of mouse and rabbit Ig, but may comprise as many as 40% of the L chains of human and 95% of chicken Ig (Hood *et al.*, 1967). The mouse λ chain employs primarily three functional V/J pairs and is mostly diversified by somatic hypermutation (see Eisen and Reilly, 1985). The human λ family, though organized similarly to that of the mouse, has many more V and J elements and employs all of the general diversifying strategies discussed above to some degree. The chicken λ locus, on the other hand, has only a single functional V and J gene segment (Reynaud *et al.*, 1985), but has 25 tightly arrayed pseudo-V gene segments just 5′ to the functional V_λ gene segment (Weill, 1986). It appears that somatic diversification of the chicken λ repertoire results almost entirely through gene conversion-like events of the functional V gene segment by the pseudogene segments. Thus, a single V_λ gene segment appears capable of generating virtually the entire L chain repertoire of the chicken. The λ chain diversity in different species can obviously arise by very different mechanisms. Presumably then, the contribution of any gene family to the total repertoire of Ig or TcR diversity is determined by its overall diversifying potential rather than merely by its size.

The number of gene segments employed by various gene families is illustrative of how combinatorial potentials can vary in a family and in a species-specific manner. For example, the mouse has >500 V_H gene segments (Livant *et al.*, 1986), but <30 V_β gene segments (Patten *et al.*, 1984; Barth *et al.*, 1985; Behlke *et al.*, 1985). The human, on the other hand, may have only about 50 V_H gene segments but 75 V_β gene segments. Both the mouse and human may have less than 100 V_α gene segments and no D_α gene segments. However, the α locus has 5 to 10 times more J gene segments than other loci (Arden *et al.*, 1985; Becker *et al.*, 1985; Hayday *et al.*, 1985a; Winoto *et al.*, 1985; Yoshikai *et al.*, 1985). As Table I illustrates, when all variations are considered,

mouse B cells and T cells are probably capable of generating comparable levels of combinatorial diversity (Goverman *et al.*, 1986). Presumably, the same is true for other mammals as well.

As great as the combinatorial diversification potential may seem, the gene segment combinatorial values of Table I imply a much more modest diversity potential for Ig and TcR than would be expected if all diversifying mechanisms are factored in. It is impossible to be rigorous in quantifying the contributions of these mechanisms, but reasonable approximations can be made. Basically, instead of simply factoring in D segments in the combinatorial calculation, it may be better to quantify the total number of potential junctional sequences that can be generated between V and J gene segments, the loop of the third hypervariable region. The sequence in this region reflects the effects of N-region diversification, joining-site variation, multiple D elements (translated in three reading frames), and nonstandard rearrangements. Diversification from these mechanisms can be so great that each V gene family has a certain junctional window that can be virtually any sequence at the protein level. This window is defined as the sequence between the most C-terminal V segment residue that is never altered from germ line by junctional variation and the equivalent most N-terminal residue of the J regions (Fig. 7). Each chain has a characteristic minimum and maximum length for this window. Examination of known Ig and TcR HV3 regions suggests that all 20 amino acids can be found at each residue position in this region. Therefore, to a first approximation the number of potential junctional sequences for any chain is

$$\sum_{N=\min}^{\max} 20^N$$

where max is the maximum length of the window and min is the minimum length. The window for known V_β sequences for example is from about 6 to 15 amino acid residues. In practice, however, not all lengths within this range are equally represented, as this distance is only seldom greater than 8 residues. The similarly limited window size of V_α sequences appears to be from 3 to 7 residues. The number of sequences between 6 and 8 residues in length with any amino acid possible at any position is almost 2.7×10^{10}. When this value (and 1.3×10^9 for V_α) is factored into the combinatorial "V $\times$ J $\times$ junctional sequences" (using values from Table I), 4.4×10^{13} V_β and 6.5×10^{12} V_α chains can be generated. Assuming that only 1% of each of these is a viable protein because of inherent constraints of protein structure (e.g., chains with eight cysteines in a row are not likely to be functional), there is still the potential for generating 2.9×10^{22} receptors. If the number of these

pairs is reduced by another factor of 100 because of the inability of certain dimers to form or removal because of self-reactivity, 2.9×10^{20} potential TcR are possible from the germ line repertoire of one mouse. During the lifetime of a mouse, probably fewer than 10^9 thymocytes will ever leave the thymus, perhaps 1% of all those actually generated there (E. Rothenberg, personal communication). The difference in the potential TcR diversity and the actual level of diversity manifested in T cells is striking, particularly considering that these estimates of potential are certainly conservative. It is interesting that poorly formed chains and/or dimers may be more responsible for elimination of maturing thymocytes before migration to the periphery than selection against autoreactive clones. This could imply a role in T cell development for a test for the functionality of the expressed TcR gene products as a major aspect of MHC restriction, rather than a test for self-recognition per se (Goverman *et al.*, 1986).

Similar calculations for Ig (a window of from 5 to 10 for V_H regions and only 2 to 4 for V_L regions) (Chothia and Lesk, 1987) would result in 3.9×10^{18} potential Ig molecules. This may seem substantially less than the number of potential TcR, but Ig undergoes the added diversification of somatic hypermutation. Calculating the effects of hypermutation is difficult, but assuming that the product of any B cell division is susceptible to the process, the potential number of unique clones must be several orders of magnitude greater than the combinatorial potential alone. Again, compare these figures to a possible 10^{11}-10^{12} total B cells generated over the lifetime of a mouse.

Calculations of the potential diversity of γ/δ TcR are even less certain because the number of V_α gene segments that may rearrange with C_δ is unclear. However, to visualize the potential combinatorials it is sufficient to merely calculate the junctional sequence diversity potential of the V_δ sequences known (Elliot *et al.*, 1988). A window length of between 5 and 16 is readily possible, much wider than any other V family and primarily the result of the frequency of D_δ-D_δ joining. A window this size could potentially generate 6.9×10^{20} V_δ junctional sequences. Though it is certain that not all of these sequences are viable and that there will be skewed distribution of certain amino acids (because of the contribution of the D segments), the true diversity potential for a γ/δ dimer must be enormous. [Using a different method, Elliot *et al.* (1988) estimate a potential diversity of 10^{17} γ/δ dimers.] The potential is amazing when it is considered that there may be fewer than 10 V_γ or V_δ gene segments.

Of course, the diversity potentials just calculated primarily reside in the HV3, critical for determining the specificity of both Ig and TcR. It seems, in fact, that the presence of D segments and/or expanded J gene

segment families is the means by which to promote the complete diversification of the junctional region and can be considered as essentially equivalent diversifying strategies. However, HV3 is only one of three or four regions of each chain that contribute most to antigen specificity. Also, even residues outside the HV regions can interact with the antigen on occasion (e.g., Sheriff *et al.*, 1987). It is possible that alteration of HV3 often adjusts the specificity of a receptor that has a more general, but still restricted specificity provided by the V segment structure. This can be seen in fine specificity differences between TcR sequences that differ only in the HV3 (Winoto *et al.*, 1986; Fink *et al.*, 1986). In other words, it is selectively important to provide not only a large number of different receptors, but to ensure as broad a range of differences between these receptors as possible. Even though there are plenty of examples of single residues having profound effects on specificity, the most reasonable assumption is that the more nearly similar two receptor sequences are, the more nearly their specificities will overlap. No matter how diverse a junctional window is, if the rest of the sequence is essentially the same as many of the other members of the V gene family, the overall range of specificities will be severely truncated. Therefore, a diverse germ line repertoire of V gene segments may have more to do with providing this range than in contributing to greater combinatorial numbers. Certainly, from our previous calculations the number of potential receptors would not be much affected relative to actual clones expressed even if the number of V segments is reduced substantially (compare V_H and V_β, for example). In Table I there is a value for the number of known subfamilies of each chain. Subfamilies are defined by members of a particular gene family that are 75% or more similar (that cross-hybridize). The number of subfamilies, therefore, is one indication of the range of differences within a family (Barth *et al.*, 1985). The V_β and V_α gene families, although they have generally smaller subfamilies than Ig V gene segment families, have many more of them relative to the total number of genes and, hence, have much greater variance by this criterion than either V_H or V_L families. [Even though the maximum difference reported between V_β gene segments and between V_H gene segments is similar (Barth *et al.*, 1985), if two randomly picked V_H gene segments are selected, they will by chance be more similar to each other than two randomly chosen V_β sequences.] Does this imply a greater need for diverse TcR than Ig? Again, it must be remembered that hypermutation diversifies the entire Ig V region, perhaps mitigating any differences in range. The generally greater number of Ig V segments should also be considered. Also, experimental sampling differences could give a false impression about the true extent of germ line diversity in these families (Barth *et al.*, 1985).

The use of such varied diversifying strategies by the different gene

families and species argues that it is the total receptor diversity and the range of that diversity that generally represents the selectable phenotype, rather than particular sequences or modes of variation. This is an important perspective when considering the functional implications of the different strategies and their evolution. Most importantly, the level of potential diversity implies that virtually every pre-B and pre-T cell generates a unique receptor structure. Given even conservative estimates, the potential number of different receptors is certainly much greater than the number of different B or T cell clonal types expressed in any individual at any given moment (10^6–10^7; Jerne, 1955; Klinman *et al.*, 1976). The potential is so great for Ig that the loss of large segments of the germ line contribution can fail to have an appreciable impact on the level of functional diversity (Vice *et al.*, 1970; Kelus and Weiss, 1977). This variation is uniquely accommodated by a basic receptor structure that essentially allows the somatic tailoring of immune receptor repertoires to the current antigenic environment.

IV. Gene Organization and Diversity of the Major Histocompatibility Complex

Class I and class II MHC molecules are both noncovalently linked heterodimers (Fig. 1). Class I molecules are constructed of a class I heavy or α chain and a non-MHC-encoded light chain, β_2-microglobulin (β_2-m). Class II molecules consist of α and β chains of similar size. Both class I and class II molecules appear to fold into paired membrane-distal and -proximal domain structures (Fig. 1). The proximal domain sequences of all four chains are composed of classical C homology units (see Hood *et al.*, 1983). The distal regions of both class I and class II molecules have totally distinct structures. Therefore, the possibility of an evolutionary relationship between the distal regions and Ig homology units must remain conjecture (Ohnishi, 1984).

It seems that MHC molecules act as low-affinity, broadly specific receptors that interact with and present short peptide antigens to TcR (see Claverie and Kourilsky, 1987). These interactions are mediated by the distal domains (see Germain and Malissen, 1986) and hypervariable sequences analogous to those of Ig (see Mengle-Gaw and McDevitt, 1985). Although not generally similar, the distal sequences of class I and class II molecules do share specific sequence and structural similarities that are probably homologous in origin (Malissen *et al.*, 1984), implying not only a direct evolutionary relationship, but also suggesting that they present antigen in a similar fashion (Germain, 1986). The presented peptides arise as products of an intracellular "processing" or degradation of protein antigens. Processed peptides have been shown to bind to a single site on class II molecules (Giuillet *et al.*, 1986).

Recently, a high-resolution crystallographic analysis has been completed on a human class I molecule (Bjorkman *et al.*, 1987a). This structural data confirm that the membrane-proximal region of the α chain and β_2-microglobulin are canonical Ig C homology units. However, the most striking feature of the structure is the nature of the antigen-presenting, distal regions. Each of the two regions consists of four antiparallel β strands in a single sheet, overlayed by an extended α-helical structure. Interdomain alignment creates essentially a single, eight-stranded β sheet that appears as a table topped with two parallel helical structures running its length, forming a groove between them (Fig. 8). The residues lining

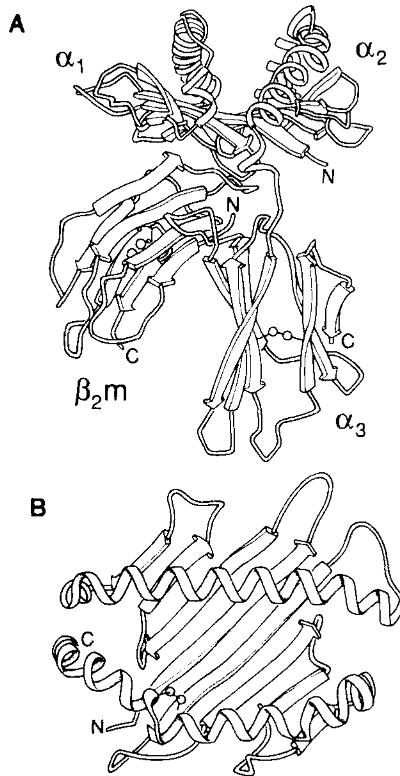

FIG. 8. Tertiary organization of an MHC class I protein. This figure (after Bjorkman *et al.*, 1987a) illustrates two views of a class I MHC molecule. A full-length side view (A) shows all three regions (α1, α2, and α3) of the class I molecules as well as their relationship to β_2-microglobulin. The β strands are shown as flattened arrows. The two long α-helical structures resting on the β-sheet platform of the αl and α2 regions and the groove between them are viewed end-on. A view directly above the αl/α2 β-sheet platform (B) looks directly into the antigen-presenting groove.

this groove as well as those on the "top" surface of the helices contain most of the previously identified hypervariable residues of class I sequences (Cowan *et al.*, 1987; Gussow *et al.*, 1987). Therefore, it appears that the helical groove is the binding site of the peptide antigens (Bjorkman *et al.*, 1987b).

In various strains of mice, there are from 25 to 36 class I genes, but only two or three (K, D/L) are generally involved in antigen presentation and are coexpressed on most somatic cells (see Hood *et al.*, 1983). There are two class II loci, I-E and I-A, each encoding an α and β chain (see Mengle-Gaw and McDevitt, 1985). The class II molecules of both loci (isotypes) are coexpressed, but usually only on specialized antigen-presenting cells. The rat and human MHC are similarly organized (see Howard, 1987; Wake, 1986). Class I and class II loci involved in antigen presentation are the most polymorphic protein coding loci known in vertebrates. At least 50-100 alleles of each expressed locus are found in both mouse and human populations, and none of these loci is dominant (see Klein and Figueroa, 1981). Analogous to the V/C dichotomy of Ig and TcR, most of the heterogeneity is found in the membrane distal regions and can reflect a variation of up to 10% between allelic proteins (see Mengle-Gaw and McDevitt, 1985; Klein and Figueroa, 1986). Although there are interesting exceptions (Streilein and Duncan, 1983; O'Brien *et al.*, 1985), most mammals studied appear to share this high level of MHC polymorphism. This heterogeneity and multigenic expression assure the presence of multiple class I and class II molecules in normal individuals. For example, 75% of Caucasians will express four different alleles at their three class I loci (Bodmer and Bodmer, 1978). Because of this heterozygosity, class II α and β proteins can contribute further to this diversity in an intraisotypic combinatorial manner analogous to that of the two chains of Ig or TcR (see Bevan, 1981). However, limitations on particular intraisotypic pairings have been seen and may account for the tight genetic linkage between pairs of α and β class II genes (Braunstein and Germain, 1987). Combinatorial products between the different class II isotypes have also been reported (Germain and Quill, 1986; Malissen *et al.*, 1986), but the frequency of functional interisotypic combinations is unknown.

MHC polymorphism and combinatorial associations directly complement the TcR repertoire by expanding the context for antigen presentation, thereby broadening the range of antigen recognition possible with a given set of TcR (Goverman *et al.*, 1986). Unlike Ig and TcR, MHC molecules must rely solely on evolutionarily acquired variation. There are two recombinational mechanisms of diversification operating on MHC genes as a result of their multigenic organization. The first is the

process of homologous, but unequal crossing over. Such recombination events lead to the expansion and contraction of multigene families and changes in the repertoire of variant members (see Hood *et al.*, 1975). The variation in the gene number of both class I and class II genes seen among mouse strains is consistent with a frequent occurrence of such events (Rogers, 1985; Stephan *et al.*, 1986). This mechanism can also generate functional hybrid variants if recombination occurs within the genes (Sun *et al.*, 1985).

"Minigene" conversion is a second, presumably recombinatorial, mechanism affecting MHC gene families (Mellor *et al.*, 1983; Pease *et al.*, 1983; Weiss *et al.*, 1983). Independent events often generate the same clustered set of point substitutions (Geliebter *et al.*, 1986) and appear to result from small, gene conversion-like events between similar sequences. These events may account for much of the high rate of mutation at some MHC loci, as high as 10^{-3}/gene/generation in mice (Melvold and Kohn, 1975). The donor sequences of analyzed examples appear to be the non-antigen-presenting class I genes of the Qa and Tla loci (Mellor *et al.*, 1983). Interestingly, certain K alleles appear to undergo spontaneous gene conversion-like events an order of magnitude more frequently than the D alleles. Thus, position in the multigene family may dictate the frequency of recombination-like events.

While miniconversion events will extend diversity between alleles, they will also maintain a higher level of similarity between gene family members in general (a conversion inherently makes the two involved sequences more similar). Therefore, while frequent minigene conversions will maintain a dynamic heterogeneity between alleles, it will also provide a force for concerted or coincidental evolution within a population, particularly if the donor and recipient genes are not extremely tightly linked and/or the correction is directional in nature (Dover, 1982). Such conversion and homologous recombination events are probably responsible for the high degree of species-specific character found in MHC gene families (Hood *et al.*, 1975; Ohta, 1982; Bregegere, 1983; Hayashida and Miyata, 1983).

Once variants have arisen, what are the forces responsible for their fixation within a population? Are there positive selective forces favoring variants or is their establishment generally neutral? Is the process of variation itself selected? These are vexing questions because, even though there are no particularly frequent alleles, the collection of alleles is relatively stable within wild populations over time (Arden and Klein, 1982; Hayashida and Miyata, 1983; Figueroa *et al.*, 1985). Studies of variation between allelic polymorphic regions of MHC sequences demonstrate that the pattern of silent and replacement base changes

more nearly resembles that expected for random substitution than that of conserved sequences like globins (Hayashida and Miyata, 1983; Gustafsson *et al.*, 1984). These values imply that MHC polymorphic regions are under relatively weak constraining pressures as to their exact protein sequence. Paradoxically, the absolute rate of evolutionary change between class I alleles and species homologs is actually less than that of most other genes analyzed, including the globins (Hayashida and Miyata, 1983). This might indicate that structural variation is sometimes less important in the effects it can have on the interaction between an allele and antigen or TcR per se, than in how concomitant variation in its specificities complement or fail to complement the collective specificities of the allelic population. Thus, the lack of frequent MHC alleles implies that there is no "best" collection of MHC genes and that selection for variation occurs in the context of a population and particular lifestyle (Streilen and Duncan, 1983). This reflects the ability of pathogens to alter their antigenic profile more rapidly than host populations can acquire new MHC alleles. Because of its degenerate specificity, mutations leading to the improvement in the interaction of an allele with one antigen may weaken its interaction with other antigens, potentially exposing the organism to a host of other infectious agents. Therefore, within a changing antigen mileu no single MHC molecule or limited collection of molecules can ever optimally present all possible antigens. It is not only advantageous for an individual to be heterozygous for MHC molecules (in order for it to be able to respond to the broadest range of antigens), but also for it not to duplicate completely the repertoire of a neighbor. Pathogens have a much more difficult time expanding within a heterogeneous population in which the neighboring host organism will have a different repertoire of antigen-presenting alleles to be circumvented than the one already successfully invaded. The negative consequences of losing this populational diversity may have recently been demonstrated in cheetahs (O'Brien *et al.*, 1985). Cheetahs are much more susceptible to viral epizootics than are other big cats. It appears that cheetahs have undergone at least two severe populational bottlenecks (O'Brien *et al.*, 1987) that, because of founder effects, have essentially eliminated diversity of the MHC locus within the species.

The mechanisms responsible for assuring MHC variation are not special to the MHC. Rather, they are inherent consequences of the organization of the multigene families encoding the MHC. We assume that analogous recombination mechanisms also play significant roles in generating the germ line variation within the Ig and TcR gene families (e.g., Krawinkel *et al.*, 1983; Hayashida *et al.*, 1984; Clarke and Rudikoff, 1984; Kodaira *et al.*, 1986). However, Ig V gene segments do not appear

to undergo homologous crossing over as frequently as do MHC genes. This may reflect the lack of conserved linear sequences in V genes that would facilitate crossing over or correction and a shorter target length. Such factors would presumably also affect the rate of gene conversion and may account somewhat for the difference in conversion rates between K and D class I loci. Therefore, differences in the organization of MHC genes and V gene segments may affect not only the rate, but also the nature and mode of their evolutionary diversification.

For both MHC and V gene families, it appears that the overall repertoire of diversity, rather than the individual variant, is generally the selectable phenotype. However, it is presumably the clonal expression (one receptor per cell) of Ig and TcR diversity versus the nonclonal expression (multiple receptors on each cell) of MHC diversity that establishes the selective constraints on the gene copy number of each family and, hence, the differences in how diversity in each system is selected and maintained. For the large Ig and TcR gene families these organizational differences reflect a selection for diversity within a family rather than between alleles. On the other hand, a single antigen-presenting cell presents multiple peptide/MHC complexes at any one time. It is the combination of peptide/MHC molecule that is the functional T cell antigen. The same peptide in association with a different MHC molecule is effectively a different antigen and would generally be recognized only by different T cells. There is certainly a practical limit on how many MHC molecules per se can be expressed on a single cell as well as a limited amount of any particular processed peptide antigen available for presentation by that cell. Too many coexpressed class I or class II variants would lower the concentration of any particular peptide/MHC combination, potentially below the threshold level required to trigger a specific T cell. With such constraints, diversity of the smaller repertoire of MHC genes is assured through the maintenance of extreme allelism and a heterozygous population. This extreme allelism ensures that the broadest possible range of antigen specificities is available to every antigen-presenting cell in a manner analogous to the contribution to the range of T cell specificities by the large number of subfamilies in TcR V genes.

V. Nonimmune Receptor Members of the IgGSF

At least 23 other distinct genes or gene families with no direct role in antigen interaction have been identified as belonging to the IgGSF. Most are single-gene members and most appear to encode distinct cell surface, receptor molecules. Although these genes are generally nonpolymorphic, the diversity of the examples is striking. For convenience,

these molecules can be loosely associated in eight categories: (1) non-antigen-presenting, β_2-microglobulin-associated molecules, (2) T-cell-associated molecules, (3) molecules expressed on both T cells and nervous system cells, (4) nervous-system-associated molecules, (5) Ig-binding molecules, (6) growth factor/kinase receptors, (7) miscellaneous and (8) uncertain examples. Note that these categories are not meant to imply necessarily functional or evolutionary relationships.

A. NON-ANTIGEN-PRESENTING, β_2-MICROGLOBULIN-ASSOCIATED MOLECULES

As mentioned, β_2-microglobulin is a single C homology unit, the light chain of the MHC class I molecule (Cunningham *et al.*, 1973). It is probably divergently related to the MHC class II α chain (McNicholas *et al.*, 1983) and may be considered functionally an orphan MHC gene. It is encoded by a single, nonpolymorphic gene (Parnes and Seidman, 1982). Besides the antigen-presenting class I sequences, β_2-microglobulin is also found in association with the Qa and Tla MHC molecules and the CDl family of antigens.

In the mouse, most MHC-linked class-I-like genes are encoded in the Qa and Tla regions and are not involved in antigen presentation. Although the Qa and Tla alleles are not as polymorphic as the K and D alleles, substantial rearrangements of the loci through unequal crossing over is seen (see Flavell *et al.*, 1986; S. Hunt, personal communication). These sequences are strongly conserved within species, but may not be conserved at all between species (Rogers, 1985). The function of putative Qa and Tla gene products is unknown, but it has been suggested that the genes serve primarily to drive the evolutionary diversification of the antigen-presenting K- and D-like alleles by providing a pool of diversity for gene conversion-like events (e.g., chicken λ; see Howard, 1987). However, the differential expression of many of these genes in distinct populations of hematopoietic cells and the relatively low frequency of obvious pseudogenes (S. Hunt, personal communication) argue that there may be selection of these sequences directly at the protein level, perhaps as elements involved in the differentiation of hematopoietic cell subsets. The chicken Vλ gene family indicates that there is no strict requirement for maintenance of open reading frames to be efficient donor sequences in gene conversion-like events. Accordingly, without selection at the protein level, one might expect a larger number of pseudogenes within this group. Also, some Qa molecules are secreted and may have regulatory functions (Soloski *et al.*, 1986).

The human CDl antigens are differentiation antigens that characterize immature thymocytes and some lymphoid malignancies (Bernard *et al.*,

1984). Like class I sequences, CDl molecules associate noncovalently with β_2-m (Ziegler and Milstein, 1979), but at least one CDl antigen (T6) is also found disulfide linked, in some thymocytes, to the CD8 molecule (Ledbetter *et al.*, 1985; Snow *et al.*, 1985), another member of the IgGSF (see below). Recent characterization of the CDl genes indicates that they have an exon and protein structure similar in organization to class I heavy chain genes, with one membrane-proximal, C homology unit and two unrelated distal regions. However, they are not linked to the MHC and they are no more similar to class I than to class II sequences (Calabi and Milstein, 1986). This family has at least five members that may be clustered. Hybridization results indicate homologous sequences are present in mice (Martin *et al.*, 1986). The role of these molecules is unknown, but their interspecies conservation, dissimilarity to MHC genes, and differential expression imply an independent protein function, possibly morphogenic in nature. The T6 molecule is expressed on all epidermal Langerhans cells. These are the only epidermal cells that express MHC class II molecules and are believed to be specialized antigen presenting cells (see Wolff and Stingl, 1983). Recent work reveals that the T6 molecule expressed on Langerhans cells is cointernalized with MHC class II molecules through the process of receptor-mediated endocytosis, indicating a potential role of the T6 antigen as a receptor involved in some immune function (Hanau *et al.*, 1987).

B. T-Cell-Associated Molecules

Besides the TcR, T cells express a host of accessory molecules that are presumably involved in signal transduction, cell adhesion, and even the facilitation of antigen/MHC targeting. The CD4 and CD8 molecules are accessory molecules of T cells that appear to play an important role in facilitating T cell interactions with target cells. The CD4 and CD8 molecules are expressed on most thymocytes together. However, most peripheral T cells fall into two mutually exclusive populations, those expressing only CD4 or only CD8 molecules. Both molecules appear to interact directly with either class I (CD8) or class II (CD4) molecules (Dembić *et al.*, 1987; Gabert *et al.*, 1987) and hence may be predominantly responsible for restricting cytoxic T cells to class-I-presented antigens and helper T cells to class-II-presented antigens (see Townsend, 1985). The human CD8 molecule is usually characterized as a homodimer of α chains, unlike the CD8 of mouse, which is a heterodimer with an α and β chain (Lyt-2 and L3T4, respectively). The CD4 molecule appears to be monomeric. However, recent evidence indicates that the human CD8 molecule may also exist as a heterodimer employing a Lyt-3 (β) homolog (J. Parnes, personal communication). Each molecule has an

N-terminal V homology unit, most like those of light chains, linked by a connecting sequence to transmembrane and cytoplasmic regions (Fig. 1) (Johnson *et al.*, 1985; Littman *et al.*, 1985; Maddon *et al.*, 1985; Sukhatme *et al.*, 1985; Zamoyska *et al.*, 1985; Johnson and Williams, 1986; Tourvieille *et al.*, 1986; Gorman *et al.*, 1987; Nakauchi *et al.*, 1987). Beyond these similarities, the CD4 and CD8 molecules have very different structures. The CD8 α and β chains have connecting sequences approximately 60 residues long. The CD8 α and, perhaps, the CD8 β chain have an immunoglobulin hingelike sequence in this region that presumably plays an important role in determining how the molecule is displayed into the extracellular environment (Sukhatme *et al.*, 1985). The CD8 β chain has a short peptide sequence that is indistinguishable from those of Ig and TcR J segment sequences (Fig. 9). Neither the CD8 α or β chain gene rearranges during differentiation. Considering the high level of identity between the CD8 β J-like sequence and those involved in rearrangement, this lack of rearrangement in CD8 β raises interesting evolutionary possibilities that will be discussed later. The CD8 α and β genes are single copy, are located within a few kilobases of each other, and are closely linked to the κ light chain locus (J. Parnes, personal communication). The CD8 homodimers and heterodimers appear to form single V domain-like receptors possibly interacting with a conserved epitope on MHC class I molecules (see Goverman *et al.*, 1986).

Even though CD4 and CD8 molecules appear to perform analogous functions with homologous ligands, class II and class I MHC molecules, respectively, they do not appear to share a recent common origin. The CD8 α and β V-like regions are more closely related to V_L sequences than the N-terminal V-like element of the CD4 molecule (Tourvieille *et al.*, 1986). Moreover, the CD4 molecule has a connecting sequence approximately 280 residues in length. Within this sequence and immediately C terminal to the first V-like sequence is a second, albeit truncated, homology unit sequence (Tourvieille *et al.*, 1986). It has

mouse J_λ con	WVFGGGTKLTVL
mouse J_κ con	.T.......EIK
mouse J_H con	D.W.Q..TV..S
mouse Lyt3	M...T......V
human CD7	N.Y.S..-..LV
rat MRC Ox-2	NM..S.-.VSGT

FIG. 9. The J-like sequences in nonrearranging IgGSF members. Shown for comparison are consensus J sequences for the three Ig families. Similarity to the J λ consensus is indicated by dots. Gaps inserted to optimize alignment between J elements are indicated with dashes.

been suggested that there are two even more extensively diverged homology units tandemly arrayed in the connecting sequence (Clark *et al.*, 1987), one without a disulfide loop. The second homology unit is more nearly similar to the first V-like region than to other IgGSF member sequences, indicating its probable origin through a duplication event involving the first V-like or related region (Tourvieille *et al.*, 1986). Therefore, although the foreshortened nature of the second homology unit might suggest that it belongs to the H class of sequences, its similarity to the V-like region implies that it should also be classified as V-like. The evolutionary implications of its truncated nature will be discussed later.

With such dramatic differences in structure, how do the the CD4 and CD8 molecules perform such apparently analogous functions? How can the CD4 molecule form a domainlike receptor structure like the CD8 chains presumably do? It is still possible that CD4 is expressed as a homodimer or heterodimer. As mentioned, it is only recently that a human CD β chain may have been identified, and a loosely coupled CD4 homodimer would be difficult to identify. Alternatively, two of the V-like units of the CD4 molecule, presumably the most N-terminal pair, could fold together to generate a pseudodimeric structure (Parnes and Hunkapiller, 1987). Monoclonal antibody epitope mapping of the CD4 chain does indicate that these two regions are in close enough apposition to generate a composite epitope (B. Jameson, personal communication). It is possible, however, that the CD4 and CD8 molecules perform their roles in completely unrelated ways. CD4 may interact monomerically with its MHC ligand through β-sheet interactions between a homology unit of CD4 and a C unit of the class II molecule in the manner of other IgGSF domain-forming interactions.

The α/β and γ/δ heterodimers are noncovalently associated on the T cell surface with the CD3 complex. The human and mouse CD3 molecules are composed of γ, δ, ϵ, and ζ chains. CD3 γ, δ, and ϵ cDNA sequences have been characterized (van den Elsen *et al.*, 1984; Krissansen *et al.*, 1986; Gold *et al.*, 1986, 1987). Each has a single H-type homology unit, a short connecting sequence, a transmembrane element, and a hydrophylic cytoplasmic tail. It is believed that an unusual asparagine residue conserved in the transmembrane region of all three chains is involved in forming a salt link with an equally conserved lysine residue in the transmembrane peptide of all four TcR chains. The γ and δ chains are each significantly more similar to each other than either is to any other IgGSF member, indicating a relatively direct evolutionary relationship (Krissansen *et al.*, 1986; Gold *et al.*, 1987). The CD3 ϵ chain, on the other hand, is not any more similar to the γ or δ chain than it is to several other members of the IgGSF, indicating a more distant

divergence. A particular relationship between the CD3 γ, δ, and ϵ chains and another H-structure IgGSF member, the neuronal-cell adhesion molecule (see below), has been argued on the basis of statistically greater similarity scores and close chromosomal linkage, each to band q23 of human chromosome 11 (Gold *et al.*, 1987). However, the scores appear more to indicate that these molecules share homologous H-structures rather than a particularly close evolutionary relationship. The Thy-1 gene maps as well to band q23, but has no particular similarity to the CD3 gene.

The CD28 (Tp44) molecule is a disulfide-linked homodimer expressed on $CD3^+$ T cells that coexpress α/β TcR (Hara *et al.*, 1985), but apparently not on peripheral T cells that express the γ/δ TcR (Poggi *et al.*, 1987). Monoclonal antibodies to the CD28 chain added in the presence of phorbol esters can cause $CD28^+$ T cell proliferation (Hara *et al.*, 1985). The normal ligand, and hence the function of the CD28 molecule, is still unknown. Its apparent absence from peripheral γ/δ TcR, however, suggests that it may play a role in as-yet undefined activation requirements of α/β T cells that might define functional distinctions between α/β and γ/δ T cells (Poggi *et al.*, 1987). The CD28 chain is encoded by a single-copy gene (Arrufo and Seed, 1987a) and has a single N-terminal V-like homology unit, a short connecting peptide, a transmembrane region, and a short cytoplasmic sequence. Immediately extracellular (N-terminal) to the transmembrane-spanning sequence is a highly distinctive hingelike region. A CD28 molecule is, consequently, organized very similarly to the CD8 molecule. It will be interesting to see whether the CD28 molecule plays an analogous accessory function.

The CTLA-4 gene was identified by analyzing a cDNA isolated from a subtracted mouse cytotoxic T-cell-derived cDNA library (Brunet *et al.*, 1987). There is a single CTLA-4 gene. The protein has not yet been identified, but the predicted protein sequence clearly suggests it is an IgGSF member. Like the CD28 antigen, the CTLA-4 protein has a rather classic V-like homology unit except for the absence of the V-region-invariant tryptophan (Fig. 3). In fact, when the CD28 and CTLA-4 sequences are compared, it is evident that they are very closely related (data not shown). The connecting sequence is particularly conserved (one stretch of 25 amino acids has 15 exact matches and 4 highly conservative substitutions). Also, all five extracellular cysteine residues are precisely conserved. It is quite possible that the CTLA-4 gene is the mouse homolog of the human CD28 antigen gene. Interestingly, the hingelike sequence in the CD28 chain is precisely deleted from the CTLA-4 chain. If the CD28 and the CTLA-4 genes are homologs, the absence of the hinge region in the CTLA-4 mRNA may reflect the deletion of an exon encoding this region during evolution or alternate RNA splicing.

The human CD7 (gp40) antigen is a highly glycosylated surface antigen of thymocytes and T cells (Palker *et al.*, 1985). It is one of the earliest and most definitive markers of cells belonging to the T cell lineage. Its function is unknown. It appears to be a single, 40-kDa chain. A CD7 cDNA sequence (Aruffo and Seed, 1987b) reveals an organization remarkably similar to that of the CD28 and CTLA-4 cDNAs, but it is not directly related to either by sequence similarity. Like the CD28 and CD8 α chains, the CD7 antigen has a pronounced hinge sequence just N-terminal to the probable transmembrane region. Also, like the CD28 and CTLA-4 chains, the CD7 chain has a classical V-like homology unit without the conserved tryptophan. The CD7 protein also shares an interesting feature with the CD8 β chain, an apparent J-region-like sequence located appropriately C-terminal to the V-like region (Fig. 9). Although the similarity of the CD7 J sequence to Ig or TcR J elements is not as dramatic as that of the CD8 β chain, it nevertheless maintains the core residues of the canonical J motif: a hydrophobic residue, an aromatic residue, a Gly, a small residue, a Gly, a Thr, any residue, and a hydrophobic residue (Fig. 9). In fact, the CD7 J sequence is more similar to that of the CD8 β chain than to any other J element: 7 of 11 residues are identical with one single residue gap and two highly conservative substitutions (Phe → Tyr, Thr → Ser). The CD7 gene, like the CD8 β gene, does not rearrange during differentiation (Aruffo and Seed, 1987b).

It seems that T cells express a whole class of accessory molecules with analogous, if not directly homologous, structures: one N-terminal V-like homology unit, a connecting sequence of approximately 50 residues, a proline-rich, hingelike sequence immediately N-terminal to a transmembrane sequence, and a relatively short cytoplasmic sequence 15–40 residues long, which is unrelated to the cytoplasmic regions of the other sequences of the IgGSF. The CD8 α and β, CD28, CTLA-4, and CD7 chains all belong to this group. Besides the hingelike sequence, each of these chains also has a conserved a cysteine residue just N-terminal to the transmembrane sequence. Cysteine residues associated with the hinge regions of immunoglobulins are involved in interchain disulfide bonds, as they appear to be in the CD8 and CD28 chains. The structural homology of these molecules suggests that they are displayed in a similar manner on the cell surface and probably interact with their ligands in a similar manner. If the CD8 molecule is typical of this group we would predict that the ligands for the CD7, CD28, and CTLA-4 proteins are superfamily members, perhaps even MHC or CD1 molecules.

C. T-Cell- and Nervous-System-Associated Molecules

The Thy-1 molecule is one of the simplest members of the IgGSF, with only a single V-like homology unit (Cohen *et al.*, 1981) attached to the

cell membrane by a glycophospholipid anchor (Tse *et al.*, 1985; Fig. 1). The murine Thy-1 molecule is found in abundance on thymocytes and neurons as well as fibroblasts and a variety of other cells. The function of Thy-1 is unknown. However, it apparently can act as a signal transduction molecule in cellular activation (Kroczek *et al.*, 1986). Although Thy-1 is generally isolated as a monomer, there is evidence that it can form multimeric complexes, probably through homophylic interactions (Xu *et al.*, 1987). This might imply a role for Thy-1 in cell–cell interactions mediated by homophylic interactions of Thy-1 molecules between cells.

The rat Ox-2 cell-surface antigen has a tissue distribution similar to Thy-1 (Barclay, 1981; Webb and Barclay, 1984) and likewise has an unknown function. Its structure resembles that of a TcR α or β chain, with V, C, transmembrane, and cytoplasmic regions (Clark *et al.*, 1985). Like the CD7 chain, the Ox-2 chain has a J-like region between the V and C regions (Fig. 9) (Barclay *et al.*, 1986). Ox-2 is presumed to be a single-chain molecule. However, its striking organizational similarity to other chains found only as dimers suggests that it is best to leave this question open.

The CD4 gene is also expressed in the brain, but the cell type(s) in which it is expressed and its functions are unknown (Tourvieille *et al.*, 1986). It is interesting that the predominant CD4 transcript in mouse brain encodes a protein with the first 200 or so N-terminal residues truncated, including the first two V-like regions (Gorman *et al.*, 1987).

D. Nervous System Molecules

The most intriguing new group of Ig-like sequences may be those expressed predominantly by cells of the nervous system. The first to be described was a neural cell adhesion molecule (N-CAM) isolated from chick embryo brain (Hemperly *et al.*, 1986a). The N-CAM gene encodes five H-type, N-terminal homology units, a long connecting sequence, a transmembrane region, and a very large cytoplasmic domain (Cunningham *et al.*, 1987; Barthels *et al.*, 1987). N-CAM belongs to a limited number of cell adhesions whose binding is involved in cell–cell interactions that coordinate patterns of migration, proliferation, and cellular differentiation during tissue development (see Edelman, 1986). N-CAM is found in several developmentally regulated and tissue-specific forms, apparently the products of alternate splicing pathways that include varying amounts of the cytoplasmic tail and determine whether membrane attachment is mediated via a transmembrane peptide sequence or a glycophospholipid anchor (Hemperly *et al.*, 1986b). These differences, along with modulation of the amount and distribution of

expression, correlate with different patterns of tissue formation. N-CAM binding is homophylic and polyvalent (Edelman, 1983) and maps to the Ig-like regions (Cunningham *et al.*, 1983). It is possible that these properties reflect Ig domainlike association between N-CAM molecules on different cells (Hunkapiller and Hood, 1986) and could have implications for other examples of interactions between other members of the IgGSF, such as the Thy-1 molecule.

Another brain protein, rat myelin-associated glycoprotein (MAG), is most similar in structure and sequence to N-CAM (Fig. 1) (Hunkapiller and Hood, 1986; Arquint *et al.*, 1987; Noronha *et al.*, 1987). MAG has two well-defined and possibly three additional relic H homology units as well as a transmembrane and large cytoplasmic region. It appears that MAG is involved in neuron–glial and glial–glial cell interactions mediating myelination and the maintenance of periaxonal space (Sternberger *et al.*, 1979; Nobile-Orazio *et al.*, 1984), possibly by providing an adhesion system in the manner of N-CAM (Riopelle *et al.*, 1986). MAG is found in low abundance in both central and peripheral nervous system myelin (Quarles, 1984). A protein has recently been described on both T and B cells as having serological and biochemical characteristics similar to those of MAG (Peault *et al.*, 1987). The protein is conserved phylogenetically and is regulated in a developmentally specific manner. The authors suggest that this protein is MAG and that it may be involved in the "homing" process of both immune and nervous system cells, mediated by its cell adhesion properties. It will be interesting to learn whether these putative homing interactions are regulated or facilitated by interactions between MAG and other immune/nervous system coexpressed members of the IgGSF such as the Thy-1 or Ox-2 antigens.

The similarity of N-CAM and MAG suggests there may be an entire family of IgGSF sequences involved in cell-to-cell interaction or adhesion in neuronal morphogenesis and raises the possibility that other CAMs also belong to the IgGSF. However, recent characterization of liver (L)-CAM reveals it to be distinct from the IgGSF (Gallin *et al.*, 1987). It is interesting to note, however, that L-CAM and several related CAM sequences (Ringwald *et al.*, 1987; Nose *et al.*, 1987) share an analogous structural motif with N-CAM and MAG. The apparent adhesive properties of each molecule is mediated by N-terminal repeat units approximately 100 residues in length. The non-IgGSF CAM sequences each have three or four such repeats that are obviously the products of internal duplication, as appears to be the case for N-CAM and MAG. These repeats, as with H-type homology units, are predicted to have secondary structures dominated by six or seven alternating β strands (data

not shown; after Chou and Fasman, 1978). Further analysis might elucidate whether the homophylic association of these CAMs is mediated by β-sheet interactions as would be predicted for N-CAM and MAG.

P_0 is a myelin-associated protein that constitutes over 50% of peripheral nervous system myelin, but is not found at all in the central nervous system. P_0 has a single external V-like homology unit (Fig. 1; Lai *et al.*, 1987) that is interestingly similar to the V-like unit of the Ox-2 antigen, suggesting an independent evolution from N-CAM and MAG. P_0 molecules are thought to facilitate the compaction of peripheral myelin by linking adjacent lamallae of Schwann cell membrane (Braun, 1984). It is suggested that P_0 brings the external membrane surfaces of adjacent lamallae together through homophylic interactions between the extracytoplasmic domains of P_0 molecules expressed on opposing membranes (Lemke and Axel, 1985), presumably through domainlike β-sheet interactions. The cytoplasmic sequence of P_0 is highly positively charged. The intracytoplasmic membrane surfaces could be compacted through electrostatic interactions of the positive charges of the P_0 cytoplasmic tail with the negative charges of the head groups of acidic lipids of the opposing membrane surface.

E. Ig-Binding Molecules

The poly(Ig) receptor (p-IgR) shuttles polymeric IgM and IgA antibodies from the blood side to the serosal side of mucous membranes (Brandtzaeg, 1981; Kühn and Kraehenbuhl, 1981; Mostov *et al.*, 1980). The external portion of the p-IgR is released during the process and is known as the secretory component (SC). The SC molecule has five V homology units (Fig. 1) (Mostov *et al.*, 1984). Comparison of the individual units indicates that they are each more closely related to each other than to other IgGSF members. Hence, they are likely to be the product of a series of internal duplication events that resulted in the expansion of a single unit sequence. It is possible that two or more units of p-IgR form a pseudodimer domain structure that acts as an Ig-like receptor for the Ig heavy chain. Alternatively, individual p-IgR homology units may interact with Ig units through domainlike β-sheet interactions, similar to the interaction of Ig units with themselves. These interactions would presumably be between p-IgR and the C-terminal regions of the heavy chain. Disulfide bonds can be generated between p-IgR and some subclasses of IgA (Brandtzaeg, 1981).

Humoral Ig/antigen complexes are bound by various cell types through receptors to a portion of the C_H region of Ig molecules known as the Fc portion. This process helps mediate cooperation between the humoral and cellular immune pathways (Springer and Unkeless, 1984; Dickler,

1982). The Fc receptors (FcR) for the mouse γ isotype have been characterized (Ravetch *et al.*, 1986). Two similar genes and alternate RNA processing are responsible for at least three different tissue-specific FcRγ. Each has an external region composed mostly of two tandem homology units, a transmembrane region, and different cytoplasmic regions. The two homology units are more similar to each other than to any other Ig-like sequence, and hence are probably the result of an internal duplication. Both domains are H unit sequences. It remains to be seen whether any of the FcR for other Ig isotypes belong to an encompassing FcR multigene family. However, at least one FcR for Ig ϵ does not appear to belong to the IgGSF (Kikutani *et al.*, 1986). The polydomain structure of the FcRγ is similar to that of p-IgR and may indicate analogous functional interactions with their Ig ligands, even though they are clearly not directly evolutionarily related.

F. Growth Factor/Kinase IgGSF Members

The receptor for platelet-derived growth factor (PDGFR) and the *fms* transforming gene have clearly related sequences and share an intracytoplasmic region similarity to *src*-related tyrosine kinases (Yarden *et al.*, 1986). The c-fms protein is thought to be the receptor for macrophage colony stimulating factor (CSF-1R) (Coussens *et al.*, 1986) and hence, both PDGFR and CSF-1R appear to be responsible for promoting cell proliferation and differentiation. Both PDGFR and CSF-1R have three and perhaps five extracytoplasmic H-type homology units (Lai *et al.*, 1987). Thus it seems that an IgGSF receptor has been coupled to a kinase activity, perhaps through an exon shuffling event, and then diverged to generate a family of growth factor receptors. The similarity of the intracytoplasmic region of CSF-1R and that of a partial cDNA of the *v-kit* oncogene (Yarden *et al.*, 1986) suggests that *c-kit* also belongs to this family. Except for antigen receptors and link protein (see below), PDGFR and CSF-1R are unusual as examples of IgGSF receptors with characterized ligand that are not also IgGSF members.

G. Miscellaneous Members

Carcinoembryonic antigen (CEA) is the most commonly used human tumor diagnostic antigen (see Shiveley and Beatty, 1985). Although it is not strictly tumor specific, its expression is greatly increased in colonic tumors and possibly those of lung and breast. CEA represents an entire family of heterogeneous, highly glycosylated proteins, including several serologically related antigens; e.g., nonspecific cross-reacting antigen (NCA), biliary glycoprotein I, and normal fecal antigen (NFA-1). The distribution of expression of members of this family is extensive,

including some within the immune system, but no functions are known. Recent protein and cDNA sequences reveal that CEA is an integral membrane protein with six external Ig homology units (Paxton *et al.*, 1987; Thompson *et al.*, 1987; Zimmerman *et al.*, 1987). These are remarkable in that they represent three tandem repeats of two H-type units each. It appears there are about a dozen CEA-related genes by hybridization. The heterogeneous nature of the proteins suggests that varying numbers of tandem repeat units may also be found in different members. (While CEA has three, it appears that NCA-55 may contain only one repeat and NCA-95 contains two.) Each CEA-related protein also has an N-terminal sequence, with no cysteine pair, about the same length as the homology units. It is possibly an evolutionary relic of a homology unit (Paxton *et al.*, 1987).

The αlB-glycoprotein (αlB) is a prevalent human plasma protein of unknown function (Schultze *et al.*, 1963). Like SC, αlB has five N-terminal homology units (Ishioka *et al.*, 1986). It has been suggested, therefore, that αlB may be the secretory component of the p-IgR for IgG antibodies (Ishioka *et al.*, 1986). Also, like p-IgR, αlB appears to be the product of multiple internal duplication events. Unlike p-IgR, however, αlB is constructed of H homology units, like most of the other polydomain IgGSF members. An interesting feature of the αlB structure, which appears to distinguish it from other polydomain IgGSF members, is the presence of a proline-rich hingelike sequence between each of the homology units. How such structures would affect the quaternary relationship of the individual units is unclear. It is interesting to note that rigid hinge sequences producing similar angles between adjacent units, could generate a spiral or circular arrangement of homology units.

The proteoglycan aggregates of cartilage are composed of proteoglycan monomers associated with hyaluronic acid (HA). Link glycoprotein stabilizes the aggregate structure by binding to both proteoglycan and HA. These interactions result in the formation of an extracellular matrix of cartilage. A prominent species of link protein has been sequenced using material from a rat chondrosarcoma and bovine nasal cartilage (Neame *et al.*, 1986; Bonnet *et al.*, 1986). Surprisingly, the link protein was found to belong to the IgGSF. This may be the only known example of an IgGSF member that may never be deployed in the context of a membrane-bound receptor (as mentioned, αlB may be a secretory component of a membrane-bound molecule). Link protein is essentially divided into two functional regions. The N-terminal 125 amino acids represent a specialized V-like homology unit. It has the repertoire of V-like residues, but is longer between the two cysteines than any other

known member by about eight residues. This length difference can be accounted for by extra residues between the N-terminal conserved cysteine and the conserved tryptophan, the region that corresponds to the HV1 of V regions. The remaining 60% of the sequence is constructed of two internally homologous non-IgGSF regions. It is probably these two repeats that interact with HA, while the V-like region binds the proteoglycan (Neame *et al.*, 1986). The sequences of rat and bovine link proteins are extremely conserved, including the HV1-equivalent loop (one mismatch in 20 residues). This contrasts dramatically with the lower level of conservation seen across species for many of the other IgGSF receptors, such as CD8 and CD4 molecules. When the nature of the interactions between these molecules is better understood, it will be interesting to determine whether the extra loop sequence is involved in generating a specilized binding pocket. Developing reasonable scenarios for the evolutionary relationship of the link protein to other V-like sequences should also prove interesting.

H. Uncertain Members

It should be noted that several other sequences have been proposed as members of the IgGSF on the basis of limited stretches of identity with other members. Several of the polydomain members (i.e., CD4, CEA, PDGFR) have sequences that may be relic homology units. This possibility is supported by statistical analysis as well as their presence in molecules with other well-defined IgGSF domain sequences. Several of these relic regions do not have the conserved cysteine pair. However, there is at least one excellent example of a functional immunoglobulin V region also lacking the disulfide bridge (Rudikoff and Pumphrey, 1986). Thus, it is clear that no single rule of membership in the IgGSF is absolute.

Whether or not the sequences mentioned above are IgGSF homology unit descendants is irrelevant to defining the molecules themselves as members of IgGSF, because each does contain rather obvious homology units. However, there are a number of sequences where the history is a bit more ambiguous. The CD2 molecule is one example. The CD2 (T11) antigen is expressed on $CD4^+$ and/or $CD8^+$ thymocytes and T cells (Reinherz, 1985). Monoclonal antibodies to the CD2 antigen in the presence of phorbol esters can initiate T cell proliferation. The CD2 molecule is involved in antigen-independent adhesion of thymocytes to thymic epithelial cells and adhesion of cytotoxic T cells to target cells. Its ligand in these reactions is the LFA-3 molecule, a rather ubiquitous cell surface marker (Takai *et al.*, 1987). On the basis of limited stretches of similarity to various members of the IgGSF, it has been claimed that

the CD2 molecule is also a member (Sewell *et al.*, 1986). The recent characterization of LFA-3 (Wallner *et al.*, 1987; Seed, 1987) indicates that the CD2 and LFA-3 chains are homologs. It is suggested that the CD2 and LFA-3 molecules are constructed of an N-terminal homology unit without a disulfide bridge immediately followed by a disulfide-defined unit, a short connecting sequence, a transmembrane peptide, and a cytoplasmic region of moderate length. The LFA-3 chain can be membrane linked by a glycophospholipid anchor (Seed, 1987). Although statistical similarity scores between one region of the CD2 chain and various IgGSF members (primarily the CD4 molecule) have values >4 SD (Williams *et al.*, 1987), the sequence does not share the conserved cysteines or many of the other IgGSF conserved residues.

Blast-1 is an activation marker of B cells (Thorley-Lawson *et al.*, 1982) and probably T cells (Staunton and Thorley-Lawson, 1987) that forms a noncovalently linked heterodimer with another, uncharacterized protein (Thorley-Lawson *et al.*, 1986). The Blast-1 molecule has also been proposed as an IgGSF member, with two external units analogous to those of the CD2 chain (one with and one without the disulfide bridge) (Staunton and Thorley-Lawson, 1987). The authors suggest that the Blast-1 sequence is homologous to the α chain of the MHC class II molecule, which also has an N-terminal domain without a disulfide bridge followed by a classical C homology unit. However, the authors indicate homology of the N-terminal region of the Blast-1 chain with those of both the CD4 chain, which is classically V-like, and the MHC class II α chain, sequences that share no apparent similarity themselves. As discussed earlier, if these chains are truly homologous, it is difficult to explain why the CD4 and class II α chains do not appear related. Also, if the crystal structure of the MHC class I molecule proves to be a good model for the structure of the class II molecule, it is apparent that the N-terminal region of the class II α chain does not share any structural homology with Ig domains.

Other molecules that have been proposed as members of the IgGSF include the CD5 molecule (Ly-1) (Huang *et al.*, 1987), a T cell differentiation marker contact site A (cs-A), a cell adhesion molecule from aggregating slime mold (Matsunaga and Mori, 1987), and adenoviral E3 glycoprotein (Chatterjee and Maizel, 1984). We cannot argue that these are not distantly diverged members of the IgGSF. However, none of them meet many of our criteria of membership so we are not convinced it is appropriate to include them as members of the IgGSF. We also believe that the arguments for including the CD2, LFA-3, and Blast-1 molecules are inconclusive as of now.

VI. Evolution of the Immunoglobulin Gene Superfamily

A. Role of the Homology Unit

Given the tremendous diversity within the IgGSF, it might be assumed that selective pressures favoring diversity have driven the evolution of the family. However, we believe that diversity is an inherent feature of the conserved protein and gene structure of the Ig homology unit and that it was the *a priori* diversity itself that shaped the evolution of the complex systems seen today. This diversity is a natural consequence of three basic characteristics of the unit. First, the tertiary structure of the homology unit is such that homology units tend to interact preferentially to form homo- or heterotypic dimers, thereby forming the domains that are the basis of the receptor structures and, at times, receptor/ligand interactions. Combinatorial associations between homology units increase both the evolutionary and somatic diversification potentials. Such interactions may also favor the establishment of new functional associations between existing members of the IgGSF. Second, except for a few conserved residues involved in direct inter- and intrachain interactions, the primary structure of these units can vary dramatically and still result in essentially the same tertiary structure (Lesk and Chothia, 1982). This is easily seen when comparing V gene segments, where even proteins encoded by members of the same gene family may be less than 20% similar. This flexibility is particularly true of the loops connecting the β strands, as seen in the HV regions of Ig and TcR. Such reduced constraints placed by structural requirements on the primary sequence would inherently promote the establishment of variants within populations. Third, in most examples of IgGSF genes for which there is genomic sequence information, the homology units are encoded by discrete exons. This allows, through relatively simple genetic events, the development of new contexts for homology unit expression. For example, most of the known polydomain sequences like N-CAM and FcRγ probably arose independently from internal duplications of unit exons rather than orthologous divergence from a common polydomain precursor. The CEA molecule illustrates that even pairs of exons may duplicate to generate polydomain structures. The number of such independent examples of polydomain sequences indicates the facility of this process. This variation is accommodated by the ½ splicing rule of IgGSF exons, which allows correct splicing between any number of tandem homology unit exons. The ½ splicing rule generally applies as well to the non-Ig-like exons (interestingly, except in the cytoplasmic regions exons) and facilitates the considerable alternative splicing of transcripts seen

throughout the IgGSF. The differential expression of alternately spliced products such as secreted versus membrane Ig and the developmentally expressed variants of N-CAM indicate that alternate RNA splicing provides a further level of functional diversity to the IgGSF. In fact, nearly all members examined to date generate alternate splicing products. Also, the kinase region of PDGFR and CSF-lR and the probable HA binding regions of link protein illustrate the construction of new gene products through the shuffling of exons encoding functionally discrete domains.

B. The Role of Diversity in the History of the IgGSF

While it is likely that many additional members of the IgGSF will be found, we believe general scenarios for the evolution of the IgGSF can be proposed from the current data. We believe that the original homology unit-based molecule was a surface-bound ligand involved in cellular adhesion through binding of like homology units, perhaps nothing more complicated than a cellular glue and probably structurally resembling the Thy-l (Williams, 1982) or CD3γ chains. The compact structural nature of the homology unit may have contributed to its stability in the extracellular environment. The self-adhesion character of N-CAM and perhaps P_0 lends support for a scenario of such homophylic interactions. [It has been suggested that the bisymmetry of the homology unit is an argument for an ancestral "half-domain" structure (Bourgois, 1975). This half-domain would presumably have to form a dimer to function similarly to a current homology unit. Alternatively, its function (or functional capacity) could have been totally unrelated to that of the full domain.] The earliest gene was presumably under selective pressure primarily to maintain the primordial antibody fold and those residues responsible for interchain interactions. Reduced constraint on the primary sequences would promote the establishment of many functionally equivalent alleles. Nonselective emergence of such markers could have been an important preadaptation for several reasons. Differential expression of multiple variants in an individual could result in tissue-specific markers, potentially allowing more complex interactions in morphogenesis to develop. A possible demonstration of a homology unit in a squid brain protein (Williams, 1982) suggests that the IgGSF has probably existed from at least the time of the earliest metazoans and may have an ancient role in morphogenesis. The morphogenic roles of N-CAM, MAG, and P_0 may also suggest similar roles for other differentially expressed IgGSF members such as the CDl, Tla, and CEA sequences.

Allelic variants will also represent genotypic markers, and once there is a marker for self, there is conversely an assay for nonself. Recognizing

nonself is important to any organism, such as many invertebrates, that competes with its own kind for space or substrate. Histoincompatibility reactions have been recorded in most multicellular phyla, particularly colonial types (see Hildemann *et al.*, 1980), raising the possibility that MHC-like genes were integral to the development of complex metazoa. Histocompatibility mechanisms could presumably also respond to any "altered-self" markers arising from the interaction of self-histocompatibility molecules and another macromolecular structure. This implies that the immune response is an outgrowth of histocompatibility defenses and could explain the present linkage of MHC and antigen recognition. It has been proposed that the inherent polymorphism was initially incorporated into systems of gametic exclusion to inhibit cross-fertilization of like genotypes and was subsequently harnessed for immune surveillance (Monroy and Rosati, 1979; Burnet, 1971). Interestingly, tunicates have a highly polymorphic gene system responsible for both histocompatibility and gametic exclusion (Scofield *et al.*, 1982). Others suggest that tissue-specific expression of alleles could act as targets for keying developmentally regulated tissue necrosis, a process critical to the development of highly complex metazoa (Williams *et al.*, 1987). Other than the relative order of occurrence, however, none of these scenarios is exclusive of the others; fundamental to each is the *a priori* existence of established polymorphism.

For early members of the IgGSF, tandem duplication of homology units may have been favored initially due to an increase in valency of interaction (i.e., N-CAM). However, subsequent specialization of duplicated regions would also become possible analogous to the divergence of entire duplicated pseudoalleles (Lewis, 1954). A fundamental specialization along these lines appears to have been the establishment of receptor and effector/structural motifs and the subsequent V/C dichotomy. Of course, V and C elements may have become associated through an exon shuffling event long after the divergence of the original elements. Classical V and C homology units have been defined through crystallographic studies of Ig molecules and there has been a tendency to define newly identified members of the IgGSF as either V- or C-like. However, many of the recently described members may not belong to either the V or C group, but rather to a third class of homology unit, H. Though most often described a V-like by primary sequence, the domains of the N-CAM, MAG, PDGFR, CSF-1R FcRγ, α1B, CEA, and CD3 molecules have intercysteine distances even shorter than those of C domains, with the concomitant loss of the extra β-strand/loop structure of V regions as well as possibly another β strand that forms an edge to one of the two β sheets (Fig. 3). The β-strand lengths of the sandwich

faces may tend to be shorter than those of C regions, however, and more nearly like those of V regions. Though more V-like, these sequences also have a distinct collection of conserved residues (Fig. 3). It will be interesting to do a thorough study of the distribution of hydrophobic residues across the faces of the putative β sheets to see if these sequences have a V- or C-like pattern. Such analysis may suggest which face mediates interchain interactions. Given that these sequences tend to share both V and C features, it is possible that they represent more nearly the prototypical homology units and may indicate a more ancient role for the IgGSF in morphogenesis.

C. The Significance of DNA Rearrangement in the Evolution of the IgGSF

The development of regulated rearrangement of V gene segments may have occurred through the opportune insertion and subsequent developmental capture of a transposable element at the 3' portion of a V-like exon (Sakano *et al.*, 1979). With only the two elements, however, this process could have contributed little to receptor diversity. It seems more probable that the initial selective significance of rearrangement was as a mechanism to provide for allelically exclusive regulation of V gene expression. Immune receptor sequences are nearly unique in vertebrates for allelic exclusion. This phenomenon is crucial to the establishment of clonally expressed receptors and, hence, the fine specificity and memory of a complex immune system. Rearrangement provides a mechanism whereby only one V gene is brought near enough to transcriptional promoters in the J–C intron or made available for proper RNA splicing to the C gene. It is also possible that the junctional flexibility of joining may have played initially a more important role in this regulation than in the generation of diversity by further attenuating the possibility of more than one functional rearrangement in one cell (Hunkapiller and Hood, 1989). Once established, allelic exclusion provided an opportunity to express a more specific repertoire of antigen receptors, increasing selective forces on diversity and diversifying mechanisms. Recent characterization of the shark heavy chain locus may support this model (Kobuku *et al.*, 1987). The shark locus heavy chain contains many tandemly repeated V-J repeats, which appear to rearrange only within the linked pairs. This rearrangement preference is essentially the same as for the V_λ loci of mammals. This organization eliminates any combinatorial diversification and reduces the potential for junctional diversity. It is possible, in fact, that it was the acquisition of DNA rearrangement that ultimately drove the divergence of the V and C homology units by allowing the expansion and specialization of the V region repertoire. Furthermore, the development of V-to-J

rearrangement fortuitously established the components that allowed for the evolution of D segments, allowing even further somatic diversification (Hunkapiller and Hood, 1989).

Gene segment rearrangement signals and switch regions also play frequent roles in major recombination events not only within families, but also between families and unrelated sequences on other chromosomes. It has been noted that transposable sequences promote duplication of neighboring sequences and that the rearrangement signal sequences might likewise promote duplicative expansion of V gene segments (Siu *et al.*, 1984). The highly recombinogenic switch sequences of the C_H genes may also lead to expansion of the C gene family as well as the production of novel, hybrid C genes (Hisajima *et al.*, 1983). Translocations involving rearrangement or switch sequences are frequently found in transformed T and B cells (Croce *et al.*, 1984; Tsujimoto *et al.*, 1985). It is unclear whether such events are mediated by the specific enzymes of normal rearrangement or are merely facilitated by the recombinogenic nature of the sequences. The J-like sequences of the CD8 β, CD7, and Ox-2 chains are interesting in this regard (Fig. 9). Unlike most of the single-gene members, the CD8 β (and CD8 α) chain seems to have directly diverged from the Ig light chain V families (Hunkapiller and Hood, 1986). On the other hand, neither the Ox-2 V or C sequence is particularly similar to any of the rearranging gene families. The J-like region of both the CD8 β and Ox-2 genes is encoded within the same exon as the V-like sequence. No germ line information for the CD7 gene is available. Such J-related sequences might be representative of V-like genes that existed before the development of rearrangement, with modern J gene segments the evolutionary descendants of the C-terminal portion of these primordial V genes (Johnson and Williams, 1986). The uniqueness of the Ox-2 (and probably CD7) gene may argue in favor of placing it in this category. The J-like sequence of the CD8 β gene, on the other hand, is nearly identical to some λ J segments, implying a much more recent divergence. Therefore, this J sequence may represent the product of VJ rearrangements in germ cells (Hood *et al.*, 1985). If so, the sequences and perhaps even enzymes involved in somatic variation may occasionally promote rearrangements in germ cells and thus contribute to evolutionary diversification. We feel that this scenario reflects the history of the CD8 β gene and raises interesting questions regarding the role of such events in the evolution of the IgGSF.

D. Implications of the IgGSF

The physical nature and organization of particular genetic information establish the limits and possibilities of its variation. What is striking about the IgGSF is the hierarchical nature of its organization and,

hence, the hierarchical potential of its evolution. The history of the IgGSF reflects both the fundamental diversifying and combinatorial properties of the Ig homology unit. Also, the multigene organization and recombinogenic nature of much of the family establishes particular diversifying potentialities on many of the members. New functional possibilities arise primarily through the duplication of the various informational units involved, e.g., nucleotides, exons, genes, and entire multigene families. Duplication of a multigene family and the attendant cis-acting control mechanisms can in a single event create the genetic basis of a complex new phenotype, suggesting the possibility of rapid evolutionary change. Duplications within the IgGSF not only generate the potential for direct evolutionary change, but also are the seeds of their own subsequent expansion and diversification by the fact that multiple copies of sequences promote further duplication and realignment of gene families. Somatic rearrangement-promoting sequences can apparently also promote such events in the germ line as well. The tendency of even divergent homology units to interact expands even further the potential for rapidly developing new functional relationships between new and existing members. This picture leads to an image of rapid, even saltational evolutionary acquisition of complex new phenotypes that share the regulatory machinery of established systems.

Until recently, members of the IgGSF had been primarily associated with immune recognition or cells of the nervous system. In these systems, complex cell interactions require a surface recognition structure with a high informational potential. The Ig homology unit provides the basic architecture upon which multiple sequence substitutions can be rapidly imposed, thus providing a diverse repertoire of recognition and target elements. The adhesins N-CAM and MAG suggest that other developmentally regulated and cell-specific adhesins may also be dependent on the informational capacity of the homology unit. Interestingly, the number of the identified single-gene IgGSF members that are expressed in both the brain and the immune system suggests the possibility of shared cell surface recognition functions and that related molecules may be involved in some of the intriguing phenomena linking mental states and immune response. It seems likely that the fruitful strategies of somatic diversification employed by well-characterized members of the IgGSF will also be used by other receptor families, perhaps involved in morphogenesis or neuronal development. At present, there is no evidence for this possibility, although candidate systems (e.g., olfaction) have been suggested (Hood *et al.*, 1985). It is thus remarkable to view the entire IgGSF in the context of its evolutionary origins: from a single gene encoding roughly 90 amino acids has arisen a superfamily of

elements with extraordinarily complex interactions both within and outside of the family structure.

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ADVANCES IN IMMUNOLOGY, VOL. 44

Genetically Engineered Antibody Molecules

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I. Introduction

The development of the hybridoma antibody technique over a decade ago by Köhler and Milstein revolutionized the discipline of immunochemistry and provided a new family of reagents with potential applications in clinical diagnosis and immunotherapy (Köhler and Milstein, 1975). The availability of monoclonally derived hybridoma antibodies provided an infinite supply of homogeneous reagents so that laboratories around the world could use standardized procedures. However, some limitations persisted. While it has become routine to produce rat and mouse monoclonal antibodies with antigen-binding specificities appropriate for use in basic research and clinical diagnosis, it has been difficult or impossible to produce human hybridoma antibodies for *in vivo* immunotherapeutic applications. Moreover, even with rodent antibodies it is frequently not possible to produce monoclonal antibodies with precisely the desired specificity and affinity. Another limitation resulting from the monoclonal origin of hybridoma antibodies is that they are monoclonal with respect to their constant region isotype and the antibody may not have the appropriate constant region for the desired effector functions.

One approach to producing hybridoma antibodies with more desirable biological effector functions or antigen-binding specificities has been to take advantage of the high rate of somatic mutation which occurs in cultured myeloma and hybridoma cell lines. Using either the fluorescence-activated cell sorter (Liesegang *et al.*, 1978; Dangl and Herzenberg, 1982) or ELISA screening in a "sib-selection" procedure (Müller and Rajewsky, 1983; Spira *et al.*, 1984), heavy chain isotype switch variants of mouse hybridoma cell lines have been identified and isolated. The limitation inherent in this approach is that it is possible only to switch to isotypes which lie downstream of the expressed heavy chain gene isotype in the heavy chain gene complex. In addition it has

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been nearly impossible to isolate isotype switch variants of human hybridoma cell lines where IgM[1] antibody production is predominant. Similar selection approaches have been used to isolate somatic variants of mouse hybridoma cell lines producing antibodies with altered reactivity with antigen; some of these variants exhibit a new antigen binding specificity (Diamond and Scharff, 1984).

An alternative approach to producing improved monoclonal antibodies is to use recombinant DNA techniques and eukaryotic gene expression methods, the objective being to produce antibodies with improved antigen specificities and effector functions. An advantage of this approach is that we are not limited to producing antibodies as they exist in nature. Instead we can design and produce antibody molecules with optimized specificities and effector functions. Additionally we can introduce into the antibody molecule novel functions not normally found there. The ability to create these kinds of reagents promises to continue the revolution in immunology begun by the introduction of hybridoma antibody molecules.

II. Antibody Production in Bacteria and Yeast

Attempts to generate intact functional antibodies by expression in bacteria until recently met with limited success (see note added in proof). Efforts were made to produce antibody binding the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) (Boss *et al.*, 1984). The hapten-specific λ light chain lacking the hydrophobic leader sequence was expressed in *Escherichia coli*; however, it ended up as insoluble material which accumulated in inclusion bodies. In addition to the full-length light chain, a number of smaller, distinct proteins reactive with anti-λ antibodies were detected. These fragments could have resulted from proteolytic degradation, from premature termination of transcription, or from internal initiation of translation. Similarly, the NP-specific μ heavy chain expressed in bacteria was insoluble. When both heavy and light chains were expressed in the same bacterium, no antigen-binding activity

[1]Abbreviations: ars, *p*-azophenyl arsonate; C_H, constant region of the heavy chain; CDR, complementarity-determining regions; DNS, dansyl; *E. coli*, *Escherichia coli*; *gpt*, guanine phosphoribosyltransferase gene; H, heavy chain; HAT, hypoxanthine/aminopterin/thymidine; HGPT, hypoxanthine guanine phosphoribosyltransferase; Hu, human; Ig, immunoglobulin; IMP, inosinic acid; kDa, kilodaltons; L, light chain; *neo*, the *E. coli* aminoglycoside phosphotransferase gene from Tn5; NIP, 4-hydroxy-5-iodo-3-nitrophenyl acetyl; NP, 4-hydroxy-3-nitrophenyl acetyl; PC, phosphocholine; TNP, trinitrophenyl; XGPRT, 5-phospho-α-ribose-1-diphosphate: xanthine phosphoribosyltransferase; XMP, xanthylic acid; V_H, variable region of the heavy chain.

was detected. Mild denaturation and treatment with reagents to promote disulfide interchange resulted in antigen-binding activity; however, this *in vitro* reassembly was very inefficient, with the specific activity of the reconstituted antibody only 1.4×10^4 g/g of IgM equivalents. Despite this, antibody produced in bacteria and reassembled *in vitro* was heteroclitic like the original mouse hybridoma antibody and bound 4-hydroxy-5-iodo-3-nitrophenyl acetyl (NIP) better than NP. Similarly, heavy and light chains and the Fd fragment from an antibody with specificity for carcinoembryonic antigen were expressed in *E. coli*. Again low levels of antigen-binding activity could be reconstituted from the insoluble products by treatment with denaturing agents and reagents to promote disulfide interchange (Cabilly *et al.*, 1984).

When a cDNA encoding the κ light (L) chain from the mouse myeloma MOPC321 was expressed into *E. coli*, light chains were found both in the bacterial cytoplasm and periplasmic space. In cells synthesizing the entire L chain, starting with the signal peptide, the periplasm contained a mature-sized light chain, indicating that the signal peptide initiated secretion and was processed appropriately (Zemel-Dreasen and Zamir, 1984). In this study there was no clear indication of what fraction of the κ light chains was soluble.

Since one potential problem of immunoglobulin production in bacteria may be the formation of incorrect disulfide bonds, attempts have been made to express isolated variable regions which have only one possible disulfide pair (A. Black, unpublished). The V_L domain from an antidextran hybridoma cell line was expressed in *E. coli* both with and without its hydrophobic leader sequence. Unlike the κ light chain of MOPC321, the polypeptides expressed with a leader sequence did not have it processed. Both the V_L polypeptides containing and those lacking the leader sequence were insoluble, although they could be solubilized. It remains to be demonstrated if the V_L will renature and assemble with its homologous heavy chain and whether antigen binding can be recovered. Expression of the V_H domain from the same antidextran hybridoma could not be detected in bacteria. Segments of V_L and V_H expressed as tryp-E fusion proteins were effective immunogens for producing antivariable region antibodies.

Bacterial expression of human IgE fragments has been reported. In the initial experiments IgE "Fc-like" fragments were produced which contained the $C_\epsilon 1$-4, $C_\epsilon 2$-4, or $C_\epsilon 3$-4 domains (Kurokawa *et al.*, 1983). These polypeptides reacted with anti-IgE antisera and were of the expected molecular weight. The amount of protein synthesized appeared low (0.5–32 μg/liter), possibly because only the proteins present in the soluble fraction were measured. No functional studies were done on these peptides.

Subsequent studies have demonstrated that these IgE fragments synthesized in *E. coli* exhibit the biologic properties characteristic of intact IgE molecules. The expressed fragments which were analyzed contained either the entire $C_{\epsilon}2$, $C_{\epsilon}3$, and $C_{\epsilon}4$ domains of the ϵ heavy chain or about two-thirds of $C_{\epsilon}2$ and the entire $C_{\epsilon}3$ and $C_{\epsilon}4$ domains (Kenten *et al.*, 1984; Liu *et al.*, 1984). When expressed in *E. coli*, the ϵ fragment constituted up to 18% of the total bacterial protein. Both polypeptides were insoluble and associated with large inclusion bodies in the bacterial cell. However, the IgE fragments could be solubilized with denaturing solvents and isolated by affinity chromatography using anti-ϵ antibodies. Following reduction and reoxidation greater than 80% of the chains formed dimers. The ability of these dimers to inhibit an *in vivo* Prasnitz-Küstner reaction compared favorably with that of an intact IgE myeloma protein, both in terms of blocking concentrations required and duration of the blocking effect (Geha *et al.*, 1985). The dimeric but not the monomeric IgE fragment inhibited the *in vitro* sensitization of human lung mast cells by intact IgE (Coleman *et al.*, 1985). IgE fragments and intact IgE molecules also had identical affinities for the IgE Fc receptors on cultured human basophils (Ishizaka *et al.*, 1986). A dose-response curve of histamine release showed that the recombinant product is equivalent to intact IgE in transducing the signal for degranulation. Since proteins synthesized in *E. coli* do not contain carbohydrate, it is clear that the carbohydrate present on the ϵ heavy chain is not required for its binding to the high affinity Fc ϵ receptor. Both the recombinant ϵ fragment and IgE myeloma proteins are denatured and lose affinity for the Fc ϵ receptor following heating at 56°C. The recombinant protein could be renatured by passage through a solution of 6 *M* guanidine hydrochloride, while the intact IgE protein could not be renatured. The renatured ϵ dimers exhibited affinity for FcϵR and their original circular dichroism (CD) spectra.

With successful expression of functional IgE, deletional mutagenesis techniques were used to map the Fc ϵ mast cell-binding site on human IgE to a stretch of 76 amino acids spanning the C_H2 and C_H3 domains (Gould *et al.*, 1987). All peptides containing the amino acid sequence Gln 301 → Arg 376 inhibit the Prasnitz-Küstner reaction and contain the target cell triggering activity characteristic of cross-linked native IgE (Helm *et al.*, 1988).

In most cases cDNAs have been used directly for expression in bacteria. However, a novel approach used retroviruses to convert genomic unprocessed genes into processed genes (Takeda *et al.*, 1985). This approach can also be used to produce chimeric immunoglobulin chains in which the variable region was derived from one species and the constant region from another.

Generally bacterial expression of antibody molecules has met with limited success. The bacterial expression of IgE Fc-like dimers remains an exception. It is clear that the *E. coli* biosynthetic environment does not support protein folding that requires specific disulfide bond formation, glycosylation, and polymeric polypeptide chain assembly. In each situation where all of these are required for protein function, the proteins synthesized did not exhibit any biological activity. The IgE Fc dimers did not require any of these activities. The homodimers could be renatured from the insoluble polypeptides synthesized in *E. coli*, complex chain assembly was not required, and the dimers were active without glycosylation.

There has been one published account of an attempt to obviate some of the problems of gene expression in *E. coli*, by expressing immunoglobulin genes in yeast. Yeast fermentation technology makes this expression system very attractive and yeast are capable of asparagine-linked glycosylation, but glycosylation is limited to the addition of high-mannose carbohydrates. Efficient expression of both the λ and μ chains from an NP-binding myeloma was achieved in yeast (Wood *et al.*, 1985). The μ heavy chain was glycosylated, albeit with a high-mannose carbohydrate. Depending on the culture condition, 5-15% of the μ chain and 10-40% of the λ chain were secreted into the culture medium. However, antibodies isolated from the yeast cell extract had a specific activity only of 0.5% and no antigen binding could be observed in the secreted antibodies. Thus, addition of high-mannose carbohydrate is not sufficient for assembly of functional immunoglobulin molecules and the high-mannose sugars added to multiple sites on the μ heavy chain may have disrupted the heavy chain structure; however, this was not determined. The decision to express a μ heavy chain was perhaps unfortunate since μ chains have more than one glycosylation site and are assembled normally into a complex pentameric structure $(H_2L_2)_5$. A systematic study to understand the problems of expressing a heteropolymeric protein in yeast has not been done.

III. Gene Transfection of Lymphoid Mammalian Cells

Gene transfection into eukaryotic recipients provides an alternative to transformation and gene expression in bacteria (Morrison, 1985). The most appropriate mammalian cells for immunoglobulin gene expression should be myeloma and hybridoma cell lines, which are known to be capable of high-level expression of endogenous heavy and light chain genes and can glycosylate, assemble, and secrete functional antibody molecules. Therefore the problems which appear to be insurmountable in bacterial expression would be circumvented by expression in myeloma

cells. However, since we have not identified most of the parameters controlling the expression of transfected genes, many formidable problems remain in effectively expressing introduced immunoglobulin genes. When all of the important parameters controlling the expression of transfected genes are understood, it should be possible to achieve high levels of Ig expression by transfection.

Even under optimal conditions gene transfection into myeloma cells is an inefficient process, with only between 1×10^{-4} to 1×10^{-5} recipient cells becoming stably transfected. Therefore, vectors must be used which contain biochemically selectible markers which permit the selection of the rare, stably transfected cell line. The most commonly used expression vectors have been the pSV2 vectors (Mulligan and Berg, 1980; 1981; Southern and Berg, 1982). These vectors contain a eukaryotic transcription unit which directs the expression of a biochemically selectible marker. This transcription unit consists of a Simian virus 40 (SV40) early promoter, splice signal, and poly(A) addition signal. Two selectible markers are frequently used: the bacterial xanthine–guanine phosphoribosyltransferase gene (*gpt*) or the aminoglycoside phosphotransferase gene from Tn5 (*neo*). Both enzymes, when expressed, endow a dominant biochemically selectible phenotype to mammalian cells so that they can be used for selection in non-drug-marked recipient cells.

The bacterial enzyme XGPRT encoded by the *gpt* gene differs from the analogous mammalian enzyme, hypoxanthine guanine phosphoribosyltransferase (HGPT), in that it can efficiently use xanthine as a substrate in nucleotide synthesis (Mulligan and Berg, 1980, 1981). When *de novo* nucleotide synthesis is blocked by mycophenolic acid, only cells expressing XGPRT can survive when provided with xanthine. The expression of the phosphotransferase gene (*neo*) confers resistance to the antibiotic G418, which interferes with the function of the mammalian 80 S ribosomes and thereby blocks protein synthesis (Davies and Jiminez, 1980; Davies and Smith, 1978). Since each enzyme involves a distinct selection mechanism, when necessary the enzymes can be used simultaneously.

IV. Antibody Expression Vectors

Several methods have been developed to introduce DNA into mammalian cells. $CaPO_4$-precipitated DNA is commonly used to transfect genes into nonlymphoid cells; however, this method of transfecting DNA works poorly in myeloma and hybridoma cell lines (Oi *et al.*, 1983). Stable transfectants can be isolated, but at frequencies of less than 1×10^{-6}. Both DNA electroporation (Potter *et al.*, 1984) and bacterial protoplast fusion (Sandri-Goldin *et al.*, 1981; Oi and Morrison, 1986)

are efficient methods for introducing DNA into lymphoid cells. Although transfection frequencies vary considerably from cell line to cell line, a number of investigators have reported that both electroporation and protoplast fusion can generate stably transfected lymphoid cell lines at frequencies as high as 1×10^{-3} cells (Potter *et al.*, 1984; Morrison *et al.*, 1988; Gillies *et al.*, 1983). Transfection frequencies between 1×10^{-4} and 1×10^{-5} cells are more reasonable expectations.

Several myeloma cell lines are available for gene transfection and expression. J558L, a heavy chain loss variant of the IgA, λ, anti-α ($1 \rightarrow 6$) dextran myeloma J558, is perhaps the easiest of all myelomas to transfect, and stable transfection frequencies of greater than 10^{-3} can be achieved (Gillies *et al.*, 1983). Unfortunately, the persistent synthesis of the irrelevant λ light chain in J558L makes it unsuitable for the expression of novel antibody molecules. All efforts to date to isolate a nonproducing variant of J558L have been unsuccessful. Hence, the cell lines routinely used to produce transfected antibody-producing cell lines are the nonproducing hybridoma cell line parents, SP2/0 and P3X63.Ag8.6.5.3. They have decreased but acceptable gene transfection frequencies.

In order to create novel immunoglobulin molecules, the two genes, encoding heavy and light chains, must be transfected and both heavy and light chain polypeptides must be synthesized and assembled. Several methods have been used to achieve this objective. Both the heavy and the light chain genes have been inserted into a single vector and then transfected (Ochi *et al.*, 1983b); this approach generates large, cumbersome expression vectors and further genetic manipulation of the vector is difficult. A second approach is to transfect sequentially the heavy and light chain genes (Morrison *et al.*, 1984). To facilitate this, one gene is inserted into a vector containing the *neo* gene, permitting selection of transfected cells expressing G418 resistance. The other gene is placed in an expression vector containing the *gpt* gene which confers mycophenolic acid resistance to transfected cells. It is possible then to transfect with one vector, isolate stable transfectants, and then transfect these selecting for expression of the second marker. The double transfectants synthesize both heavy and light chains. Sequential selection of transfectants is time consuming.

We have used another strategy to transfect both heavy and light chain genes into lymphoid cell lines. We developed a novel pair of expression vectors (Oi and Morrison, 1986) which are used to introduce both genes simultaneously into lymphoid cells using protoplast fusion methods. The first of the pair is the pSV2 vectors, containing either the *gpt* or *neo* gene for mammalian cell biochemical selection; the second of the pair contains either of the same selectable genes, but these genes are inserted

into another *E. coli* plasmid, pACYC184, containing a different plasmid origin of replication. The pBR322-derived pSV2 vectors encode bacterial ampicillin resistance; the pACYC184-derived pSV184 vectors encode chloramphenicol resistance. Both plasmids can be maintained at high copy number in the same bacterium because they have compatible replication origins. The phenotype of bacteria containing both plasmids is ampicillin and chloramphenicol resistance. Protoplasts made from these bacteria fused to mammalian cells introduce the *gpt* gene on one vector and the *neo* gene on the other, thereby permitting selection for both vectors in the recipient mammalian cells using G418 and mycophenolic acid. Figure 1 schematically depicts two such vectors. We customarily put immunoglobulin light chain genes into pSV184 *neo* vectors, and heavy chain genes into pSV2 *gpt* vectors.

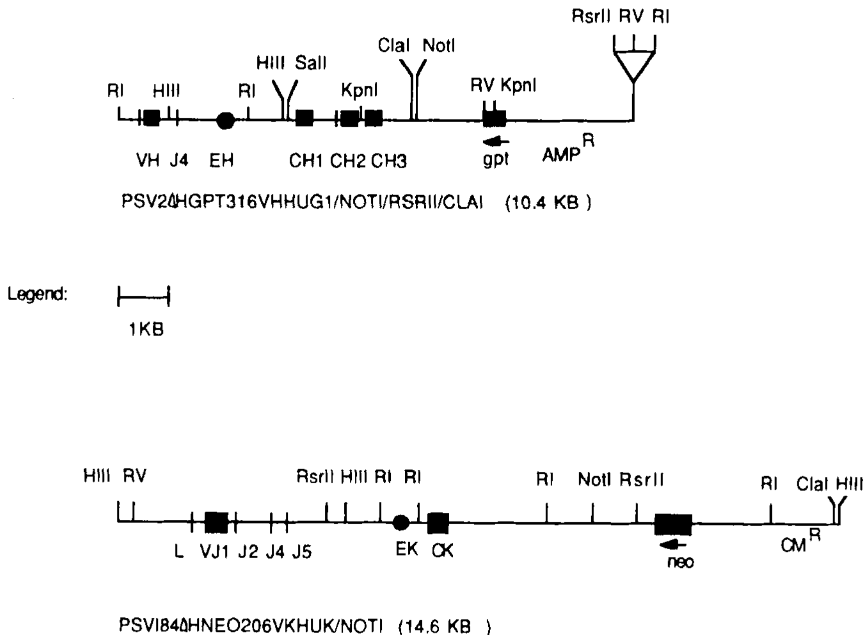

FIG. 1. Schematic representation of a heavy (HuG1) and light (HuK) chain expression vector. The heavy chain is contained in the pSV2 vector with a pBR322 origin of replication, a β-lactamase gene (*Amp*^{R}), and with the *gpt* gene encoding the marker selectible in eukaryotic cells. The light chain is in a pSV184 vector with a pACYC184 origin of replication, with a chloramphenicol resistance gene (*Cm*^{R}), and with the *neo* gene providing the selectible markers for eukaryotic cells. The restriction sites are as indicated: HIII, *Hin*dIII; RV, *Eco*RV; and RI, *Eco*RI.

V. Cloning and Manipulating Antibody Genes

The variable regions desired for expression can be obtained from the appropriate hybridoma antibody-producing cell line as either cDNA or genomic clones. Variable regions must be rearranged to be expressed, so the expressed V regions are juxtaposed next to a J region while the unexpressed V regions are not. Therefore J region probes can be used to identify restriction fragments containing the expressed variable region exons and to screen the recombinant library constructed in λ phage. Thus one does not need to determine the amino acid sequence of a rearranged V region before cloning. Unfortunately, hybridoma cell lines often contain aberrantly rearranged variable region gene segments, some of which are transcribed; in these cases further characterization of the expressed variable region is required so that the correct gene can be identified. An advantage in using genomic clones is that the variable region is obtained as a complete transcription unit, with its own promoter and splice junctions.

Genomic clones of immunoglobulin variable and constant region genes are easy to manipulate using genetic engineering techniques because of their division into exons. The distinct immunoglobulin polypeptide domains are each encoded by a discrete exon. Conveniently, all splice junctions, except that between the two IgD membrane exons (Tucker *et al.*, 1981), show the same splicing pattern; the codon at the splice junction consists of one nucleotide from the donor splice site and two nucleotides from the acceptor splice site. Therefore, it is easy to manipulate Ig structure by deleting, exchanging, or altering the order of exons and have the resulting mRNA encode a complete polypeptide. Immunoglobulins with deletions, duplication, and internal insertions of other genes have been produced and characterized (Morrison *et al.*, 1988) and will be discussed later.

We have exploited the exon structure of the immunoglobulin genes to design and construct antibody cassette expression vectors. Oligonucleotide linkers were used to flank the constant region exons with unique restriction sites (Oi and Morrison, 1986). The available vectors contain the constant regions of both mouse and human light and heavy chains (Oi and Morrison, 1986). In addition, we have constructed a rabbit γ constant region cassette vector as well. These vectors were constructed so that once a particular variable region is cloned in an expression vector, it can easily be placed next to any constant region gene, thus facilitating isotype switching. A family of switch vectors is illustrated in Fig. 2. In more recent gene constructs, linkers have been placed between the domains of the human IgG heavy genes, making exon and domain shuffling of these straightforward (Morrison *et al.*, 1988).

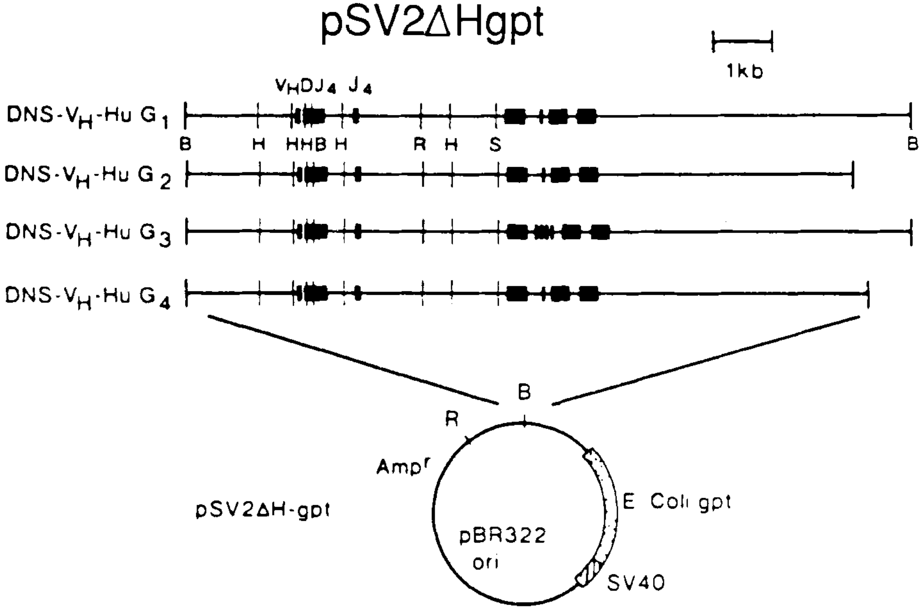

FIG. 2. A family of vectors in which the same variable region derived from a hybridoma specific for the hapten dansyl is expressed with the four different human γ chain constant regions. The heavy chain constant regions are all contained on *Sal*I-*Bam*HI fragments. Therefore, once the variable region is expressed with one constant region it can be expressed with any of the others merely by substituting the *Sal*I-*Bam*HI fragment in the expression vector. The restriction endonuclease sites are shown: B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; and S, *Sal*I.

Two approaches have been used to construct vectors to express immunoglobulin cDNA clones. One approach substituted the variable region from a heavy chain cDNA clone for the corresponding variable region in a genomic clone (Morrison *et al.*, 1988). This substitution was possible because the cDNA and the genomic clones shared restriction sites at amino acid four and in the J3 region. In the engineered clone, the leader sequence and the first four amino acids of the variable region were from the genomic clone; however, they were identical to those present in the cDNA. The engineered variable region was part of an active transcription unit with the immunoglobulin promoter, splice signals, and DNA regulatory sequences derived from the genomic clone.

In the second approach to expressing immunoglobulin molecules using cDNA derived gene sequences, a heterologous transcription unit was created. An entire cDNA gene was generated by joining a cDNA variable

region with a cDNA constant region (Liu *et al.*, 1987a,b) using restriction enzyme recognition sites created in the cDNA sequences at the V/C junction. Appropriate restriction endonuclease cleavage sites were also created on the 5′ side of the ATG codon by *in vitro* mutagenesis using oligonucleotides. The immunoglobulin genes were inserted into a transcription unit containing the promoter, splice donor/acceptor, and transcription termination/polyadenylation signals of SV40 and the immunoglobulin heavy chain enhancer element. This approach was used to create chimeric immunoglobulin genes in which the variable regions of heavy and light chains from mouse hybridoma cell lines were joined to human γ_1 and κ constant region genes (Liu *et al.*, 1987a,b).

In a third approach, instead of using the entire cDNA variable region sequence or the entire variable region genomic exon, the variable region gene segment is sequenced first and the complementarity-determining regions (CDRs) are synthesized and inserted into framework residues. Mosaic variable regions are produced with the framework residues from one source, the CDRs from another (Jones *et al.*, 1986; V. T. Oi, unpublished). Both mouse and human heavy and light chain variable region mosaic gene cassette vectors have been generated (V. T. Oi, unpublished). These vectors are currently being used to examine how extensively the framework residues of the variable region domains affect antigen binding and represent the first generation of vectors with which the antibody-combining site will be dissected.

Obtaining the desired expressed variable regions from the relevant hybridoma cell lines using cDNA cloning methods has obvious advantages. Since specific oligonucleotides can be used as primers for cDNA synthesis, cDNA libraries enriched for the variable region of interest can be produced. It is usually easier to produce a cDNA of a desired variable region than it is to isolate the variable region from a genomic library. However, transcripts from aberrantly rearranged variable regions present problems as they do in identifying rearranged variable regions from genomic clones. The disadvantage in using cDNAs is that they are more difficult to manipulate genetically than the corresponding genomic sequences. Special attention must be paid so that any amino acid changes introduced as a consequence of cloning or any subsequent manipulations do not alter the functions of the resulting proteins.

VI. Expression of Antibody Molecules

The first immunoglobulin genes expressed by transfected myeloma and hybridoma cell lines were light chain genes from mouse myeloma cells (Rice and Baltimore, 1982; Oi *et al.*, 1983; Ochi *et al.*, 1983a).

In all cases the proteins produced were found to be a faithful representation of the gene sequence that was used for transfection. When the appropriate light chain gene was transfected into a light chain loss variant of an antigen-binding myeloma, the expressed transfected light chain restored the secretion of antigen-specific antibody molecules (Ochi *et al.*, 1983a,b). Transfection of the appropriate heavy chain gene also restored antigen-binding antibody production in heavy chain loss variant cell lines. In each case, the transfected heavy chain was shown to be glycosylated. Heavy and light chain genes, when transfected together, generated cell lines producing complete, glycosylated, assembled tetrameric antigen-binding antibody molecules with appropriate interchain disulfide bonds.

A persisting problem has been the expression level of the transfected immunoglobulin gene. The expression of transfected heavy chain genes is frequently seen to approach the level seen in myeloma cell lines; however, efficient expression of transfected light chain genes is more difficult to achieve. This difference in expression level between heavy and light chains is seen both when genomic genes with their normal controlling elements are used, and when the genes are placed in viral transcription units (Liu *et al.*, 1987c). Thus the different expression levels cannot be directly attributed to differences in the strength of either the promoter or the enhancer elements.

At this time, cell lines synthesizing large amounts of the transfected genes are usually selected by brute force. Transfection procedures are so efficient that a sufficient number of independent transfectants can be screened to ensure recovery of a "good producer" cell line.

Gene amplification has been used to increase the level of immunoglobulin expression. Amplification has been achieved directly (Dorai and Moore, 1987) and by sequential transfection (Liu *et al.*, 1987a–c). To study the effect of gene amplification on expression levels, mouse/human chimeric heavy and light chain genes were cloned adjacent to the gene for dihydrofolate reductase, which provides resistance to methotrexate (Dorai and Moore, 1987). Stable transfects were selected in methotrexate and the integrated immunoglobulin genes amplified by selection of transfectants in increasing concentrations of methotrexate. Amplification of the heavy chain gene lead to little increase in heavy chain production. Light chain gene amplification increased light chain expression in some, but not all, transfects; however, even after amplification, the level of light chain produced remained less than the amount of heavy chain.

It remains a puzzle as to why heavy and light chain genes are expressed to different levels. In normal and malignant B lymphocytes and plasma cells, the endogenous heavy and light chains are expressed to similar

levels; in fact, there is frequently production of excess L chain which appears to facilitate Ig assembly (Morrison and Scharff, 1979). Thus there appears to be no inherent difference in the transcriptional capability of the two gene families. It is possible that the recipient cells used for gene transfection are deficient in a factor necessary to fully activate light chain expression. The requirements, both cis and trans, for immunoglobulin expression have not yet been fully defined. Cis-acting enhancer elements have been demonstrated to be required to activate the transcription of a gene introduced by transfection (Gillies *et al.*, 1983; Queen and Baltimore, 1983). However, variant cell lines have been characterized in which the heavy chain enhancer element has been deleted but in which the synthesis of the heavy chain remains unchanged (Eckhardt and Birshtein, 1985; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985). Thus, the enhancer element appears to be required to activate gene expression, but may be dispensable once transcription is occurring. In the myeloma S107, which is deficient in the trans-acting regulatory factor NFkB (Atchison and Perry, 1987), the high-level expression of the endogenous light chain remains unchanged but a light chain gene introduced by gene transfection is not expressed. Thus NFkB appears to be necessary to initiate light chain gene expression but does not appear necessary to maintain it. It is possible that additional as-yet-unidentified factors may be important for achieving high-level expression of transfected genes.

A major goal for developing immunoglobulin gene transfection and expression systems was to be able to engineer novel hybrid, chimeric, and mosaic genes using recombinant DNA techniques, and to transfect and express these genetically engineered genes in mouse myeloma and hybridoma cells. Once the feasibility of combining these methods was established and reduced to practice, this new technology would have a significant impact on our understanding of the structure and function of antibody molecules in two distinct areas: (1) understanding the structures and mechanisms involved in the biological effector functions of antibody molecules; and (2) determining the structural basis of antigen-binding and antibody idiotypes. In addition to establishing a fundamental understanding of antibody functions this technology can be used to produce modified hybridoma antibodies that will be more suitable for *in vivo* immunotherapeutic applications. Generation of antibody variants using site-directed mutagenesis techniques will enable us to refine our understanding of antibody structure and function. Additionally, research and diagnostic applications and *in vivo* therapeutic applications based on antibody-fusion proteins should expand the potential application of antibody-related molecules.

In order to study the antibody structures involved in mediating biological effector functions, hybrid antibodies have been produced where the V_H gene segment from an antibody-producing myeloma or hybridoma cell line is cloned and juxtaposed, using recombinant DNA techniques, to a heavy chain constant region gene different from that found in the original antibody-producing cell line. The engineered gene that joins intraspecies gene segments is defined as a hybrid gene encoding a hybrid protein. Hybrid antibodies are particularly useful when it is difficult to produce directly antigen-specific antibodies of the appropriate isotype and have been used to study the biological effector functions and structural dynamics of a variety of mouse immunoglobulin isotypes. A phosphocholine (PC)-specific mouse IgE antibody (Gritzmacher and Liu, 1987) was constructed and expressed with a myeloma PC antigen-binding site originally associated with an IgA heavy chain isotype. This hybrid mouse IgE has the expected reaginic biological properties of naturally occurring IgE molecules and is being used to dissect the relationship between IgE structure and function. Mouse, human, and rabbit IgG antidansyl (DNS) antibodies, sharing the same V_H gene segment joined to each of these heavy chain genes, and the same mouse κ light chain were produced in order to extend a previous study demonstrating a correlation between the segmental motion of different antibody isotype Fab arms and certain biological effector functions [see Section VII and Dangl *et al.* (1988)].

In most cases direct manipulation of the antibody genes is used to produce hybrid proteins. However, a novel approach used recombination in *E. coli* to generate hybrid gene sequences. Mouse γ_1 and γ_{2a} gene segments were introduced together on separate plasmids into *E. coli*, where recombination occurred between the plasmids.

Fifteen hybrid genes that were either γ_1:γ_{2a} or γ_{2a}:γ_1 were generated, i.e., they were γ_1 in the 5′ part of the gene and γ_{2a} in the 3′ portion and vice versa. The exact recombination cross-over site of each hybrid gene was determined by DNA sequencing. Each of these 15 hybrid genes was expressed with the same anti-DNS V_H and κ light chain producing 15 hybrid anti-DNS antibodies. These molecules were used to identify the differences in *Staphylococcus aureus* protein A binding sites between mouse IgG_1 and IgG_{2a} as being due to amino acid differences (EU numbering) at positions 252 (Thr → Met) and 254 (Thr → Ser) of the heavy chain (Schneider *et al.*, 1987). Analysis of these molecules identified the C_H1:C_L domain as a structural feature which, in addition to the antibody hinge, determines the range of motion of the antibody molecule's Fab arms [see Section VII and Schneider *et al.* (1987)].

The nature of V_H and V_L domain interactions were examined by constructing an unusual antibody light chain dimer. A V_H gene segment

cloned from an anti-*p*-azophenyl arsonate (ars)-specific myeloma cell was joined to the mouse κ constant region gene. When this gene construct was transfected into a heavy chain loss variant of the original anti-ars myeloma cell line, a new anti-κ reactive polypeptide of 25 kDa was synthesized and assembled with the endogenous κ light chain, forming a κ light chain heterodimer which bound the ars hapten (Sharon *et al.*, 1984). Complete characterization of the recreated hapten-binding site was not done, but it is clear that noncovalent interactions were strong enough to ensure formation of a light chain dimer pairing heterologous "light chain" polypeptides.

Gene transfection has been used coordinately with the study of somatic cell mutants to identify regions within mouse IgM important for its biologic properties. A mutant IgM was identified which lacked amino acids 550-562 and did not form pentamers (Shulman *et al.*, 1982). Site-directed mutagenesis was used to construct a μ gene lacking the nucleotides encoding these amino acids; cells expressing this altered μ gene produced monomeric IgM, verifying that this deletion is sufficient to account for the deficiency in polymerization observed in the original mutant protein (Baker *et al.*, 1986). A similar approach of identifying somatic mutants with changes in their constant region and then using site-directed mutagenesis and transfection to produce molecules with only a single change in their constant region has shown that the third constant region domain of IgM is critical for complement activation (Shulman *et al.*, 1986, 1987).

VII. Chimeric Antibody Molecules

Chimeric human/mouse antibodies, in which the variable region is derived from a mouse myeloma or hybridoma and the constant region is from a human gene, are potentially useful for many applications. They should avoid some of the deleterious immune response which occurs when murine antibodies are used for *in vivo* imaging or immunotherapy. Chimeric antibody technology also makes accessible the vast antigen-binding repertoire which is now available from the many mouse hybridoma cell lines and which has not been duplicated in human hybridoma antibodies.

The first mouse/human chimeric antibodies created consisted of variable regions derived from mouse myeloma cell lines producing antibodies specific for haptenic groups (Morrison *et al.*, 1984; Boulianne *et al.*, 1984; Neuberger *et al.*, 1985). When the variable regions from the antiphosphocholine (PC) myeloma S107 were joined to the human γ_1 heavy chain and κ light chain constant regions, the resulting chimeric proteins assembled into appropriately glycosylated H_2L_2

molecules which bound the PC hapten, reacted with *S. aureus* protein A, and expressed idiotopes present on the original S107 myeloma protein (Morrison *et al.*, 1984). Comparably, a hybridoma antitrinitophenyl (TNP) specificity was joined to human μ heavy chain and κ light chain; the resulting chimeric protein bound TNP, assembled into pentamers, and was capable of activating the complement cascade (Boulianne *et al.*, 1984). Similarly, a chimeric IgE anti-NP antibody, consisting of a human ϵ heavy chain expressed with a mouse λ light chain, bound NP and the Fc ϵ receptor on human basophils. Cross-linking the Fc ϵ-bound chimeric IgE antibodies triggered degranulation and histamine release (Neuberger *et al.*, 1985).

Subsequently a family of chimeric proteins has been produced in which the same variable region from an NP-specific hybridoma protein was joined to different human constant regions (Bruggemann *et al.*, 1987). These were expressed in the mouse plasmacytoma J558L, which synthesized the NP-specific λ mouse light chain. As expected, the human IgM, IgE, and IgA antibodies were glycosylated extensively relative to the chimeric IgG_1, IgG_2, IgG_3, and IgG_4 molecules. Each immunoglobulin isotype except IgA was assembled and secreted as expected. The IgA antibody-producing cell line secreted a complex mixture of H, HL, H_2L_2, and $(H_2L_2)_2$ forms. The IgA was of the A2m(1) allotype and so would be expected to noncovalently assemble H and L chains. IgG_1, IgG_2, and IgG_4 molecules bound to protein A as expected; no protein A binding was detected with IgM, IgE, IgA_2, or IgG_3 molecules. IgM, IgG_1, and IgG_3 but not the IgG_2, IgG_4, or IgE antibodies bound Clq. IgG_3 bound more Clq than IgG_1; however, IgG_1 was more effective in activating the complement cascade than was IgG_3. IgG_1 was also more effective than IgG_3, the only other effective isotype in mediating antibody-dependent cell-mediated cytotoxicity. Similar results characterizing the biological effector functions of antigen-specific human antibodies were obtained when a family of human IgG antidansyl chimeric antibodies were analyzed (Dangl *et al.*, 1988; and unpublished results).

Using a combination of isotype switch variants and genetically engineered antibodies we have made a complete study of the relationship between the segmental flexibility of the Fab arms and of the antibody molecule biological effector functions, in particular complement fixation. Mouse, rabbit, and human hybrid and chimeric antibodies possessing identical antigen-binding sites were studied. In our initial study isotype switch variants of mouse anti-DNS hybridoma were identified and cloned. A correlation was found between the segmental flexibility of the Fab arms of mouse IgG_1, IgG_{2a}, IgG_{2b}, and IgE anti-DNS

antibodies and their ability to activate the complement cascade (Oi *et al.*, 1984). This study was extended to include other immunoglobulin isotypes by joining the same anti-DNS variable region to other mouse isotypes (Dangl *et al.*, 1988). The correlation between Fab segmental flexibility and the capacity to activate complement was corroborated. Moreover, it was found that Fab segmental flexibility correlated with the length of the hinge from the end of the C_H1 to the first cysteine forming an interheavy chain disulfide bridge (Dangl *et al.*, 1988). These results point to a structural basis for two critical properties of antibody molecules, Fab motion and complement activation. Surprisingly, human IgG_2, which does not activate human or guinea pig complement, was able to activate rabbit complement. Relatedly, mouse IgG_3, which activates both rabbit and human complement, did not activate guinea pig complement. Perhaps these incongruities will prove to be informative in understanding the molecular mechanism of antigen- and antibody-mediated complement activation.

Another study compared the capacity of mouse and chimeric mouse/human IgM which shared specificities for the hapten TNP to bind Cl and to trigger complement-dependent cytolysis (Boulianne *et al.*, 1987). Surprisingly human Cl was bound more efficiently by mouse IgM than by chimeric IgM, whereas rat and rabbit Cl bound to human chimeric IgM more efficiently. It is noteworthy that the human chimeric IgM binds to human Cl less well than mouse IgM, indicating that homologous effector systems are not necessarily the most effective. The difference in Cl binding is reflected in the ability of the mouse and human IgMs to activate complement from different species. In addition, despite all indications that the TNP-binding sites are identical in the mouse and human chimeric IgM, the agglutination titer of the human chimeric IgM on TNP-SRBC targets is 17-fold higher than with the mouse IgM antibody. The reason for this difference is unclear.

It has also been possible to use gene transfection to express a chimeric gene with a human VDJ_H gene segment joined to mouse $C\kappa$ (Tan *et al.*, 1985), as well as a completely human heavy chain gene (L. K. Tan, unpublished) and a human κ light chain gene (Potter *et al.*, 1984). Thus both human heavy and light chain promoters are functional in mouse cells. Therefore it is feasible to use gene transfection to produce totally human antibody molecules; it may be possible to use this approach to rescue low-producing human hybridoma cell lines.

For the genetically engineered chimeric antibodies to be of maximum utility, they must be assembled and secreted and must retain their ability to specifically bind antigen. To investigate the requirements for assembly and secretion of antibody molecules, a series of molecules with altered

heavy chains was produced. It was found that a chimeric mouse/human IgG_3 gene in which C_H1, hinge, and C_H2 are duplicated was expressed; it assembled with a mouse λ light chain and was secreted (Morrison *et al.*, 1988). Thus immunoglobulins with heavy chains of increased size can be produced. In a related series of experiments, mouse/human IgG_3 heavy chain genes encoding proteins with deletions of C_H2, hinge + C_H2, C_H1 + hinge, and C_H1 + hinge + C_H2 were constructed and transfected into myeloma cells. The gene with the deletion of C_H2 directed the synthesis of shortened heavy chains, which assembled into H_2L_2 molecules that were secreted. When the hinge and C_H2 were deleted, the resulting heavy chain apparently assembled into HL half molecules which were secreted. However when C_H1 + hinge were deleted no assembly of the heavy chain occurred, probably because of the absence of the cysteine in C_H1 which forms the interchain disulfide bond. Similarly, when the variable region was jointed directly to C_H3, no assembly took place. Even in the absence of interchain disulfide bonds both shortened heavy chains were secreted.

VIII. Biological Properties of Genetically Engineered Antibodies

With the availability of the transfectoma technology, the study of structure-function relationships within the antibody molecule can now be approached systematically. In particular it is now possible to manipulate and study human immunoglobulins. To facilitate our study of human IgG constant regions, we have inserted unique sites for cleavage by restriction endonucleases between the exons encoding the constant region domains. These constructs can be used to shuffle and delete domains. A pair of such vectors is illustrated in Fig. 3. It can be seen

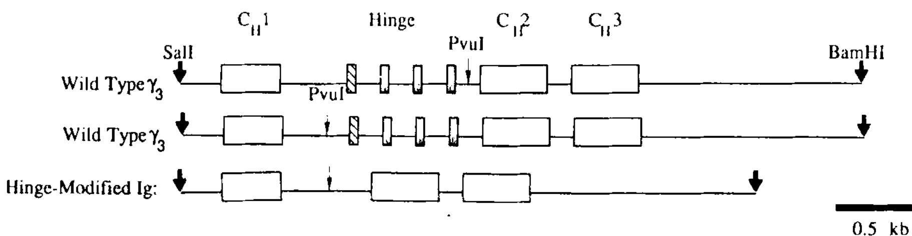

FIG. 3. Cloning vectors with unique restriction endonuclease cleavage sites inserted between domains. *Pvu*I cleavage sites were inserted either before the first hinge exon or after the four hinge exons of IgG_3 using oligonucleotide linkers. Cleavage of these two vectors with *Pvu*I and ligation of C_H1 from one vector with C_H2–3 of the other vector readily produces a hinge deleted IgG_3 gene.

how these two vectors facilitate the deletion of the hinge region from the IgG_3 constant region in this example.

Currently we are investigating the extent to which the hinge region of the IgG molecule modulates its effector function. It has been suggested by several groups including ourselves that hinge length (i.e., the number of amino acid residues in the hinge region) determines the degree of Fab segmental flexibility and affects Clq and Fc receptor binding by IgG molecules (Oi *et al.*, 1984; Klein *et al.*, 1981; Beale and Feinstein, 1976). The four human IgG subclasses share extensive sequence identity in the three constant region domains; the hinge is the one region of the antibody molecule that shows considerable variation in length and amino acid composition. IgG_3, a very flexible molecule which fixes complement very well, has an extended hinge encoded by four exons, the last three of which are identical. On the other hand, IgG_4 is a rigid molecule, does not fix complement, and has a short hinge encoded by a single exon.

To address the question of the role played by the hinge in determining different biologic effector functions, we constructed, expressed, and characterized a panel of IgG_3 molecules with different hinge lengths. Our results demonstrate that presence of at least one of the hinge segments is essential for complement activation, consistent with the idea that interheavy chain disulfide bonds are critical for maintaining complement-binding activity. It is noteworthy that increasing the length of the γ_3 hinge beyond four hinge segments actually reduces complement activation efficiency. In fact, a molecule with a single repeated hinge exon can activate complement better than wild type IgG_3. On the other hand, substituting the γ_3 hinge with the shorter γ_4 hinge reduced the ability of the modified IgG_3 to activate complement; providing γ_4 with the hinge of γ_3 did not endow it with the ability to activate complement. Clearly, the Fc portion of the molecule must play a significant role in determining the biological properties of antibody molecules.

Another property of antibody molecules critical for their *in vivo* function is the ability to bind to cellular Fc receptors. Using a novel assay (S. Canfield, unpublished), the affinity of chimeric human IgG for the high-affinity Fc receptor has been determined. Chimeric IgG_1 and IgG_3 bind with the highest affinity, approximately $1 \times 10^{-9}\ M^{-1}$; chimeric IgG_4 binds with approximately 10-fold reduced affinity and binding by chimeric IgG_2 was not detectable. Experiments are in progress to construct and analyze a series of exon-shuffled IgG molecules. In this way we will be able to determine precisely which exons interact with the Fc receptor. Ultimately, using site-directed mutagenesis, it should be possible to pinpoint the constant region sequence required for the reaction with the Fc receptor binding site.

IX. Genetically Engineered Antigen-Combining Sites

In the course of sequence studies of antidextran antibodies with different binding affinities for antigen, it was observed that high binding affinity correlated with the presence of the amino acid sequence that is the recognition site for the attachment of asparagine-linked carbohydrate in the second complementarity-determining region of the heavy chain (Akolkar *et al.*, 1987). Since the antibodies were IgMs and IgAs, both of which contain an asparagine-linked carbohydrate in the C_H1 domain, it was difficult to prove that there was an additional carbohydrate moiety present in the variable region of the Fab fragment. To circumvent this problem, the variable regions from these antidextran antibodies were transferred to human γ constant regions, which do not contain any carbohydrate in the C_H1 domain (Wallick *et al.*, 1988), and the chimeric gene was transfected and expressed; presence of carbohydrate in the Fab of these molecules demonstrated that the glycosylation site indeed was utilized. Analysis of the binding constants of antibodies produced in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation, showed that the carbohydrate was required for the higher binding constant. This observation is extraordinary as it demonstrates that carbohydrate groups in the variable regions of an antibody molecule, in addition to its primary amino acid sequence, can contribute to the antigen-binding affinity.

Sequence analysis of murine antibodies specific for the hapten ars revealed an invariant serine at the junction of the variable V_H and diversity (D) gene segments of the heavy chains. Oligonucleotide-directed mutagenesis of the V_H domain was used to convert this invariant serine to threonine or alanine (Sharon *et al.*, 1986). Analysis of the antibody synthesized following transfection of the mutated heavy chain genes into a myeloma producing the specific light chain showed that both variant proteins maintain the same idiotopes as do wild-type molecules, as determined by reactivity with a panel of antiidiotypic antibodies. It is intriguing that the serine-to-threonine variant retained full antigen-binding activity, while no antigen-binding activity could be detected with the serine-to-alanine mutant.

A potential limitation in the *in vivo* use of chimeric mouse/human antibodies is the possible immune response to the murine variable regions. One way to minimize this response would be to make the mouse variable regions more human. The variable region contains framework residues which determine the position of the complementarity-determining residues which form the antigen-binding site. The three-dimensional structure of the frameworks are similar when different antibodies are compared. This general structure suggests that it should be feasible to

transfer the CDRs from one variable region to another. In fact, when the CDRs from the V_H domain of a mouse monoclonal antibody specific for the NP hapten were placed in the framework of the human myeloma protein NEWM, and expressed with the NP-specific mouse light chain, an antibody is produced that retains antigen binding, albeit with reduced affinity (Jones *et al.*, 1986). The "humanized" antibody also failed to express many of the antigenic determinants recognized by a polyclonal rabbit antiidiotypic antiserum reactive with the original mouse myeloma antibody. It remains to be determined if this approach is generally applicable and if it is possible to substitute both light chain and heavy chain framework residues.

X. Antibody Fusion Proteins

Novel proteins that possess the binding specificity of antibody molecules can be created by replacing the constant region DNA sequence of an immunoglobulin molecule with a sequence derived from another molecule, such as an enzyme or toxin. Such molecules have potential use in immunoassays, in imaging, and in immunotherapy. Antibody fusion proteins also have been suggested as a means to purify proteins that are difficult to manufacture.

Several examples now exist in which enzymes have been used to replace the constant regions of immunoglobulin molecules. In one case, the nuclease from *S. aureus* (SNase) was joined in the C_H2 domain of a mouse γ_{2b} gene heavy chain containing a variable region specific for the hapten NP (Neuberger *et al.*, 1984). When this gene was transfected into a mouse myeloma synthesizing the NP-specific light chain, a fusion protein was synthesized which assembled with the light chain to produce an antigen-binding molecule that was secreted. The fusion protein was heterogeneous in size and appeared to assemble into both $F(ab')_2$-SNase and Fab-SNase. The fusion protein had nuclease activity similar to authentic *S. aureus* nuclease, requiring Ca^{2+} ions but not Mg^{2+} ions; however the catalytic activity of the fusion protein was about 10% of that of authentic *S. aureus* nuclease. Another antibody fusion protein consisted of the Klenow fragment of *E. coli* DNA polymerase I and the same NP-specific mouse heavy chain polypeptide (Williams and Neuberger, 1986). In this case the heavy chain also was heterogeneous in molecular weight and the Fab-polymerase fusion protein had reduced catalytic activity (i.e., about 1.1×10^3 units/mg compared to a value of 7×10^3 units/mg for the native enzyme).

Antibody fusion with proteins also can be used to obtain increased amounts of proteins that are usually available only in limited quantities.

Expression of an NP-specific heavy chain gene in which most of the C_H2 domains and the C_H3 domain were replaced by the third exon of the *c-myc* gene in a cell line producing the NP-specific light chain resulted in the production of a Fab-myc protein, which retained both its ability to bind NP and its reactivity with a monoclonal anti-*c-myc* gene antibody (Neuberger *et al.*, 1984). Again the fusion protein was heterogeneous in size, possibly as a result of proteolysis.

In another study antibody specificity and a new effector function were combined to produce an antibody-targeted pharmacological reagent. Covalent attachment of tissue-type plasminogen activator (t-PA) to a fibrin-specific monoclonal antibody results in an agent more potent than t-PA alone; however, current cross-linking chemistries are inefficient and irreproducible and result in poor yields and a heterogeneous assortment of cross-linked antibodies and tPA molecules. To produce joined molecules homogeneous in structure, the variable region from the heavy chain of an antifibrin hybridoma antibody was joined to a mouse γ_{2b} constant region that had most of the constant region exons replaced by the DNA sequences encoding the catalytic β chain of the t-PA molecule (Schnee *et al.*, 1987). The fusion heavy chain gene was transfected into a heavy chain loss variant of the original mouse antifibrin hybridoma. The transfectants synthesized a 65-kDa heavy chain t-PA fusion protein that was secreted associated with the specific light chains as a 170-kDa H_2L_2 molecule. The polymeric antibody fusion protein possessed 70% of the peptidolytic of the native t-PA. Surprisingly, the native antifibrin antibody binds fibrin monomer 100 times better than the fusion protein. Success in producing biologically active fusion proteins probably requires that the joined molecules can fold independently to reconstitute active domains.

In a slightly different approach, the exons which encode the main body of the constant regions of the α, β, or γ polypeptides of the T cell receptor were inserted into the intronic regions between the variable regions and the κ constant regions exons (Traunecker *et al.*, 1986). When the fused genes were transcribed, they were spliced so that an exon of the T cell receptor was inserted into the κ light chain. The fusion proteins were purified by passage of secretions from transfectoma cell lines expressing the fused gene through a monoclonal rat antimouse κ antibody affinity column and were used to immunize rabbits. The resulting antisera precipitated the T cell receptor from a T cell hybridoma cell line. Thus the constant region of the T cell receptor β chain assumes at least a partially native conformation, even though placed between two light chain domains. Immunoglobulin light chain may be useful generally as a carrier to express other protein sequences which are available in limited quantities and difficult to isolate.

XI. Therapeutic Chimeric Antibodies

As noted earlier, chimeric mouse/human antibodies consisting of mouse variable regions from a myeloma or hybridoma cell line and human constant regions should be useful for many therapeutic applications because of their reduced immunogenicity when compared to totally mouse antibodies. Chimeric antibodies can be made using any of the binding specificities currently available in mouse hybridoma cell lines.

Among the mouse hybridoma antibodies extensively used in therapy is the 17-1A antibody (Herlyn *et al.*, 1979), which reacts with cells of human gastrointestinal malignancies. It has been used successfully in radioimmune imaging studies in man (Moldofsky *et al.*, 1983) as well as in phase I and phase II therapy trials in patients with colon and pancreatic cancer (Sears *et al.*, 1986; Lobuglio *et al.*, 1986). As with all mouse antibodies, however, its effective clinical use is limited by the antimouse immunoglobulin immune response that is elicited when it is administered to humans.

To circumvent this limitation, a chimeric human antibody was constructed in which the V_H from the mouse protein was joined to the human γ_3 heavy chain and the V_L was joined to human C_κ (Sun *et al.*, 1986). The resulting protein was a functional antibody molecule which exhibited the same antigen specificity as the original hybridoma antibody and had the same apparent molecular weight as normal human IgG_3 (Sun *et al.*, 1987). Studies have shown that both native and chimeric 17-1A antibodies have identical reactivities with colon cancer cell lines, adenocarcinoma cells lines, and other nonadenocarcinoma cell lines (Shaw *et al.*, 1987). Both native and chimeric 17-1A antibodies exhibit antibody-dependent cell-mediated cytotoxicity for SW1116 tumor cells when used with human lymphoid effector cells. This was expected because human IgG_3 and mouse IgG_{2a} antibodies, the isotype of the original mouse hybridoma antibody, compete equally for binding to Fc receptors on the activated human monocytic cell line U937 (Lubeck *et al.*, 1985). The human IgG_3 chimeric antibody did not mediate complement-dependent lysis of target tumor cell lines, even though human IgG_3 is efficient in activating the complement cascade.

A human IgG/(κ) chimeric antibody was produced using the variable regions from the murine hybridoma cell line B6.2, which reacts with a cell surface antigen found in human breast, lung, and colon carcinomas (Sahagan *et al.*, 1986). This chimeric antibody retained the same reactivity pattern to human carcinoma cells as the original mouse hybridoma antibody (Brown *et al.*, 1987). When isotopically labeled chimeric and murine B6.2 antibodies were injected into mice bearing human tumors, they showed a similar biodistribution with essentially

identical kinetics of localization in tumors and clearance from other tissues, such as liver and blood. In addition, the chimeric antibody could be utilized for the *in vivo* imaging of tumors in athymic mice.

Likewise, a human/mouse IgG/(κ) chimeric antibody was constructed with specificity for the human common acute lymphocytic leukemia antigen (CALLA), a tumor-associated antigen, using the variable regions from the mouse hybridoma cell line NL-1 (Nishimura *et al.*, 1987). The antigen-binding reactivity of the chimeric antibody was comparable to that of the original mouse hybridoma antibody. In this case the chimeric antibody exhibited complement-dependent cytotoxicity and was more efficient in mediating antibody-dependent cell-mediated cytotoxicity than the parental murine antibody when human peripheral blood mononuclear cells were used as effector cells. The amount of chimeric antibody produced by the transfectants (10–30 μg/ml) was comparable to that produced by the murine hybridoma cell line.

An advantage provided by human/mouse chimeric antibodies should be their ability to interact more effectively with the human immune system than can a totally mouse antibody. Despite some data indicating that human antibodies may not always be most efficacious when used in conjunction with human effector molecules (see above), recent results suggest that this is in fact the case. A human IgG/(κ) chimeric antibody was constructed from the mouse hybridoma L6, which reacts with a cell surface antigen found on many carcinoma cells (Liu *et al.*, 1987b). The chimeric antibody and the mouse L6 antibody bound to carcinoma cells with equal avidity and both mediated complement-dependent cytolysis. However, when human effector cells were used to mediate antibody-dependent cell-mediated cytotoxicity, the chimeric antibody was effective at concentrations 100 times lower than the mouse antibody. In addition, the chimeric antibody, but not the mouse antibody, was effective in complement-dependent cytolysis of a melanoma line expressing low amounts of the antigen.

Another study also demonstrated that human chimeric antibodies are more effective than mouse antibodies in interacting with human effector cells (Liu *et al.*, 1987c). The mouse IgG_{2b} hybridoma antibody 2H7, reactive with the CD20 cell-surface phosphoprotein expressed in normal and malignant B cells, does not mediate antibody-dependent cell-mediated cytotoxicity with human effector lymphocytes or complement-dependent cytotoxicity with human serum. The human chimeric IgG_1/(κ) antibody had the same binding reactivity to the CD20 antigen and was very effective in mediating antibody-dependent cell-mediated cytotoxicity with human effector cells and complement-dependent cytotoxicity with human complement.

XII. Expression of Surface Immunoglobulin

Although gene transfection has been most frequently used to produce and analyze secreted immunoglobulin, it can also be used to study the production and function of surface Ig. On B lymphocytes, antibody serves an important function as the antigen-specific cellular receptor for activation and antigen presentation. Cross-linking of membrane IgG_{2b}, expressed in a lymphoma cell line by DNA-mediated transfer of the γ_{2b} H chain, leads to an increase in intracellular Ca^{2+} ions; thus transfected heavy chain genes can be used to study the structural requirements for activation by receptor cross-linking (Mizuguchi *et al.*, 1986). Antigen-specific B cell lines produced by transferring both the heavy and light chain genes from an anti-TNP-specific myeloma function to present specific antigens via Ia molecules (Watanabe *et al.*, 1986) and can be activated by antigen-specific cross-linking of surface receptors. Gene transfection has been used to identify sequences important in determining whether the transcripts are processed to the membrane or the secreted form (Kobrin *et al.*, 1986; Danner and Leder, 1985).

XIII. Conclusions and Perspectives

Standard techniques of genetic engineering and gene transfection make it feasible to produce antigen-binding molecules with widely varying structures. Antigen-binding specificities can be joined to both immunoglobulin and nonimmunoglobulin sequences to provide antibody and antibody-like molecules for use in diagnosis and immunotherapy.

Perhaps most importantly, genetically engineered antibody molecules can be used to systematically investigate structure–function relationships within the antibody molecule. The challenge is to define which structure is optimal for a desired function. We will then be in a position to use the existing technology to produce modified molecules with improved functional properties; ultimately it should be possible to produce totally synthetic antibody-like proteins with optimal binding specificities and effector functions.

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NOTE ADDED IN PROOF: Recently, the bacterial expression of fully functional L6 Fab and McPC603 Fv fragments was described [Belter, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988). *Science* **240**, 1041; Skerra, A., and Pluckthun, A. (1988). *Science* **240**, 1038]. The production of functional recombinant McPC603 Fv fragment was achieved by fusion of the V_L and V_H coding sequences to prokaryotic signal sequences which directed the secretion of the eukaryotic proteins into the periplasm of the bacteria (Skerra and Pluckthun, 1988). The induction of the genes arranged in a dicistronic operon led to the secretion of the soluble, assembled Fv fragments. A similar approach was used in the production of recombinant L6 Fab fragments (Belter *et al.*, 1988).

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Antinuclear Antibodies: Diagnostic Markers for Autoimmune Diseases and Probes for Cell Biology

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I. Introduction

In the 6 years since the last review of this subject in these volumes (1), there has been a considerable accumulation of knowledge concerning the molecular identity of autoantigens and further consolidation of the use of autoantibodies as diagnostic markers in clinical medicine. Important historical landmarks which have led to our present state of knowledge include the pioneering work of Hargraves *et al.* (2) and Kunkel and his colleagues (3–5). Hargraves described, with careful technological precision, the phenomenon of the LE cell and equated this phenomenon with clinical systemic lupus erythematosus (SLE). Kunkel and his colleagues elucidated the immunochemical basis of this phenomenon, showing that it was related to the activity of circulating antibodies and that the target antigens included both DNA and deoxyribonucleoprotein. These early studies pointed to the directions for subsequent investigations and there has been continuing identification of other circulating autoantibodies, characterization of their respective cellular antigens, and demonstration of the relationship between autoantibodies and clinical syndromes.

The initial observations were followed by a series of studies identifying several autoantibodies which supplied key reagents for elucidation of the molecular nature of autoantigens. Knowledge of the structure of the antigens led to studies of function and this has provided insights into important cellular processes. The Sm antigen–antibody system was characterized in patients with SLE (6) and the nuclear ribonucleoprotein (nRNP) antigen–antibody system was characterized in patients with mixed connective tissue disease (MCTD) and SLE (7–9). The antibodies identified in these studies have been used extensively by investigators in molecular and cell biology as powerful probes for understanding precursor mRNA splicing (10–12). Autoantibodies designated SS-A or Ro and SS-B or La were identified in patients with Sjögren's syndrome (SS) and SLE (13, 14) and have been shown to target intracellular proteins which may be involved with regulation of RNA polymerase III

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function (15, 16). Autoantibody to Scl-70 found in patients with scleroderma (17) has been shown to target DNA topoisomerase I as antigen (18–20) and the antigen which reacts with autoantibody to proliferating cell nuclear antigen (PCNA) in SLE (21) is now known to be auxiliary protein of DNA polymerase δ (22, 23).

Investigators in the field had long perceived that these spontaneously occurring autoantibodies in human disease would turn out to be useful reagents in cell biology. It was also appreciated that it was necessary to characterize the molecular nature of the autoantigens so that in addition to their immunochemical properties, their structure and function might be determined. Steitz and her colleagues (10) initiated studies which showed that Sm antibody immunoprecipitated small ribonucleoprotein particles resident in the nucleus and that the particles consisted of a class of small nuclear RNAs, called U RNAs, which were complexed with several nuclear proteins. These small nuclear ribonucleoprotein (snRNP) particles were shown to be involved in splicing of mRNA (24). Since then, numerous investigators have contributed to elucidation of the structure and function of intracellular autoantigens and among those which have been characterized are cellular components participating in DNA replication, DNA supercoiling, transcription related to RNA polymerases I and III, splicing as mentioned above, and aminoacylation of certain tRNA species and organelles such as centromeres involved in mitosis.

For the clinical biologist, this new information has placed on a more molecular basis the classification of autoantibodies and their corresponding antigens. Conglomerations of autoantibodies, which have been called profiles, have been shown to be uniquely related to certain diseases. For several years, the continuing discovery of more and more autoantibodies, although of great utility as diagnostic markers of disease, was beginning to confound efforts to understand processes underlying autoimmunity. One of the purposes of this presentation will be to show that the new molecular biology of cellular antigens and autoantibodies could now be providing insights into comprehending some features of autoimmunity. In addition, there appears to be a need for synthesis of the wealth of information which has accumulated and an evaluation of what it might signify. The first portion of this paper will deal with individual diseases and their related autoimmune manifestations and the second portion with hypotheses based on these data.

II. Systemic Lupus Erythematosus (SLE)

SLE is the prototype of a generalized or systemic autoimmune disease in many respects. It is characterized by an exuberant immune response

which is manifested by both the presence of multiple autoantibodies (Table I) and by their presence in high concentrations in many cases. In addition, many of the expected physiopathologic and histopathologic markers which might result from *in vivo* immune reactions are observed in this disease. They include detectable circulating immune complexes, decreased levels of complement caused by reaction of antibody and antigen either in the circulation or at tissue sites, tissue damage as a result of antigen-antibody reaction and the resultant activation of complement and other inflammatory mediators, and infiltration of damaged tissues with cells mediating immune reactions such as lymphocytes and monocytes. The immune response to intracellular antigens detectable in serum is depicted in Table I. Most antigen-antibody systems were initially detected with standard immunological assays and the clinical designation of the antigen is given first, followed by what is known of its structure and function. The clinical designation of antigens might in some cases employ the code name of the serum used in the study, such as Sm (*6*), and, in others, the disease with which the antibodies are associated, such as SS-A and SS-B (13) for Sjögren's syndrome-related antigens. It was anticipated by some that when the molecular structure and function of antigens were identified, it would be possible to replace the clinical designation with molecular nomenclature. However, this might not be possible in some cases, since certain antibody systems such as anti-Sm recognize proteins of 29, 28, 16, and 13 kDa, and the sera of patients show heterogeneity in their reaction with these proteins. Furthermore, as will be discussed in more detail later, many autoantigens are components of multimolecular assemblies which form subcellular particles, and autoimmune responses may be directed at different regions of the same particle.

Table I also depicts the approximate autoantibody frequencies for each antibody system. It can be appreciated that the frequencies cover a wide range, varying from 70% for antibodies to single-strand DNA and histones to 3% for antibody to PCNA. These frequencies hold true in studies of SLE patients from different racial and ethnic backgrounds and are therefore not related to overt genetic factors. As will be seen later, varying frequencies of autoantibodies are also observed in other autoimmune diseases. The reasons for this are a complete mystery and it is particularly intriguing that in a murine model of SLE, the MRL/Mp-lpr/lpr strain also develops anti-Sm antibody at approximately 25% frequency (50, 51). A study of this murine model led the authors to hypothesize that ability to make anti-Sm antibody was under genetic control but that the expression of this capability in an individual animal was governed by stochastic (random) events (51). It is difficult to understand how stochastic processes could operate with such constancy and

TABLE I

CELLULAR ANTIGENS AND AUTOANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS

Antigen			
Clinical designation	Molecular identity	Autoantibody frequency (%)[a]	Reference
Native DNA[b]	Double-strand DNA	40	25,26
Denatured DNA	Single-strand DNA	70	27
Histones	H1, H2A, H2B, H3, H4	70	28,29
Sm[b]	Proteins, 29 (B'), 28 (B), 16 (D), and 13 (E) kDa, complexed with U1, U2, and U4-U6 snRNAs; spliceosome component	30	6,10,30,31
Nuclear RNP	Proteins, 70, 33 (A), and 22 (C) kDa, complexed with U1 snRNA; spliceosome component	32	10,30,31
SS-A/Ro	Proteins, 60 and 52 kDa, complexed with Y1-Y5 RNAs	35	13,14,32-34
SS-B/La	Phosphoprotein, 48 kDa, complexed with nascent RNA pol III transcripts	15	15,16,35-38
Ku	Proteins, 86 and 66 kDa, DNA-binding proteins	10	39-42
PCNA/cyclin	Protein, 36 kDa; auxiliary protein of DNA polymerase δ	3	21-23,43-47
Ribosomal RNP	Phosphoproteins, 38, 16, and 15 kDa, associated with ribosomes	10	48,49
Hsp 90	Heat-shock protein, 90 kDa	50	122,123

[a]In this and subsequent tables, frequencies are best approximations.
[b]Disease-specific autoantibodies and diagnostic markers.

fidelity for each of the 11 autoantibodies described in Table I. Elucidation of this interesting phenomenon might be of help in understanding lupus and other autoimmune diseases.

Another general feature of systemic autoimmune diseases is the presence of certain antibodies which are disease specific. In SLE, they are antibodies to double-strand DNA and anti-Sm. Other antibodies such as anti-single-strand DNA, antihistones, antinuclear RNP, anti-SS-A/Ro, and anti-SS-B/La are not specific for SLE but may be detected in other diseases. Insufficient information is as yet available to make statements concerning anti-Ku, anti-PCNA, and antiribosomal RNP. Disease-specific autoantibodies are also present in scleroderma and in polymyositis and this is another special feature of systemic autoimmune diseases where its elucidation might be expected to advance our understanding of clinical autoimmune diseases.

Patients with SLE have a very characteristic polyclonal immune response in that the usual patient simultaneously has an average of three different autoantibodies of the types listed in Table I (52). In the more severely ill patients, there might be six or more autoantibodies detectable in serum. These features have been confirmed recently in another study performed in a group of 100 SLE patients (53). The polyclonality of the autoimmune response in SLE appears to be in contrast to other autoimmune diseases where the autoantibody response is more restricted and appears to be directed at one or two molecular species of antigen(s). This feature of SLE patients has made it difficult to correlate special clinical symptoms with specific antibodies and attempts to "subset" SLE on the basis of antibody specificities has met with problems. Studies of this nature have not taken into consideration that a particular antibody under study is not standing alone but is associated with many others which may vary from one patient to another. It is suggested that future studies of this type should subject each serum to a complete analysis of antibody specificities before conclusions are made relating an antibody to special clinical features.

A. Antibodies to Double- and Single-Strand DNA

Antibodies to DNA were the focus of much research in the 1960s and 1970s and the epitopes recognized by these antibodies have been carefully analyzed. This subject has been reviewed previously in these volumes (1) and there has been a more exhaustive review recently (26). In general, antibodies defined as reactive with single-strand DNA recognize polymers of purine and pyrimidine bases which are available for reaction with antibody in the single-stranded form of DNA but are not accessible in the double-stranded form. On the other hand, antibodies defined as

reactive with double-strand DNA recognize mainly the deoxyribose phosphate backbone and are therefore reactive with both double- and single-strand DNA. In certain cases, antibodies recognize helical structures on double-strand DNA and are also reactive with single-strand DNA where the latter has folded back to form secondary helical structures caused by base pairing of complementary nucleotide sequences. Antibodies to DNA arise spontaneously in different strains of murine lupus (54) and in recent years hybridomas have been generated from the spleens of such mice which produce a variety of anti-DNA monoclonal antibodies (55-57). These promise to be useful reagents for immunochemical and DNA structural studies.

Antibodies to DNA are of central importance in our understanding of one mechanism whereby autoantibodies may be related to pathogenesis. In certain patients, it can be demonstrated that antibody to DNA was followed in sequence by appearance of circulating DNA antigen and the precipitous disappearance of antibody, suggesting the formation of *in vivo* circulating immune complexes (25). Compelling evidence that such immune complexes were deposited in vascular organs such as the kidney was the demonstration that glomerular eluates were highly enriched for anti-DNA antibodies compared to circulating levels (58). Subsequently, other studies comparing circulating levels of anti-DNA with clinical symptoms have shown that decreasing titers of antibody correlate closely with worsening symptoms (59), further implying that in such situations the antibody might have been consumed in immune complex-mediated pathogenesis. These and many other studies support the evidence that antibody to DNA plays a role in disease pathogenesis in SLE. However, it should be noticed that this particular antibody-mediated pathogenic mechanism requires that intracellular DNA has to be exteriorized into the circulation or in extracellular tissue sites where it can react with antibody to form immune complexes. Such a situation has been demonstrated for DNA in that antigenically reactive macromolecular DNA can be demonstrated in significant concentrations in SLE sera (60, 61). One should be cautious in extrapolating these observations into the general statement that autoantibodies of other specificities could also be pathogenic. In this presentation, we are dealing with intracellular and, in most cases, intranuclear antigens, and with the exception of DNA, there is little evidence that other antigens have been detected in extracellular compartments where they can react with autologous autoantibodies.

B. Antibodies to Histones

Histones occur in the nucleus in a highly organized nucleosomal structure which consists of three subunits, two H2A-H2B dimers which flank an H3-H4 tetramer and with this entire protein complex surrounded

by the third subunit which comprises approximately two turns of DNA (62, 63) (Fig. 1). Connecting one nucleosome to the next is a variable length of linker DNA which is associated with histone H1. In the nucleus, certain physiologic environments may promote internucleosomal interactions so that there is polynucleosomal compaction and supercoiling which result in even higher ordered structures that are visible in the light microscope as chromosomes.

Antibodies to all classes of mammalian histones, H1, H2A, H2B, H3, and H4 are present in SLE but are not restricted to this disease. They have also been detected in drug-induced autoimmunity, rheumatoid arthritis (64, 65), and an undifferentiated form of connective tissue disease (66). This presentation will not cover rheumatoid arthritis and undifferentiated connective tissue disease and more information concerning these areas can be obtained by reference to the studies cited. The relationship between antibodies to histones and SLE is of historical and classical interest because all current evidence points to the conclusion that it is autoantibody to histone which is responsible for the LE cell phenomenon. The older studies had demonstrated that autoantibodies to deoxyribonucleoprotein, defined only as DNA-histone complexes, were capable of producing LE cells *in vitro* (4, 67). Since the introduction of newer techniques for detecting and identifying antinuclear antibodies, the LE cell assay is now rarely performed. However, the precise type of antihistone antibody which is capable of producing the LE cell phenomenon should be of more than historical interest, since it is possible that only antibodies reactive with certain

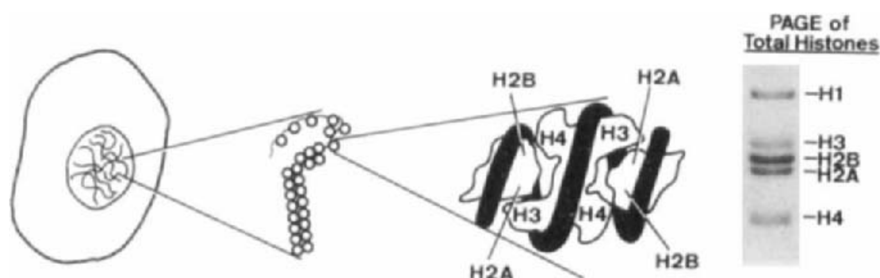

FIG. 1. Organization of histones in the nucleus. Chromatin fibers in the nucleus consist of nucleosomes interconnected by DNA. The core particle of the nucleosome is a tripartite structure held together by histone-histone interactions and stabilized by an entwining cord of DNA 146 bp long. The four core histones along with H1 can be extracted from chromatin and separated biochemically or by polyacrylamide gel electrophoresis (PAGE). One molecule of H1 is presumably associated with the linker DNA between adjacent nucleosomes (from Ref. 126).

classes of histones or certain histone subunits may have this property. When investigators were studying the mechanism of the LE cell phenomenon. it was noted that sera with this type of activity contained antibodies which reacted with broken cell nuclei to cause swelling of nuclear deoxyribonucleoprotein (68). This phenomenon could be visualized by time-lapse photomicrography. Other findings included changes in metachromasia of the deoxyribonucleoprotein (69). The application of the newer knowledge on antihistone antibody specificities to previous observations on the LE phenomenon might provide further insights into several important questions. Is there a possibility that antihistone antibodies capable of producing the LE cell phenomenon may be recognizing epitopes on the nucleosome which destabilize ordered structures resulting in unravelling of nucleosome structure? Information of this kind might be useful in understanding the interaction of different histone proteins in nucleosome formation since antibodies may be reacting with domains involved in protein-protein interaction. There is abundant evidence for the presence of antihistone antibodies which react with amino and carboxy termini of different histones and for other antibodies which react with more central core regions or with dimer (H2A-H2B) or tetramer (H3-H4) forms (70-73).

C. Antibodies to Sm and Nuclear RNP

The new developments in the molecular biology of autoantigens were centered around studies which elucidated the structure and function of Sm and nuclear RNP. After DNA and histones, Sm was perhaps the first of a succession of intranuclear autoantigens which were identified and characterized at the immunological and biochemical levels (6). Antibodies to Sm were found in patients with SLE (6), and antibodies to nuclear RNP were also found in patients with SLE (9) but even more frequently in patients with mixed connective tissue disease (8), a clinical syndrome which had not been recognized as a distinct entity until the autoantibody had been identified. At this stage of the studies, it was already evident that there was an association between Sm and nuclear RNP antigens in that nuclear RNP could not be biochemically isolated free of Sm, and that, as the name implied, nuclear RNP was nuclear in location and comprised a complex of RNA and protein (7, 9). These immunological and biochemical features of Sm and nuclear RNP antigens were elucidated by the important studies of Lerner and Steitz (10), who introduced the tools of molecular biology into this field and showed that these autoantigens were subcellular particles composed of a species of small nuclear RNAs (U RNAs) complexed with proteins. This set of autoantibodies was critically instrumental in showing that

small nuclear RNPs were involved in the splicing of precursor messenger RNAs (24, 74, 75) and has helped to advance our understanding of this important process in mammalian cell biology. Several comprehensive reviews of this subject have been published (11, 12, 76, 77) and describe in detail how these antibodies have been used to study assembly, structure, and function of snRNPs.

Although the molecular structure of Sm and nuclear RNP antigens recognized by SLE autoantibodies have been shown to be complexes of U RNAs and proteins, the antigenic determinants are the protein and not the RNA components. This can be clearly demonstrated by Western blotting and by the fact that snRNAs dissociated from proteins are not precipitable by autoantibodies. The antigens of Sm consist of at least four proteins, conventionally called B′ (29 kDa), B (28 kDa), D (16 kDa), and E (13 kDa). Affinity-purified anti-B′/B antibodies cross-react with the D protein and vice versa, but there are less frequently occurring sera where no cross-reaction is observed (78), indicating that there are at least two epitopes on the B′/B proteins recognized by anti-Sm sera. Occasional SLE sera immunoblot the E protein and one such autoantibody has been used to isolate a cDNA clone encoding the protein (79). The antigens of nuclear RNP are proteins of 70, 33 (A), and 22 (C) kDa. In addition, certain rare sera contain antibodies which react with A′ and B″ proteins (80, 81). This array of autoantibodies has been used to show that the core proteins of U1, U2, U5, and U4/U6 snRNPs are the B′, B, D, E, F, and G polypeptides. U1 snRNP contains, in addition, A and C polypeptides, and U2 snRNP contains A′ and B″ polypeptides. The available evidence currently indicates that the 70-kDa polypeptide is a nuclear matrix protein and not an intrinsic component of U1 snRNP, although it may be involved in the binding of U1 snRNP to the nuclear matrix (82). It would thus be incorrect to define the antigen(s) of antinuclear RNP as U1 RNP since this snRNP particle does not contain the 70-kDa protein. It also appears from analysis of these data that the immunogenic stimulus giving rise to antibody to nuclear RNP should consist of the U1 RNP particle associated with 70-kDa protein of the nuclear matrix.

This new information concerning the molecular identity of the proteins which constitute the antigen(s) recognized by anti-Sm and antinuclear RNP autoantibodies, and the knowledge that they are in assemblies of interactive proteins and RNAs engaged in mRNA splicing, provide challenging ideas about the nature of the immunogens in these diseases. In SLE, anti-Sm and anti-nRNP antibodies are frequently present together. In molecular terms, the immunogen should contain the core proteins DEFG and proteins A, B′, and B of U1 RNP and

proteins A′ and B″ of U2 RNP. In other patients, anti-Sm is present alone, and, in this situation, the immunogen may consist only of core proteins DEFG. The question arises as to the possibility that in these cases, the immunogen might be U5 and U4/U6 snRNP particles with the immune response limited to the core proteins only or that antibodies to proteins special to U5 and U4/U6 particles have not yet been detected. These considerations bring up the possibility that in SLE, the Sm and nRNP immune responses are induced either by the spliceosome, which is a superassembly of subcellular particles engaged in mRNA splicing (12, 76, 77, 83), or by component parts of the spliceosome.

D. Antibodies to SS-A/Ro and SS-B/La

Autoantibodies to subcellular particles SS-A/Ro and SS-B/La are found in Sjögren's syndrome and SLE. The Ro antigen was first isolated from extracts of human spleen and was identified in an immunodiffusion precipitin reaction with antibodies from SLE patients (84). It was reported to be a cytoplasmic antigen on the basis of its partitioning with cytoplasmic fractions in cell homogenates. These studies were extended and the La antigen was also identified in human spleen extracts with lupus sera (13). This initial report also indicated that the La antigen was cytoplasmic. Shortly thereafter, sera from patients with Sjögren's syndrome were shown to contain antibodies which reacted with soluble extracts from a human lymphocyte cell line and the antigens were designated SS-A and SS-B (14). Both antigens were reported to be nuclear in origin and there were in addition certain other reported biochemical characteristics which suggested that Ro and La were different antigen-antibody systems from SS-A and SS-B. However, the previous observation that the La antigen was thought to be cytoplasmic could be explained on the basis of its high solubility in physiological saline, so that it was easily leached out of the nucleus and appeared in the "cytoplasmic" fraction during cell fractionation. Thus, when it was shown that the La antigen was identical with SS-B and the Ro antigen with SS-A (85), these apparent differences could be reconciled. In addition, there is other evidence to suggest that these antigens are present in nuclear and/or cytoplasmic compartments, depending on the functioning state of the cell. Antibodies to these antigens are present in Sjögren's syndrome in higher frequency than in SLE (see C, Table II).

In addition to SLE and Sjögren's syndrome, autoantibody to SS-A/Ro is present in high frequency in a clinical "subset" of lupus called subacute cutaneous lupus (86, 87). Many of these patients have been falsely labeled antinuclear antibody (ANA) negative. The SS-A/Ro antigen is present in low abundance in cells (88), estimated to be about one-tenth the abundance of SS-B/La and Sm, and if adequate precautions are not taken to preserve the antigen in the nucleus, it can be eluted from nuclei in

TABLE II

CELLULAR ANTIGENS AND AUTOANTIBODIES IN DRUG-INDUCED AUTOIMMUNITY, MIXED CONNECTIVE TISSUE DISEASE, AND SJÖGREN'S SYNDROME

Antigen		Autoantibody frequency (%)	Reference
Clinical designation	Molecular identity		
A. **Drug (procainamide)-induced autoimmunity**			
Denatured DNA	Single-strand DNA	80	124,125
Histones	H1, H2A, H2B, H3, H4, and H2A-H2B dimer	>95	126-129
B. **Mixed connective tissue disease**			
Nuclear RNP	Proteins, 70, 33 (A), and 22 (C) kDa, complexed with U1 snRNP; spliceosome component	>95	8,10,30, 31,131
C. **Sjögren's syndrome**			
SS-A/Ro	Proteins, 60 and 52 kDa, complexed with Y1-Y5 RNAs	60	32-34,90, 97,132,133
SS-B/La	Phosphoprotein, 48 kDa, complexed with nascent RNA pol III transcripts	40	15,16, 35-38,132

the staining and washing procedures associated with tissue immunofluorescence. Further, the SS-A/Ro antigen appears to vary in abundance in cells of different species (89) so that if the substrate used for immunofluorescence had a low content of the antigen, the ANA test could be interpreted as negative. Anti-SS-A/Ro is also highly associated with neonatal lupus, a form of lupus which is related to transplacental transfer of IgG autoantibody from mother to infant and manifested in the infant as skin rash and congenital complete heart block (90, 91). The cause and effect relationship of autoantibody and clinical symptoms has not been proved because many mothers with anti-SS-A/Ro give birth to normal offspring. Several other clinical syndromes have been associated with anti-SS-A/Ro antibodies, including the lupus of homozygous complement C2 and C4 deficiencies (92, 93), primary biliary cirrhosis, and chronic active hepatitis (discussed in Ref. 94).

In contrast to the heterogeneity of clinical syndromes seen in association with anti-SS-A/Ro antibodies, anti-SS-B/La autoantibodies have been detected only in Sjögren's syndrome (see C, Table II) and SLE. An interesting similarity between the immune responses to SS-A/Ro and SS-B/La and those to Sm and nRNP should be noted. Antibodies to Sm are frequently associated with anti-nRNP but anti-nRNP antibodies are frequently detected without concomitant anti-Sm (see B, Table II). Similarly, anti-SS-B/La is frequently associated with anti-SS-A/Ro but not vice versa, as in the many clinical situations described above where anti-SS-A/Ro was detected without anti-SS-B. It would be of interest to unravel the reasons for the special feature associated with these immune responses.

As depicted in Table I, the subcellular particle immunoprecipitated by anti-SS-A/Ro antibody consists of two proteins of 60 and 52 kDa complexed with four to five small RNAs called Y RNAs (Y standing for cytoplasm) ranging in size from 80 to 112 bases (95). The SS-B/La particle is a 48-kDa protein (16, 37, 38) complexed with nascent RNA polymerase III transcripts. Analogous to the Sm and nRNP antigens, the antigenic determinants recognized by autoantibodies reside on the proteins and not on the RNAs. Further data on the structure and function of these two antigens will be discussed in relation to C in Table II.

E. Antibodies to Ku and Ribosomal (r) RNP

There has existed some confusion concerning the Ku antigen-antibody system. At about the same time that Ku autoantibodies were described, other investigators in the same laboratory (39, 96) described a precipitating antibody system which was labeled Ki. Classification of these autoantibodies was on the basis of immunodiffusion precipitin lines

and whether or not they demonstrated "identity" with reference standards. This could sometimes be misleading since nuclear protein antigens are present in cell extracts as aggregates of several components in the form of subcellular particles, and several antigen species could be present in such particles. Further compounding this difficulty is the well-known observation that autoimmune sera are rarely monospecific and, in fact, may contain several autoantibodies. This situation may have caused some temporary confusion concerning the Ku and Ki sera used as reference standards. However, the Ku antigen system has now been clarified by investigators in the above-mentioned laboratory, in conjunction with parallel investigations done in other laboratories (40, 41). The Ku antigen system consists of a pair of proteins of 60/66 and 80/86 kDa, which has for convenience also been called p70/p80. Unlike Sm, nRNP, SS-A/Ro, and SS-B/La, the p70/p80 proteins are not tightly bound to RNA, so that no RNAs are detected by immunoprecipitation and subsequent SDS gel electrophoresis. However, they have been shown to be DNA-binding proteins (41, 97) both *in vitro* and *in vivo*. Their association with DNA *in vivo* was demonstrated by colocalization with condensing chromosomes in early prophase and late telophase (41). Interestingly, in late prophase, metaphase, and anaphase, p70/p80 proteins were disengaged from the condensed chromosomes (41). In the interphase cell, anti-p70/p80 immunolocalize in the nucleoplasm as well as the nucleolus. Their function has not been established.

There are antibodies recognizing different determinants on p70/p80 in sera with the Ku specificity. It has been shown by use of antigen affinity-purified antibodies that there is a common determinant shared between the p70 and p80 proteins, and at least one distinct determinant on each p70 and p80 protein (40). As with most, if not all autoantigens, the antigenic epitopes on these proteins are conserved in evolution and have been detected in man, monkey, calf, sheep, rabbit, and rat (40). In our laboratory, autoantibody to Ku or p70/p80 was found in 10% SLE sera and was not detected in approximately 100 scleroderma sera examined (40).

Although the majority of autoantibodies to intracellular antigens in SLE are intranuclear in location, it has long been recognized that there are autoantibodies to ribosomal proteins of the cytoplasm (98-103). In general, about 10% of an unselected population of SLE patients appear to have antibodies which stain the cytoplasm in a somewhat homogeneous pattern by indirect immunofluorescence. Frequently the nucleoli are also stained. However, because of the multiplicity of autoantibodies in SLE, the cytoplasmic and nucleolar immunofluorescence are frequently accompanied by nucleoplasmic fluorescence caused by other autoantibodies

reactive with nucleoplasmic antigens. Of the latter, there is a high association with antibody to Sm (48). The structure, and by analogy to related proteins in other eukaryotes which have been studied extensively, the function of the ribosomal protein antigens have been well characterized (48, 49). The antigens are present in ribosomal ribonucleoprotein (rRNP) and are three phosphoproteins of 38, 16, and 15 kDa which are components of the large ribosomal subunit. From the early studies, it was shown that a related or identical antigenic determinant was present on all three proteins, since antibody eluted from any electrophoresis protein band was reactive with the other two (48). Human autoantibodies reactive with 16- and 15-kDa proteins were also reactive by Western blotting with analogous protein species in the large ribosomal subunit of such diverse species as rat (protein subunits pl/p2), brine shrimp (*Artemia*, eL7/eL12), and yeast (Al/A2) (48, 103–107). The 16- and 15-kDa human ribosomal protein antigens are acidic and are quite similar in molecular weight to the Al/A2 acid ribosomal proteins of yeast, which have functional equivalents in other eukaryotes and have been shown to participate in the elongation step of ribosomal protein synthesis (108, 109). The antigenic determinant recognized by a population of human autoantibodies has been shown to be present on a peptide of 22 amino acids at the C-terminus of *Artemia* ribosomal protein eL12 (110).

There has been a report showing an association between psychotic syndromes in SLE and autoantibodies to rRNP (111). It would be important to have a confirmation of this finding by other laboratories. In this report a total of 32 patients with SLE were determined to have antibodies to rRNP and 18 (56%) had psychotic syndromes whereas 14 (44%) did not. It appears that there could be other factors associated with anti-rRNP which might contribute to psychosis.

F. Antibodies to PCNA (Proliferating Cell Nuclear Antigen) and HSP 90

Antibodies to PCNA are detected in 3% of patients with SLE and there appear to be no distinctive clinical features in such patients that would set them apart from other SLE patients. Although these antibodies might never achieve significance as diagnostic markers in clinical medicine, they have been extremely useful probes in the study of events regulating DNA replication, cell proliferation, and blast transformation. These antibodies were first observed as a novel precipitin line in double-diffusion assays against soluble extracts of calf and rabbit thymus (21). In attempts to correlate this novel antibody with patterns of immunofluorescent localization in tissues, it was noted that the antibody showed some distinctive features. In tissue sections of mouse kidney, renal tubular and glomerular cells were not reactive whereas

scattered cells in the renal interstitium showed strong nuclear localization of the antibody. In the thymus, areas containing dividing cells such as the cortex were positive but quiescent areas such as medulla were negative. When nonsynchronized but rapidly dividing tissue culture cells were examined, some cells were weakly positive whereas others were strongly positive and showed a wide variety of nuclear staining morphology (see Fig. 2). It became apparent that the nuclear antigen recognized by the antibodies was expressed in a differential manner and was probably cell-cycle related. A series of studies showed that the nuclear antigen could be induced in resting lymphocytes by mitogen stimulation and in established tissue culture cell lines, peak expression of the antigen was in late G and early S phases of the cell cycle, during a period immediately preceding full DNA synthesis (21, 43, 46). The antigen was therefore named proliferating cell nuclear antigen.

Shortly after the initial observations on PCNA but proceeding

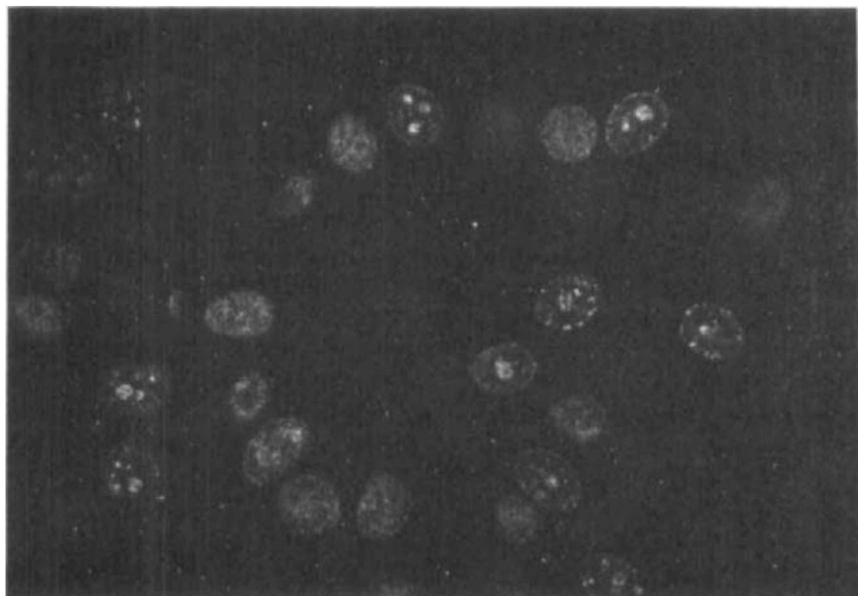

FIG. 2. Localization of PCNA on human amnion AMA cells by immunofluorescence using human autoantibodies. In nonsynchronized cells, there is characteristically a variable pattern of immunolocalization. PCNA is seen in nucleoplasm with relative absence of nucleolar localization in some cells and prominence of nucleolar localization in other cells. Note that in some cells there is also localization at the nuclear membrane. Some of these areas of PCNA localization have been shown to be associated with DNA synthesis at these sites.

independently, Bravo and Celis were surveying changes in cellular proteins which might differentiate malignant from normal cells. In two-dimensional gel electrophoresis, they detected a protein of 35–36 kDa and isoelectric point 4.8–4.9 which stood out and was present in virally transformed but not normal cells. Its expression was cell-cycle related and they called the protein cyclin (44, 112). These two lines of investigation might have progressed in parallel for some time had not Mathews *et al.* (45) showed that PCNA and cyclin were identical.

Progress in characterizing both the structure and function of PCNA/cyclin has been very rapid. The pure protein was isolated, the first 26 residues of the amino terminus of rabbit PCNA were sequenced (47), and two murine monoclonal antibodies to the purified protein have been produced and used in flow cytometry to study cell-cycle events and activation of human T lymphocytes (113–115). The published N-terminal amino acid sequences of rabbit PCNA was used by Matsumoto *et al.* to synthesize oligonucleotides for hybridization screening of a rat thymocyte library, and a clone of cDNA encoding rat PCNA has been isolated (116). With a different strategy using rabbit antibodies to purified human PCNA, Almendral and colleagues (117) have also isolated an apparent full-length cDNA encoding human PCNA. There is good sequence similarity in amino acids deduced from these cDNA clones with only 4 amino acid changes in a total protein of 261 amino acids.

In 1986, a report from the laboratory of Downey and So described a nuclear protein of 36 kDa which was required for chain elongation or processivity in DNA synthesis mediated by DNA polymerase δ (118). In rapid succession, it was reported from two laboratories that the auxiliary protein of DNA polymerase δ is identical to PCNA/cyclin (22, 23). Currently, many laboratories are pursuing studies to define the role of PCNA in DNA replication, synthesis, and repair and PCNA autoantibodies have provided powerful probes to observe modification or inhibition of such activities. A comprehensive and up-to-date review of this field has recently been written by Mathews (119). Regarding the epitope(s) on PCNA recognized by human autoantibodies, it is of interest that in an *in vitro* system of oligonucleotide synthesis mediated by DNA polymerase δ, human autoantibodies were inhibitory whereas two murine monoclonals were not (120). It had been determined previously that the murine monoclonal antibodies recognized different epitopes on PCNA from human autoantibodies (121). This observation reinforces other findings showing that human autoantibodies are capable of inhibiting the function of the cognate antigen and in this instance the autoantibody epitope appears to be related to the active site whereas other immunogenic epitopes are not.

Recently, there has appeared an interesting report of autoantibody to a mammalian heat-shock protein of 90 kDa which is a cytoplasmic and plasma membrane protein (122, 123). The autoantibody was present in 50% of patients with SLE and two of six patients with polymyositis.

III. Drug-Induced Autoimmunity

A complication of treatment with certain drugs is the development of an autoimmune response characterized by autoantibodies to single-strand DNA and nuclear histones (124, 125) (see A, Table II). Several drugs have this propensity but the most common offenders are by far procainamide and hydralazine, with quinidine, chlorpromazine, and acebutalol running distant seconds (126). Studies with procainamide have been more extensive than with other drugs and it has been shown that after 1 year of therapy, approximately 50% of patients will develop antidenatured (d) DNA and antihistone antibodies. The majority will be asymptomatic but 15-20% of those with autoantibodies might develop lupuslike symptoms consisting of arthritis/arthralgias, fever, weight loss, rash, splenomegaly, and pleural inflammation but absence of renal disease (reviewed in Ref. 126). Since this is an autoimmune disease where the inciting agent is known, there has been interest in elucidating the mechanism of induction of this phenomena.

The immune response is restricted and characteristic. Antibodies to dDNA are present in 70-80% and antibodies to histones in over 95%. A striking feature is the absence of autoantibodies to other nuclear antigens detected in patients with the idiopathic form of the disease or SLE. For some reason, antibody to double-strand or native DNA is absent in spite of the universal presence of antibody to histone. The immune response to histone was first demonstrated with indirect immunofluorescence (127) using cell substrates from which histones and other proteins had been extracted and only histones reconstituted by virtue of their spontaneous reassociation with unextracted DNA. Virtually all patients with procainamide-induced lupus had antibodies reactive with reassociated histone-DNA. This study was prompted by the observation that since patients with drug-induced lupus had a high prevalence of LE cells, it might be expected that their autoantibody specificity would be related to histones.

Although the mechanism of induction of antihistone antibodies in procainamide-induced autoimmunity is as yet unclear, equally intriguing is the observation that only 15-20% of patients with autoantibodies will develop symptomatic disease. Undoubtedly, many factors may be operating, but the acetyltransferase phenotype appears to play an important

role (128). Acetyltransferase activity resides mainly in the liver and its level is genetically controlled. Slow acetylators are homozygous for a recessive gene controlling hepatic acetyltransferase activity and will tend to have approximately two-fold higher blood levels of unacetylated drugs at equivalent therapeutic doses than rapid acetylators. A strong association has been observed between acetylator phenotype and the emergence of drug-induced lupus symptoms, with slow acetylators developing ANAs and clinical symptoms more quickly and after lower total drug dose than rapid acetylators. In addition to genetic factors which appear to determine whether a patient will develop clinical symptoms, a clear difference in the character of the immune response to histone has been documented (129). The antihistone response in asymptomatic individuals is predominantly IgM with some IgG and is heterogeneous in specificity, with antibodies reactive with individual histones of all classes. In contrast, symptomatic individuals make IgG antibodies which are most reactive with histone dimer H2A-H2B. The data are shown in Table III, where antibody binding was determined by ELISA. Reactivity with the native H2A-H2B dimer was greater than with the denatured dimer and was negligible with individual H2A or H2B. In contrast, IgG antibodies in asymptomatic patients showed equal reactivity with the native dimer, denatured dimer, and individual histones, indicating that reaction with native H2A-H2B dimer was due to its component parts and not a special property of the dimer complex.

TABLE III
DISTINCTION BETWEEN ANTIBODY REACTIVITY WITH THE H2A-H2B DIMER AND THE COMPONENT HISTONES H2A AND H2B[a]

		Antibody binding to histone (OD)			
		H2A-H2B complex			
Patient status	Patient	Native	Denatured	H2A	H2B
Symptomatic	10	5.52	1.81	0.17	0.06
	8	2.98	0.81	0.19	0.07
	16	5.85	1.62	0.13	0.04
	13	4.34	1.70	0.16	0.21
Asymptomatic	21	1.80	1.68	1.85	1.74
	22	1.28	1.11	0.90	1.80
	23	1.77	1.40	0.33	2.99
	24	1.12	0.51	0.66	2.88

[a]From Rubin (126).

These studies have also shown that IgG antibody to H2A-H2B has the potential of being a prognosticator of impending clinical symptoms. Furthermore, symptomatic patients recovering after withdrawal of procainamide have shown gradual fall in titers of IgG anti-H2A-H2B. In addition to their clinical relevance, these findings should serve as guideposts for investigations into the mechanisms underlying this form of autoimmunity.

IV. Mixed Connective Tissue Disease

Mixed connective tissue disease is a clinical entity which was recognized partly on the basis of a specific autoantibody, antinuclear RNP (8). The name implies that it is a clinical disease with features of several connective tissue diseases which are SLE, scleroderma, and polymyositis. MCTD should not be confused with "overlap syndromes," which should be reserved for some patients who have overlapping features of two diseases, such as combinations of SLE and scleroderma, or SLE and rheumatoid arthritis, or scleroderma and dermatomyositis (130). In order to define MCTD clinically, it would be of help to use the criteria established in a multicenter study in Japan (Table IV). It can be noted that anti-nRNP is a required item together with either Raynaud's phenomenon or swelling of the fingers/hand. In addition, other clinical findings are necessary items and they include symptoms and/or signs derived from at least two of the three diseases, SLE, scleroderma, and polymyositis (131).

The autoimmune manifestation in MCTD is strikingly restricted to autoantibody to nuclear RNP and defined more precisely as antibodies which immunoblot 70-kDa protein of the nuclear matrix and the A (33 kDa) and C (22 kDa) proteins of U1 snRNP. In immunoprecipitation of labeled cell extracts, U1 RNA is the only snRNP species detected. It will be recalled in the earlier discussion that core proteins of U1 snRNP are B, D, E, F, and G and that certain SLE patients make antibodies to B, D, and E core proteins (anti-Sm specificity). The lack of autoantibodies to snRNP core proteins in MCTD has been proved beyond reasonable doubt. The reason for immune responses that can be so exquisitely differentiating is now totally unclear and herein must lie some clues to the cause of autoimmunity.

V. Sjögren's Syndrome

Autoantibodies to Sjögren's syndrome are restricted to SS-A/Ro and SS-B/La antigens. In this respect, this disease is similar to MCTD and

TABLE IV
PRELIMINARY DIAGNOSTIC CRITERIA FOR MIXED CONNECTIVE TISSUE DISEASE[a]

I. Common Symptoms
  1. Raynaud's phenomenon
  2. Swollen fingers or hands
II. Anti-nRNP antibody
III. Mixed findings
  A. SLE-like findings
    1. Polyarthritis
    2. Lymphadenopathy
    3. Facial erythema
    4. Pericarditis or pleuritis
    5. Leukocytopenia (less than 4000/mm^3) or thrombocytopenia (less than 100,000 mm^3)
  B. PSS-like findings
    1. Sclerodactyly
    2. Pulmonary fibrosis, restrictive change of lung (%VC less than 80%) or reduced diffusion capacity (DL_{co} less than 70%)
    3. Hypomotility or dilatation of esophagus
  C. PM-like findings
    1. Muscle weakness
    2. Increased serum level of myogenic enzymes (CPK)
    3. Myogenic pattern in EMG

Diagnosis: MCTD will be diagnosed when all three of the following conditions are fulfilled:
  1. Positive in either one of two common symptoms
  2. Positive anti-nRNP antibody
  3. Positive in one or more findings in two or three disease categories of A, B, and C.

[a]From Kasukawa (131).

drug-induced autoimmunity in the limited heterogeneity of the autoimmune response, which is in contrast to SLE. These antibodies are present in higher frequency than in SLE as noted previously. Of more than passing interest, one should notice that all autoantibody specificities described in Table II have been detected in SLE and yet the selective expression of one or a limited number of these autoantibodies is associated with distinctively different diseases. In this context, it would be of interest to refer to Tables V and VI, where it can be seen that autoantibodies in dermatomyositis, polymyositis, and scleroderma are directed against totally different sets of intracellular antigens.

There have been several studies devoted to the characterization of the SS-A/Ro antigen and reports describe protein or proteins varying from 50 to 150 kDa (discussed in Ref. 133). The best consensus appears to be a polypeptide of 60 kDa. Recently (133), it has been shown that the SS-A/Ro antigen is a complex of two proteins, the 60-kDa protein and

TABLE V
CELLULAR ANTIGENS AND AUTOANTIBODIES IN DERMATOMYOSITIS AND POLYMYOSITIS

Antigen		Autoantibody frequency (%)	Reference
Clinical designation	Molecular identity		
Jo-1[a]	Protein, 50 kDa; histidyl-tRNA synthetase	25	142-146
PL-7[a]	Protein, 80 kDa; threonyl-tRNA synthetase	4	147
PL-12[a]	Protein, 110 kDa; alanyl-tRNA synthetase and alanyl-tRNA	3	148,149
PM-Scl	Complex of 11 proteins, 110 to 20 kDA	8	152,153
Mi-2	Proteins, 61 and 53 kDa	5	154
SRP	Signal recognition particle protein, 54 kDa, complexed with 7 SL RNA	Rare	155-157

[a]Disease-specific autoantibodies and diagnostic markers.

another 52-kDa protein, which can be distinctly demonstrated with Western blotting techniques (Fig. 3). Selected sera from patients with Sjogren's syndrome were analyzed in Western blots with HeLa cell extract. Lanes 1-4 demonstrate sera which blot both 60- and 52-kDa proteins, in addition to SS-B/La (48-kDa native protein and 43-kDa degradation product). Lanes 5-6 demonstrate sera which blot only 60 but not 52 kDa, and lanes 7-10 demonstrate sera which blot only 52 and not 60 kDa (other polypeptides are also blotted in some of these lanes). Affinity-purified antibodies from either 60- or 52-kDa bands were not cross-reactive, demonstrating that these were at least two separate antibody populations. Either affinity-purified antibody immunoprecipitated the identical spectrum of Y1 to Y5 small RNAs, indicating that at least a certain proportion of 60- and 52-kDa proteins were in complex either with one another or with Y RNAs. When affinity-purified antibodies were reacted in indirect immunofluorescence with human HEp-2 cells, both antigens were localized in the nucleoplasm in punctate distribution (Fig. 4).

SSB/La is an antigen which is a binding protein for a number of small RNA species. The RNAs which have been identified include RNA polymerase III transcripts which are precursors of tRNA, 5 S RNA, VA RNA, EBER RNA, 4.5 *I* RNA, 7 S RNA, Y RNA, and U6 RNA (15, 16, 35, 38, 132). In addition, it has been reported that the antigen binds to U1 RNA (134), and to vesicular stomatitis virus leader RNA (135). It appears that the 3′-oligouridylate tail of these small RNAs is

TABLE VI
CELLULAR ANTIGENS AND AUTOANTIBODIES IN SCLERODERMA

Antigen		Autoantibody frequency (%)	Reference
Clinical designation	Molecular identity		
Scl-70[a]	100-kDa native protein and 70-kDA degradation product; DNA topoisomerase I	70% in diffuse scleroderma	17-20, 158,159
Centromere[a]	Proteins, 17, 80, and 140 kDa, localized at inner and outer kinetochore plates	70-80% in CREST	160-167
RNA Pol I[a]	RNA pol I complex of subunit proteins, 210 to 11 kDa	4	171,172
Fibrillarin[a]	Protein, 34 kDa; component of U3 RNP particle	8	174,175
PM-Scl	Complex of 11 proteins, 110 to 20 kDa	3	144,145
To	Protein, 40 kDa, complexed with 7-2 and 8-2 RNAs	Rare	176,182, 183
NOR-90	Protein, 90 kDa, localized in nucleolus organizer region	Rare	184

[a]Disease-specific autoantibodies and diagnostic markers.

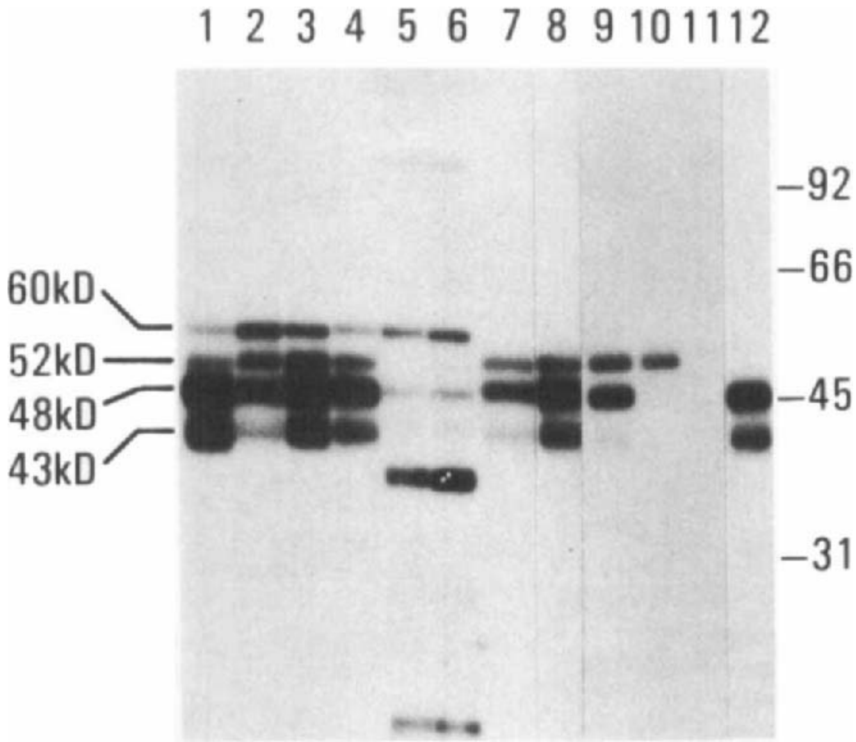

FIG. 3. Western blot analysis of representative sera from patients containing autoantibodies to SS-A/Ro using extracts from HeLa cells. Lanes 1-4 demonstrate the presence of antibodies to bands of 60, 52, 48, and 43 kDa. Lanes 5 and 6 show antibodies to the 60-kDa protein and not to the 52-kDa protein. Lanes 7-10 show antibodies to the 52-kDa band and not the 60-kDa band. Lane 11 is normal serum and lane 12 a monospecific anti-SS-B prototype serum. The 43-kDa polypeptide is a degradation product of the 48-kDa SS-B protein (from Ref. 133).

required for binding with the antigen (35, 136, 137). SSB/La is a phosphorylated protein with several isoelectric species which are related to degrees of phosphorylation (138, 139). Phosphorylation occurs on serine and threonine but not on tyrosine residues (140). The 48-kDa protein consists of two structural domains which can be generated by controlled digestion with *Staphylococcus aureus* V8 protease, a 28-kDa domain rich in methionine and a smaller 23-kDa domain which contains all the detectable phosphorylated amino acids. These domains can be visualized using [^{3}]leucine-labeled cell extracts (Fig. 5) which were first digested with *S. aureus* V8 and then immunoprecipitated with autoantibodies (38).

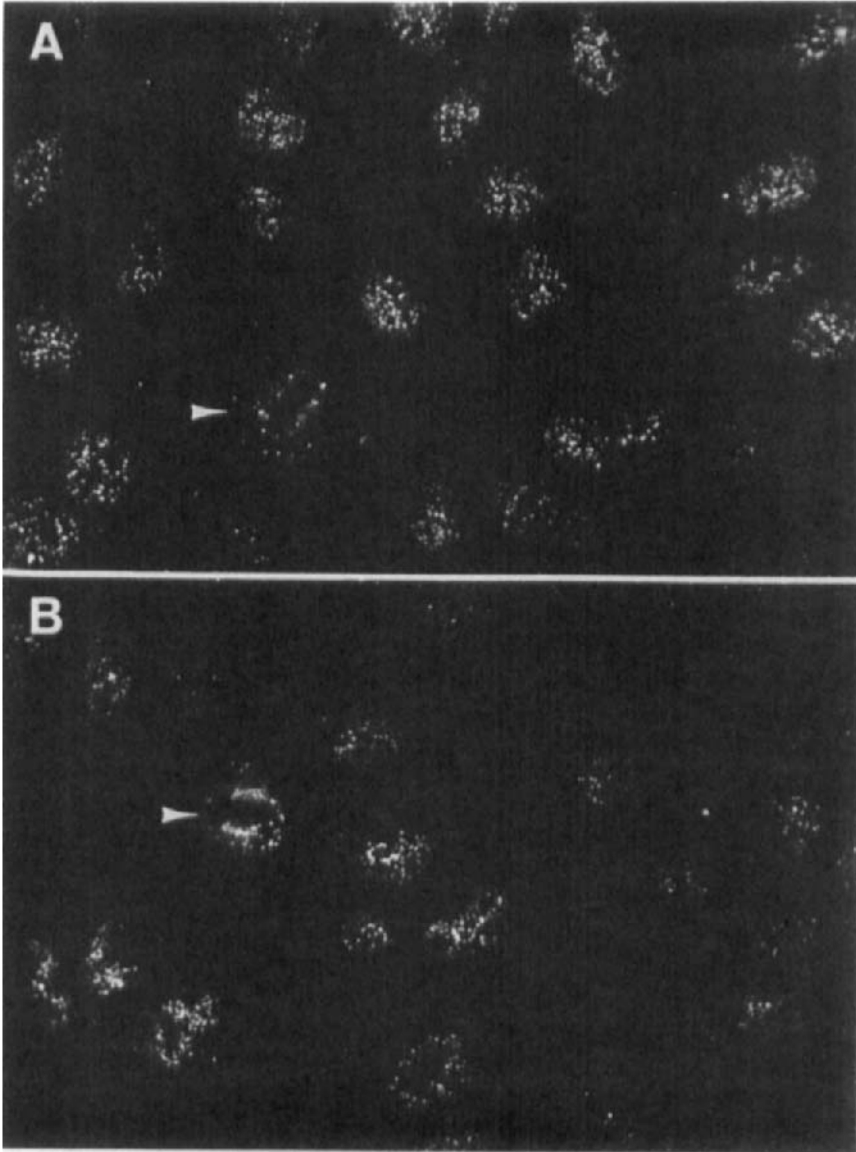

FIG. 4. Indirect immunofluorescence using prefixed HEp-2 cells as substrate. (A) Affinity-purified anti-60-kDa antibodies. (B) A serum which was monospecific for anti-52-kDa antibodies. Note the similar punctate nuclear staining patterns in both cases, the relative absence of cytoplasmic staining, and the absence of the antigens in the area of the condensed chromosomes of mitotic cells in prophase (arrows) (from Ref. 133).

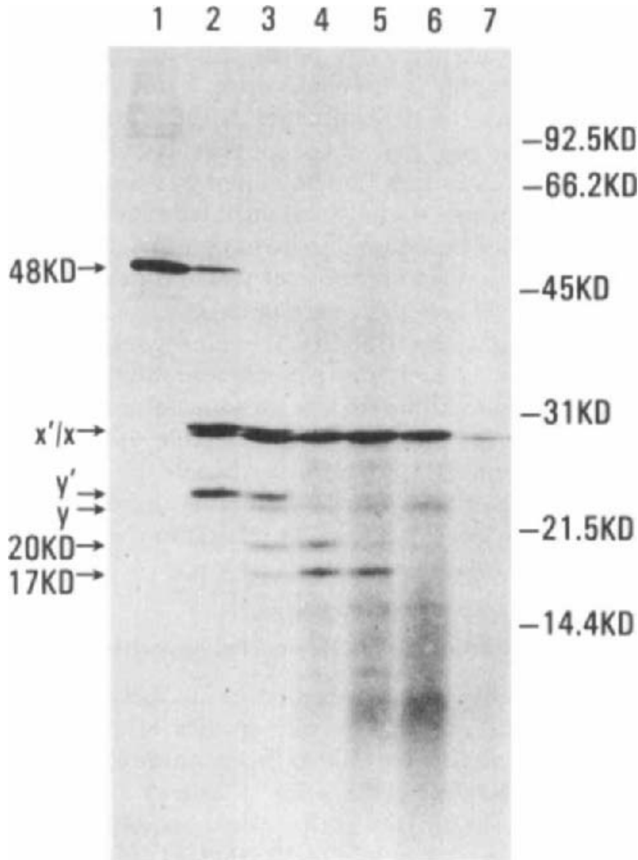

FIG. 5. Immunoprecipitation of extracts from [^{3}H]leucine-labeled HeLa cells. Labeled extracts were first digested with *Staphylococcus aureaus* V8 before immunoprecipitation with serum containing antibody to SS-B. Lanes 1-7 correspond to extracts digested with 0, 10, 30, 90, 270, 810, or 2430 μg protease/ml, respectively. Two sets of partially protease-resistant polypeptides (X/X′ and Y/Y′) were precipitated by this serum. The X/X′ polypeptides were considerably more resistant to protease than the Y/Y′ polypeptides, which were further degraded to the 20- and 17-kDa polypeptides. There were also less well-defined fragments precipitated at the lower molecular-weight range (lanes 5 and 6) (from Ref. 38).

When UV irradiation was used to cross-link preexisting RNA-bound antigen before *S. aureus* V8 digestion and immunoprecipitation, it was shown that the RNA-binding site of the antigen resided in the non-phosphorylated methionine-rich 28-kDa fragment.

The above studies also demonstrated that there are at least two epitopes on SS-B/La and at least one each on the 28- and 23-kDa fragments. These epitopes are highly conserved, since human autoantibody is capable of recognizing the determinants in cells from man, monkey, bovine, rabbit, hamster, rat, and mouse species (141). On the other hand, when purified SS-B/La from a bovine source was used as immunogen in mice and several murine monoclonal antibodies were tested for their range of reactivity, they were found to be more restricted, reacting with man, monkey, bovine, and rabbit but not with hamster, rat, and mouse cellular protein (141). These data emphasize the feature that epitopes recognized by human autoantibodies are more conserved than other immunogenic sites on the same protein, as exemplified by ability of the less conserved epitopes to stimulate host immune responses in the mouse. If a functional assay for SS-B/La were available, it would clearly be of interest to determine if autoantibodies would be more capable of inhibiting function than monoclonal antibodies, based on the assumption that evolutionary conservation was related to the need to preserve important cellular activities.

VI. Dermatomyositis and Polymyositis

A fascinating group of autoantibodies in dermatomyositis and polymyositis target as antigens certain species of tRNA synthetases (Table V). Rosa *et al.* isolated RNAs from immunoprecipitates and identified them as $tRNA^{His}$ (143), while Mathews and Bernstein and Yang *et al.* identified the antigen as the protein moiety of histidyl-tRNA synthetase with a molecular weight of 50,000 (144, 145). Clinically, this autoantibody was described by Nishikai and Reichlin (142) in approximately 25% of patients with polymyositis and was identified originally as a precipitating antibody in immunodiffusion with soluble cell extracts. Many of these myositis patients also have associated interstitial lung disease (146). A second myositis-related autoantibody was also described by Mathews and collaborators (147), labeled PL-7 to identify its immunodiffusion precipitin line, and shown to be antibody to threonyl-tRNA synthetase, recognizing a protein of 80 kDa. The third autoantibody system in this series (PL-12) was identified as at least two antibodies, one of which recognized naked alanyl-tRNA and the other the alanyl-tRNA

synthetase (148, 149). Some important insights into the nature of the autoimmune reaction have emerged from these studies, which are extremely reminiscent of other autoantibodies described previously. The myositis autoantibodies all are capable of inhibiting specific aminoacyl charging of their respective tRNAs, so that antihistidyl, antithreonyl, and antialanyl tRNA synthetases inhibit only the charging of their respective tRNAs (144, 145, 147, 148). In the case of the PL-12 system, a second autoantibody is reactive with naked alanine tRNA (149). By ribonuclease protection and oligonucleotide binding experiments, the antibody-binding site was narrowed down to a seven- to nine-nucleotide sequence containing the anticodon loop of tRNA (149). In this respect, the PL-12 antibody system may be unique and different from the other myositis autoantibodies since no antibodies to tRNAs have yet been reported for Jo-1 and PL-7.

The insights provided by examining the reactivity of autoantibodies with tRNA synthetases come from the consistent demonstration that autoantibodies do not immunoblot or react weakly with denatured synthetases, such as those treated by boiling in sodium dodecyl sulfate preparatory to gel electrophoresis and transfer to nitrocellulose for Western blotting (148, 150, 151). If nondenatured histidyl- or threonyl-tRNA synthetase is tested in direct immunoblotting, the respective autoantibodies react well (150, 151). However, experimentally induced antibodies to purified synthetases, which presumably react with linear or sequential epitopes, were strongly reactive with synthetases displayed in Western blot formats (147, 150, 151). These observations implied that autoantibodies, in contrast to experimental antibodies, might be recognizing conformational epitopes. Further studies showed that autoantibody to the histidyl synthetase showed greater inhibition of function when synthetase had been preincubated with tRNA (150). Finally, in a threonyl-tRNA synthetase functional assay, experimentally induced antibody which recognized both the native and denatured form of the synthetase had no effect on aminoacylation, but autoantibody which recognized only the native form of the synthetase and not the denatured form was able to specifically inhibit threonyl-tRNA synthetase activity (151). Taken together, all the evidence described above suggests that the epitopes recognized by autoantibodies to tRNA synthetases appear to be the catalytic sites of these synthetases, a theme which appears to be saying that spontaneously occurring antibodies may have the general property of recognizing active sites on proteins or nucleic acids.

PM-Scl antigen is the name given to a complex of proteins which are immunoprecipitated by sera of patients who have clinical features which overlap between polymyositis and scleroderma (152). It was first

recognized as an immunodiffusion precipitin line against tissue extracts and sera showed nucleolar staining in immunofluorescence. Immunoprecipitation of [^{35}S]methionine-labeled cells demonstrate a complex of 11 proteins ranging from 110 to 20 kDa (153). The 80- and 20-kDa polypeptides in this complex are phosphorylated. In actinomycin D-treated HeLa cells, where nucleoli are segregated into granular and fibrillar components, the PM-Scl antigen was localized to the granular regions. This was confirmed by immunoelectron microscopy of rat liver hepatocytes. Since the granular component of nucleoli is thought by some to be the site of ribosome assembly and packaging, the location of the antigen suggests a possible relationship of the PM-Scl antigen with preribosomes. The antigenic determinants were detected on polypeptides of 100 and/or 80 kDa (153).

Other antigen-antibody systems identified are the Mi antigen in calf thymus extracts consisting of two proteins of 61 and 53 kDa of yet uncharacterized structure and function (154) and a protein of 54 kDa of the signal recognition particle (SRP) (155–157). SRP is a complex of a cytoplasmic RNA called 7SL RNA and six polypeptides ranging from 9 to 72 kDa. The 54-kDa protein, which contains the antigenic epitope, has been shown to be required for elongation arrest of polypeptide synthesis and for translocation across the endoplasmic reticulum. These sera demonstrate nucleolar and cytoplasmic staining in tissue immunofluorescence.

VII. Scleroderma

In contrast to SLE, in which there is ample evidence for immune-mediated pathogenesis of tissue injury, such as infiltration of lymphocytes and deposition of immunoglobulin and complement in tissue lesions, and presence of circulating immune complexes and hypocomplementia in the blood, there is scarce evidence of such findings in scleroderma. It is therefore of great interest that the autoantibody response in scleroderma is as exuberant in this disease as in SLE. As depicted in Table VI, several different types of autoantibodies have been characterized, and if assays are done correctly, at least 95% of all scleroderma patients would have an identifiable autoantibody. Included in the classification of scleroderma is a large subset of patients who have a form of the disease described with the acronym CREST (*c*alcinosis, *R*aynaud's phenomenon, *e*sophageal dysmotility, *s*clerodactyly, and *t*elangiectasia). In most large clinics, CREST patients may comprise 20 to 30% of all scleroderma. A second feature which distinguishes scleroderma from SLE is the restricted heterogeneity of autoantibody

types which are present in each patient, so that the individual patient will rarely have more than one autoantibody detected in serum, in contrast to the occurrence of three or more antibodies in patients with SLE. This has been well documented for antibodies to Scl-70 and centromere antigens, where the presence of one autoantibody appears to exclude the presence of the other (17, 158, 159).

A. Antibodies to Scl-70 and Centromere Antigens

Scleroderma sera were observed to react in immunofluorescence with a nucleoplasmic antigen which was visualized as minute and evenly distributed punctate staining (158). The antigen was readily extracted from cells in physiological buffers and was precipitable in double immunodiffusion against such sera. Further analysis with antigen extracted from rat liver showed that it appeared to be a protein of 70 kDa by SDS gel electrophoresis and it was named Scl-70 (17). An exciting development was the independent discovery in three different laboratories that the cellular antigen reactive with this group of scleroderma sera was DNA topoisomerase I with a native molecular weight of about 100,000 (18–20). If protease inhibitors were not included in extraction buffers, the 100-kDa protein was degraded spontaneously to polypeptides of lower molecular weight, one of which was an antigenically reactive fragment of about 70 kDa. Autoantibodies were shown to be capable of inhibiting the relaxation of supercoiled DNA induced by topoisomerase. In the early studies, this antibody was detected by immunodiffusion in approximately 20% of an unselected scleroderma population (158), but in later studies using the same assay system, it was reported to be detected in 75% of patients with the diffuse form of scleroderma (159). This increased sensitivity was probably related to better extraction or preservation of topoisomerase I in the extracts (thus preventing spontaneous proteolytic degradation to smaller nonreactive fragments) and also related to its higher prevalence in the diffuse or more severe form of scleroderma.

Autoantibodies to centromere antigens were detected on the basis of a distinct pattern of immunofluorescent localization on tissue culture cell substrates (160, 161). A finite number of punctate spots in the nucleoplasm was observed to react with certain sera but the distinguishing feature was that in dividing cells, the antigen segregated with and colocalized in the region of condensing metaphase chromosomes. Subsequently, immunolocalization was achieved with isolated metaphase chromatin, which were not treated with acetic acid because it destroys their antigenicity; staining was observed in the primary constrictions of isolated chromatin whether metacentric or acrocentric. By

subjecting the preparation to a series of chemical treatments and enzyme digestions, the antigen was deduced to be proteins tightly bound to centromeric DNA. By immunoelectron microscopy, centromeric antigens were localized to the inner and outer layers of the trilaminar kinetochore structure with no reaction with centromeric DNA (162), confirming the earlier observations in fluorescence microscopy and further showing that well-formed kinetochores were present in the interphase nucleus, an issue of some debate before autoantibodies were available. Immunoelectron-microscopy also demonstrated that in the interphase nucleus, kinetochores were frequently observed adjacent to nuclear membranes (163), a feature which might suggest a mechanism whereby chromosomes during interphase are attached to nuclear membranes. Several laboratories have shown that the centromere antigens consist of three proteins of 17/19, 80, and 140 kDa (164-166), and a cDNA clone which encodes about 95% of the 80-kDa protein has been isolated (167). Three independent antigenic epitopes have been recognized on this protein by subcloning of cDNA restriction fragments and analysis of expressed fusion proteins.

Autoantibodies to centromere are present in 70-80% of patients with the subset of scleroderma called CREST. With a few exceptions, this autoantibody is highly specific for the disease. It has been observed that approximately 25% of patients with idiopathic Raynaud's phenomenon (without other signs or symptoms of CREST) have anticentromere antibodies (168). These patients may have an early variant of CREST or a forme fruste.

B. Antibodies to Nucleolar Antigens

Autoantibodies to nucleolar components are part of the spectrum of antibodies present in autoimmune sera and, as described in Table VI, antigens such as RNA polymerase I, fibrillarin and To are confined to the nucleolus. Previously, antinucleolar autoantibodies were used in immunofluorescence microscopy by several investigators to gain insight into the structural organization of the nucleolus (169, 170), and with the current knowledge of the identity of the antigens, studies of this kind should be even more informative.

Autoantibodies to RNA pol I are present in at least 4% of patients with scleroderma (171) and might probably be present at an even higher percentage since this figure was obtained by a process in which scleroderma sera with high-titer antinucleolar immunofluorescent staining were preselected and analyzed for anti-RNA pol I. Thus, sera with lower concentrations of antibodies would have been missed. Careful examination by immunofluorescence showed a characteristic punctate

staining limited to interphase nucleoli, but, in dividing cells, prominent staining of the nucleolus organizer regions were observed (171). In actinomycin-D or 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB)-treated cells, immunolocalization was observed in the fibrillar portion but not in the granular portion of the segregating nucleolus. This was confirmed in immunoelectron microscopy of regenerating rat liver cells (171, 172). These results also confirmed earlier studies showing the immunolocalization of rabbit antibodies to RNA pol I in the fibrillar centers of nucleoli (172). Human autoantibodies immunoprecipitated a complex of proteins in HeLa cells consisting of 13 polypeptides ranging from 210 to 12.5 kDa (Fig. 6). The complex contained four

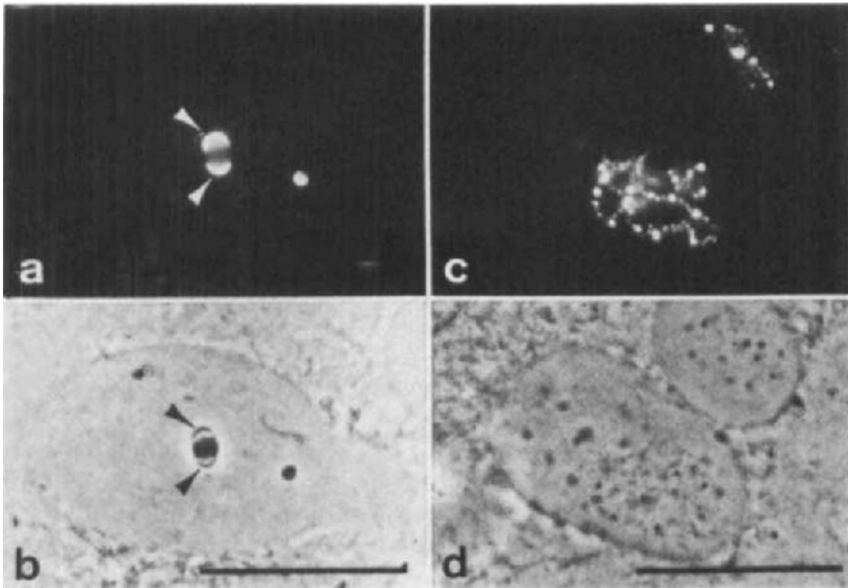

FIG. 6. Vero cells were treated with actinomycin D (a and b) or DRB (c and d), which cause nucleoli to segregate into fibrillar and granular regions. In actinomycin-D-segregated nucleoli, scleroderma serum containing antibody to RNA polymerase I selectively stained the fibrillar region as shown in (a). Immunofluorescence staining was most prominent in two crescents (arrows) in the fibrillar regions. In phase contrast (b) the granular component is in the center of this compact nucleolus, and by reference to immunofluorescence, this area is unstained. In Vero cells treated with DRB, anti-RNA polymerase I antibodies give a necklace-type of staining which is dispersed throughout the nucleoplasm (c, immunofluorescence; d, phase contrast). Bar, 20 μm (from Ref. 171).

phosphoproteins of 180, 80, 64, and 18 kDa, but no small RNAs were coprecipitated. It is conceivable that this protein complex contains not only the genuine subunits of RNA pol I but also other factors which might be associated with RNA pol I transcription. When purified IgG from a serum with high-titered anti-RNA pol I was microinjected into oocytes of *Xenopus laevis*, new synthesis of 28 and 18 S RNA was markedly inhibited (171). On the basis of immunoprecipitation of the polypeptide complex, anti-RNA pol I was detected only in patients with scleroderma and not in other autoimmune diseases, including SLE, MCTD, drug-induced lupus, and polymyositis.

The nucleolar antigen fibrillarin is a 34-kDa protein which reacts well in Western blots with about 8% of scleroderma sera (174). The antigen was called fibrillarin on the basis of its immunolocalization in the fibrillar centers or dense fibrillar component of nucleoli by electron microscopy (172, 175). Several investigators have shown that fibrillarin is a component of the U3 RNP particle of the nucleolus (176, 177). Autoantibodies coprecipitate U3 RNA, and the entire protein complex contained four nonphosphorylated proteins of 36, 30, 13, and 12.5 kDa and two phosphorylated proteins of 74 and 59 kDa (177), of which the 36-kDa protein most likely corresponds to fibrillarin. This protein is basic (p*I* 8.5) and is rich in N^G, N^G-dimethylarginine and glycine (178). It has many of the features of a previously described nucleolar protein, B36, which was isolated from the slime mold *Physarum polycephalum* (179). The epitope on fibrillarin recognized by human autoantibody is also highly conserved, being detectable in nucleoli of many animal species as well as of plants (174, 180).

As described previously in the section on polymyositis, autoantibodies to the Pm-Scl antigen are detected in a clinical syndrome called polymyositis-scleroderma overlap disease (144, 145). The antigen is immunoprecipitated as a complex of 11 proteins ranging from 110 to 20 kDa and is of unknown identity and function. By both immunofluorescence and immunoelectron microscopy, the antigen is present in nucleolus and nucleoplasm.

If tissue culture cell lines such as HeLa or HEp-2 are used as substrates for immunofluorescence, anti-RNA pol I demonstrates punctate nucleolar staining, antifibrillarin clumpy nucleolar staining, and anti-PM-Scl homogeneous nucleolar staining associated with diffuse nucleoplasmic staining. These different morphological patterns of nucleolar staining may not be readily demonstrated for every serum since several factors tend to influence this kind of descriptive characterization, including differences in titers of antibodies. Definitive characterization will depend on immunoprecipitation and Western blotting.

Classification of the specific type of antinucleolar antibody may be important because a preliminary study has shown that anti-RNA pol I tends to be associated with diffuse scleroderma characterized by a high prevalence of internal organ involvement, including renal crisis (181).

Rare scleroderma sera contain autoantibodies to a 40-kDa protein of the nucleolus which is complexed to a 7-2 RNA of the nucleolus (176, 182, 183). The 7-2 RNA was shown to be an RNA pol III transcript and to be sequentially associated with SS-B/La and a second autoantigen, which is most likely the 40-kDa protein (182, 183). The 7-2 RNP particle has been localized by immunoelectron microscopy in the granular regions of the nucleolus (183). Recently an autoantibody in scleroderma has been shown to blot a 90-kDa protein and appears to correlate with immunolocalization in the nucleolus organizer region by immunofluorescence (184). Its identity and function have not been characterized.

VIII. Autoantibodies of Rare Occurrence or Uncertain Disease Association

Table VII describes a number of autoantibodies which occur either more infrequently than those which have been discussed or where the specific disease associations are still uncertain.

Autoantibodies to nuclear lamins are targeted at three proteins of 74, 68, and 60 kDa, which are also called A, B, and C lamins, respectively (185, 188). Because of strong amino acid sequence similarity between lamin proteins and intermediate filaments (IFs), lamins are now considered to be a new class of IF proteins which are part of the cytoskeleton (189, 190). The nuclear lamina is a meshwork of these IFs that lines the inside or nucleoplasmic surface of the inner nuclear membrane, and it is postulated that the nuclear lamina provides an anchoring site for interphase chromosomes (189). Autoantibodies are detectable by Western blotting, and in immunofluorescence react with a characteristic linear staining of the nuclear envelope which disintegrates during the mitotic phase of cell division. In contrast, autoantibodies to DNA and histones which may at times simulate this type of immunofluorescence localization would show strong staining of condensed chromosomes during mitosis. At first analysis, it might appear that autoantibodies to nuclear lamins are present in a variety of diseases, including linear scleroderma (185), SLE (186), and a heterogeneous group of patients with thrombocytopenias, neutropenias, and liver disease (187, 188). Closer analysis tends to suggest that the underlying common clinical feature in all patients might be chronic autoimmune liver disease, either chronic active hepatitis or chronic biliary cirrhosis with other associated clinical features (191).

TABLE VII

CELLULAR ANTIGENS AND AUTOANTIBODIES OF RARE OCCURRENCE OR UNCERTAIN DISEASE ASSOCIATION

Antigen		Disease association	Reference
Clinical designation	Molecular identity		
Nuclear lamins	Lamin proteins, 74, 68, and 60 kDa	Linear scleroderma, SLE, hepatitis	185-188
Poly(ADP-ribose) polymerase	Protein, 116 kDa; poly(ADP-ribose) polymerase	Sjögren's syndrome, neuropathy	193,195
Alu-RNA protein	Protein, 68 kDa, complexed with Alu-RNA	SLE	196
MSA	Proteins of mitotic spindle apparatus	Miscellaneous diseases	197,198
Sp100	Protein, 100 kDa	Miscellaneous diseases	199
Centriole/centrosome	Not identified	Scleroderma	200-202
Golgi body	Not identified	Miscellaneous diseases	203,204
Midbody	Not identified	Miscellaneous diseases	205
NSp I and II	Not identified	Miscellaneous diseases	206
Metaphase chromosome core	Not identified	Scleroderma	207

Poly(ADP-ribose) polymerase (ADPRP) is a single polypeptide of 116 kDa which is a DNA-binding protein and catalyzes the DNA-dependent transfer of ADP-ribose from nicotinamide adenine diphosphate (NAD) to nuclear proteins to form elongated and branched poly(ADP-ribose) chains (192). This function is postulated to be involved in DNA repair and cell differentiation in eukaryotes. Recently, autoantibodies to ADPRP were identified in several patients, some of whom had Sjögren's syndrome and others having ill-defined clinical syndromes associated with neuropathy (193). ADPRP has been extensively characterized as to its structure and functional domains, and recently a full-length cDNA clone has been isolated (194). Further studies with the autoantibodies have shown that a major epitope lies within the NAD-binding domain of the enzyme, and autoantibodies are capable of inhibiting the catalytic activity of the purified enzyme, as measured by the transfer of ADP-ribose from [^{32}P]NAD to either histones or autocatalytically to ADPRP (195).

It has been demonstrated repeatedly in this presentation that autoantibodies which are present spontaneously in many diseases have the potential of becoming extremely useful tools for studies of structure and function of molecules involved in intracellular biology. There are therefore good reasons for observing and recording the presence of other autoantibodies described in Table VII. With some of these autoantibodies, the target intracellular organelle can be easily identified on the basis of classical morphology, such as the mitotic spindle apparatus (MSA) (197, 198), centriole (200–202), and Golgi body (203, 204), although more precise information concerning the antigens is not available. In other cases, the antigens have been described on the basis of patterns of intracellular localization. The associations of these autoantibodies with specific diseases will need to be further clarified but the present state of uncertainty may in part be related to their relative rarity.

IX. Functions of Some Autoantigens

It is fairly clear that many autoantigens are proteins or subcellular particles which are involved in important or even essential biological functions in the cell. Table VIII lists in tabulated summary some of these functions. The biological functions which have been confirmed by the critical demonstration that autoantibodies were inhibitory are splicing, DNA replication, DNA repair, transcription, and aminoacylation of certain species of tRNAs. Functions of other autoantigens have not been as clearly demonstrated but there is good evidence that they might be involved in the following functions: processing of RNA pol I and pol III transcripts, DNA binding, maintenance of nuclear architecture, cell

TABLE VIII
FUNCTIONS OF SOME AUTOANTIGENS

Antigen	Function
I. Demonstrated functions	
Sm (U1, U2, U4–U6, and snRNPs)	Splicing of pre-mRNA
nRNP (U1 snRNP)	Splicing of pre-mRNA
PCNA (DNA pol δ auxiliary protein)	DNA replication
Poly(ADP-ribose) polymerase	DNA repair, cell differentiation
Scl-70 (DNA topo I)	Transcription, DNA replication
RNA pol I	Transcription of rDNA
tRNA synthetases	Aminoacylation of $tRNA^{His,Thr,Ala}$
II. Probable functions	
SS-B/La	Processing of RNA pol III transcripts
Fibrillarin (U3 RNP)	Processing of RNA pol I transcripts
Ku	DNA-binding proteins
Nuclear lamins	Nuclear architecture and chromosome-binding proteins
Centromere proteins	Cell division and spindle attachment
rRNP	Ribosomal protein synthesis
Signal recognition particle	Protein translocation across endoplasmic reticulum

division, ribosomal protein synthesis, and protein translocation across endoplasmic reticulum. The functions of other autoantigens such as DNA and histones are now too global to assess, but in the case of histones, it might be possible to address this question if the antigenic epitopes on histones can be more precisely localized so that the function of these regions can be determined. It is indeed amazing that so many structures related to processes regulating essential functions such as cellular replication, transcription, and translation can invoke autologous immune responses. Elucidating some of these mechanisms should be challenging as well as stimulating.

X. Molecular Cloning of Autoantigens

Although sera from human autoimmune disease patients generally tend to contain multiple autoantibodies, by diligent search it is not unusual to find some sera which are relatively monospecific or contain a desired type of autoantibody in high titer. Such sera have been useful in the molecular cloning of cDNA from cDNA expression libraries. In addition to the application of the cDNAs for study of the structure and function of the expressed protein, the availability of molecular clones

provides an approach to the study of antigenic determinants recognized by the autoantibodies.

This approach has also been used to isolate genomic clones which encode for RNAs complexed to proteins, although the proteins and not RNA contain the epitopic determinants (208). Ro RNAs were selected from unlabeled cell sonicates by immunoprecipitation with human autoantibody, were phenol extracted, and were labeled at the 3′ end with [^{32}P]pCp and T4 RNA ligase. This was used as a highly selective probe to screen a human placental DNA library and a genomic clone was obtained which contained the RNA-coding regions for two human Ro RNAs, hY1 and hY3. The genes were shown to be transcribed *in vitro* by RNA polymerase III and in contrast to other mammalian class III genes, they appeared to be present as single copies in the human genome.

Autoantibody to the La antigen was used to isolate cDNA clones from human liver λgt 11 expression libraries (209). Recombinant clones were used to hybrid select HeLa cell mRNA that was translated *in vitro*, producing an apparently authentic La protein, which reacted with anti-La sera and was identical to isolated cellular La protein by peptide mapping. By analyzing the protein products of overlapping cDNA clones, an autoantigenic site of La protein was identified in the region of the terminal 12% of the carboxyl end of the nucleus.

One of the core proteins of snRNPs is the E protein, of approximately 11 kDa, which is less reactive with lupus sera than other snRNP core proteins. A cDNA clone, p281 was identified in a HeLa cell cDNA library with the use of a lupus autoantibody (210). Clone p281 hybridized with poly(A)+ mRNA of about 600 nucleotides in human and marmoset cells, and Southern blot analysis indicated the presence of 6-10 copies of p281 homologous sequences in human, baboon, cat, and mouse genomic DNA but no cross-hybridization was obtained with frog or *Drosophila* DNA. This moderate degree of evolutionary conservation of the E antigen was interpreted as consistent with previous observations that the snRNP E proteins elicited weaker autoimmune responses than other snRNP core proteins, such as B and D.

Other cDNAs encoding the Sm and U1 RNP autoantigens have also been cloned. The B″ protein is a protein specifically associated with U2 snRNP, and a full-length human cDNA has been shown to express a polypeptide with antigenic determinants and peptide map identical with isolated cellular protein (211). From the deduced polypeptide sequence, three hydrophilic regions were identified which were suggested to be RNA-binding sites or antigenic determinants for the autoantibodies. This laboratory has also cloned cDNA encoding the human U1 snRNA-associated A protein (212). By comparison of the deduced amino acid

sequence of the A protein with the B″ protein, two regions of extensive sequence similarity were observed, one located in the carboxyl-terminal region (86% sequence similarity) and the other in the amino-terminal region (77% sequence similarity). It was postulated that this might infer identical functions for the two proteins, although they are located on different snRNP particles.

The 70-kDa protein, a target antigen of sera from MCTD patients, has been cloned by two laboratories. The cDNA clones were isolated with antisera from a human liver cDNA expression library. Further screening of a HeLa cDNA library with the original clone yielded an overlapping clone which contained the complete coding sequence of the 70-k protein, in addition to 3′-unstranlated and 5′-upstream sequences (213). Northern blot analysis of RNA from a hepatoma cell line and from HeLa cells with nick-translated cDNA revealed RNAs of 3.0 kb from hepatoma cells and 3.0 and 1.7 kb from HeLa cells, suggesting that the 70-k protein may be polymorphic. Hydrophilic regions were identified at the carboxy-terminal half of the protein which could function in RNA binding. The second report of cDNA cloning of the 70-k protein was a partial sequence derived from a human brain-stem library (214). Attention was called to a degree of sequence similarity between a region containing 23 deduced amino acid residues and a region of murine leukemia virus group-specific antigen $p30^{gag}$. Anti-$p30^{gag}$ antibodies were shown to be reactive with the recombinant 70-k–lac Z fusion protein. On the basis of these findings, it was suggested that retroviruses may play a role in the initiation of autoimmunity.

The centromere antigens consist of three proteins of 17, 80, and 140 kDa, also called CENP-A, CENP-B, and CENP-C. cDNA clones encoding 95% of CENP-B have been isolated (167). Two nonoverlapping epitopes recognized by human autoantibodies have been identified by overlapping cDNA subcloning, but only one of these is the major epitope. Analysis of structure of CENP-B from the deduced amino acid sequence revealed interesting features, including the presence of highly acidic domains at the carboxy terminus which was thought to be unusual for a chromatin protein. However, it was suggested that these domains might function to interact with basic proteins such as histones in order to unravel the higher order structure of chromatin.

The N-terminal amino acid sequence of PCNA (47) was used to construct oligonucleotide probes to clone PCNA cDNA from a rat thymocyte library (116). A different strategy was used by another laboratory by using antibody raised in a rabbit immunized with purified PCNA from MOLT-4 cells to screen an expression library (117). The cDNAs of both species contain the entire coding sequence but the nucleotide sequences

are divergent in the untranslated regions. In the coding sequences, both rat and human clones encode polypeptides of 261 amino acids with estimated molecular weight of 29,000 compared to values obtained by SDS-polyacrylamide gels of molecular weight 33,000 to 36,000. This discrepancy is likely due to aberrant migration of the protein and not to posttranslational modification. The amino acid sequence between human and rat protein are strikingly similar, with only four amino acid changes. The sequence contains a helix-turn-helix motif that is a feature of many DNA-binding proteins (117).

Recently, cDNA cloning of the C protein of the U1 RNP complex has been reported (215). Even though the clone was not a full-length cDNA, the expressed fusion protein appeared to contain the entire antigenic region of the C protein.

In addition to published reports, many laboratories have been successful in isolating cDNA clones which encode other human autoantigens. These include DNA topoisomerase I, the B′ and D proteins of the Sm antigen, and SS-A/Ro (personal communications).

XI. The Autoimmune Response—A Hypothesis

The growing number of autoantibodies which are being identified is posing a challenge to investigators interested in elucidating biological mechanisms underlying autoimmunity. It is obvious that hypotheses for or models representing the type of autoimmunity discussed here will have to address the factual observations reported. The key observations include the findings that certain autoantibody responses are highly specific and disease restricted. These include anti-Sm in SLE, anti-DNA topoisomerase I in diffuse scleroderma, anticentromere in CREST, and anti-tRNA synthetases in polymyositis. A second feature is the clustering of certain antibodies in each disease, so that there is a unique autoantibody profile associated with an individual disease. SLE and scleroderma are characterized by a great diversity of antibodies whereas MCTD and drug-induced lupus represent relative homogeneity of antibodies. The new information on the structure and function of many autoantigens may be providing some insights into these questions. The nature of the Sm and nuclear RNP antigen was perceived at the immunochemical level as physically related antigens, with autoimmune responses in SLE being often coupled. The demonstration that these antigens are components of different snRNP particles which assemble into spliceosomes for splicing of pre-mRNA makes it possible to consider that the immune response might be directed against such subcellular units of known structure and function. Based on the current knowledge

of the molecular identity of many autoantigens and the considerations just discussed, a hypothesis is advanced to explain the specificity of the autoimmune response.

A. The Autoimmune Response is also Antigen Driven

One approach to analyzing the nature of the autoimmune response is to determine its clonality. In the case of systemic autoimmunity, this can be done by studies of the epitopes represented on target antigens. At the individual protein level, several studies have shown that more than one epitope is present on a single protein antigen. CENP-B antigen has been cloned and analysis of proteins produced by truncated cDNAs in expression vectors has shown the presence of two different epitopes (167). Using a different approach, PCNA and SS-B/La antigens were purified and shorter length degradation fragments were produced by limited proteolysis (37,121). In PCNA, different epitopes were identified on fragments derived from the amino and carboxyl domains of the protein, and, similarly, two different epitopes were defined on SS-B/La. In an *in vitro* lymphocyte proliferation assay, several different proteolytic fragments were capable of stimulating proliferation of lymphocytes from patients with SLE (216). Thus even at the level of the individual protein antigen, the immune response is polyclonal and directed at more than one determinant. When the disease is considered as a whole, polyclonality is further reflected in the great diversity of separate proteins, nucleic acids, or RNA-protein complexes which are antigenic. This polyclonality exists at the same time as specificity of certain antigens for an individual disease. Taken together, the data indicate that whatever the nature of the disturbance in immunoregulation, it must also be influenced or driven by highly specific antigens. The concept of molecular mimicry, which proposes that an autoantibody arises as a result of an immune response to an exogenous agent bearing an epitope identical or similar to host protein, may be attractive for diseases associated with one species of autoantibody. It would be more difficult to envisage the process of molecular mimicry being exercised several times for each disease, which would be required to produce the multiple autoantibodies detected in SLE, scleroderma, and polymyositis.

B. Immunogens are Dynamic Subcellular Particles

Some difficulties with the hypothesis that the autoimmune response is antigen driven are observations that antigens in diseases such as lupus (Table I) appear to be resident in different structural domains. Although the majority of antigens in lupus are nuclear in location (for DNA, histone, snRNPs, SS-B/La, Ku, and PCNA), some are cytoplasmic, such as ribosomal RNP and Hsp 90. Conceptually, it would be more

attractive if antigens in SLE were in proximity with each other or even in the same structural domain and more complicated to comprehend if they were in different domains and apparently unrelated to each other biologically or structurally. However, rRNP antigen has also been immunolocalized in the nucleolus in addition to the cytoplasm (102) and it is becoming increasingly clear that the SS-A/Ro antigen may be present in both cytoplasm and nucleus (89, 217).

Cursory examination of the total autoimmune response in scleroderma also appeared to suggest that antigens were segregated in at least two different structural domains, the nucleoplasm for Scl-70 (topoisomerase I) and centromere antigens, and the nucleolus for RNA polymerase I, 34-kDa protein of U3 RNP, and 7-2 RNA. PM-Scl antigen was detected in both nucleolus and nucleoplasm. Recent studies with immunofluorescence have shown that DNA topoisomerase I is strongly localized in the nucleolus of avian MSB-1 cells (218) and in certain preparations of human HeLa cells (26). In MSB-1 cells, nucleolar topoisomerase I was associated with ribosomal DNA genes and was catalytically active in transcription (218), so that it might have been enriched in the nucleolus in response to a specific functional requirement. These data merely emphasize known observations that some biologically active intracellular molecules have the ability to migrate to different domains of the cell, a property which might be related to cell function or cell cycling. A further interesting observation concerning scleroderma antigens is that both topoisomerase I and RNA polymerase I have been colocalized by immunofluorescence microscopy (19, 20) and by immunoelectron microscopy (218, 219) in the nucleolus-organizing regions (NORs), organelles which are visualized during mitosis, and are the initiation sites of nucleolar reorganization following cell division. There is also preliminary evidence that centromere antigens were colocalized with topoisomerase I in certain subcellular particles of mouse 3T6 cells during anaphase (19).

In the light of knowledge that many autoantigens are enzymes or active proteins engaged in important cell functions, it would be necessary to revise our prevailing concepts concerning the strict segregation of autoantigens into nuclear, nucleolar, or cytoplasmic domains. There is evidence that at different phases of cell growth and differentiation, some biologically active proteins may be segregated in certain cell domains to meet the need to participate in a specific type of cellular response. Autoantibodies and murine antibodies to Sm, 70kDa protein associated with U1 RNP, centromere, and other nuclear antigens have been used to track the distribution of these antigens in interphase and mitotic cells to documents the dynamism of the particles which contain the antigens (220–225).

The total repertoire of autoantigens for a given disease could therefore be localized on a single or a limited number of dynamic subcellular particles, since each particle is an assembly of multiple proteins with or without associated nucleic acids. The immune response to the particle or particles could be limited to a few epitopes or to the total repertoire of epitopes on each particle, but the extent of antibody diversity for any given disease would be restricted to determinants available on the particle. Such an immune response could explain both the diversity and specificity of autoantibodies which have been observed. Other factors must also have roles in determining the total outcome of the autoimmune response. For example, genetic factors regulating T cell involvement or macrophage processing might decide whether the immune response is made to Sm or to SS-B/La antigens, since only 30 and 15%, respectively, of patients with SLE make these autoantibodies, while others do not throughout the lifetime of the disease.

C. Epitopes are Active Sites, Catalytic Centers, or Functioning Regions

It has been convincingly demonstrated that many autoantibodies inhibit function when incorporated into *in vitro* or *in vivo* assays which measure specific cellular activities. Autoantibodies to Sm and nuclear RNP inhibited splicing of adenoviral early RNA sequences and other forms of pre-mRNA (24, 74, 75). Every type of autoantibody to tRNA synthetase ($tRNAs^{His,Thr,Ala}$) has inhibited the aminoacylation of its cognate tRNA (144, 145, 147, 148). Autoantibody to DNA topoisomerase I inhibited untwisting or relaxation of supercoiled DNA (18, 19) and autoantibody to RNA polymerase I inhibited 28 and 18 S RNA synthesis in frog oocytes (171). The studies provided the critical data as to the nature and identity of the antigens recognized by the autoantibodies and in the case of splicing, also gave further insights by helping to dissect this mechanism as a multistep process in which autoantibody to Sm or nuclear RNP would inhibit certain steps in the process (226-228). The further significance of these findings has come from the following studies which indicate that autoantibodies may be special in that they recognize the active sites or catalytic centers of the molecules which are engaged in these functions.

In order to map the epitopes on PCNA which were recognized by autoantibodies, the strategy adopted was to analyze the specificities of three different groups of antibodies: murine monoclonal antibody, rabbit antisynthetic peptide antibody, and human autoantibody. Two murine monoclonal antibodies, 19A2 and 19F4, were obtained by immunization of BALB/c mice with purified rabbit thymus PCNA (113). A 13-mer

peptide which comprised the eleventh to the twenty-third residue of the amino terminus of PCNA was synthesized (47) and antibodies induced in rabbits by immunization in Freund's complete adjuvant. Both monoclonal and antipeptide antibodies were strongly reactive in immunoblotting with PCNA separated on one- and two-dimensional gel electrophoresis. PCNA was then subjected to controlled proteolysis with *S. aureus* V8 protease and degradation fragments were probed for reactivity with autoantibodies versus experimentally induced antibodies. The rabbit antipeptide antibody provided the marker to denote that a degradation fragment contained the amino terminus of PCNA. With this strategy, it was shown that human autoantibodies recognized at least two epitopes, one toward the amino terminus and the other toward the carboxy terminus, but these epitopes were different from the 13-mer region of the synthetic peptide or the epitopes recognized by murine monoclonal antibodies. The two monoclonals recognized identical or overlapping epitopes in the central region of PCNA, between the mapped human epitopes (121). The three groups of antibodies were analyzed in an *in vitro* assay system where poly(dA)/oligo(dT) (20:1) was used as the template/primer in auxiliary protein-coupled DNA polymerase δ-regulated nucleotide synthesis (120). Only human autoantibody neutralized the activity of auxiliary protein in this nucleotide replication system. It was noticed that undenatured auxiliary protein was immunoprecipitated only by human autoantibody and not by antipeptide or monoclonal antibodies, suggesting that only the autoantibody epitope was exposed in the native protein.

A strikingly similar observation was made in a study comparing the effect of human autoantibody and rabbit polyclonal antibody to purified threonyl-tRNA synthetase (151). When both antibodies were probed for reactivity in Western blots with crude or purified synthetase which had been subjected to SDS-acrylamide gel electrophoresis and transferred to nitrocellulose, rabbit antibody was strongly reactive whereas human autoantibody was not. However, human autoantibody was positive in immunoblotting against undenatured synthetase dotted on nitrocellulose and was also able to immunoprecipitate the 80-kDa synthetase protein from extracts of ^{32}P-labeled reticulocytes. The interesting observation was that human autoantibody inhibited ability of synthetase to aminoacylate tRNA^{Thr}, whereas rabbit antibody was ineffective.

It is a widely accepted opinion that evolutionary conservation of nucleotide sequences is related to the fact that such sequences or their corresponding polypeptide products were subserving important cellular functions. The frequently made observation that epitopes recognized by human autoantibodies are highly conserved was reemphasized in a

study of the SS-B/La antigen. Five murine monoclonal antibodies were produced from a mouse immunized with purified calf thymus SS-B antigen (141). All five monoclonals reacted positively in Western blots with the 48-kDa SS-B/La protein, but only three of five immunoprecipitated pre-tRNAs from ^{32}P-labeled cell extracts reacted, indicating that only these three monoclonals were recognizing "exposed" or native determinants on the pre-tRNA–protein particle. However, even these monoclonals were recognizing determinants which were less highly conserved than human autoepitopes. In immunofluorescence on tissue culture cells, monoclonals reacted with SS-B/La in human, monkey, rabbit, and bovine cells, but not with hamster, rat, and mouse, whereas human autoantibodies reacted equally well with the entire range of species tested.

Further insights into the possible mechanisms underlying these observations have come from the laboratory of Luhrmann (229, 230). Native snRNP particles were affinity purified by using antibodies to 2,2,7-trimethylguanosine, which is part of the 5′-terminal cap structure of snRNAs U1 to U5, and elution of snRNPs from affinity columns was made with excess trimethylguanosine nucleoside. An isolated native U1 snRNP particle was used for mouse immunization and monoclonal antibodies analyzed for epitope specificity. This was compared to epitope specificity of human autoantibodies to Sn/RNP by cross-inhibition in solid-phase immunoassay. Monoclonal antibodies defined as reactive with A and B′ antigens of U1 RNP and with 70-kDa antigen could be competed out effectively by human autoantibodies of the same specificities. Attesting to the validity of the observations was the finding that a fourth monoclonal, reactive with a determinant on A but shared by B″ protein, was not inhibited by these autoantibodies.

In summary, many observations indicate that epitopes recognized by human autoantibodies are the active sites of proteins or catalytic centers of particles. These epitopes are not the only determinants on the antigens which are capable of inducing immune responses, but the epitopes which induce autoantibodies appear to be those which are expressed on native proteins or subcellular particles, and in many cases are lost in dissociated or denatured particles.

XII. Clinical and Biologic Significance of the New Biology of Autoantibodies

Antinuclear antibodies, or, more properly, autoantibodies, to intracellular antigens have already established their importance in clinical medicine as diagnostically specific markers for SLE, scleroderma, Sjögren's syndrome, mixed connective tissue disease, drug-induced lupus, and dermatomyositis/polymyositis. In terms of their practical applica-

tion, there is a significant lag in transfer of this knowledge from the bench to the bedside. It has become apparent that there is a need for a system of tests which can detect a wide spectrum of ANA specificities since these diseases are characterized by profiles, each of which consists of many antibody specificities. Some individual autoantibodies may be present in sufficiently high frequency to reach statistically significant sensitivity but this is usually not attended with specificity for the disease. With the currently available immunochemical assays, the goal of developing an assay system capable of displaying a meaningful spectrum of ANAs might be beyond reach because of the complexity of some assays and because of their high costs. However, as more and more of the genes encoding autoantigens are cloned, it might be expected that purified antigens can become more readily available and assays could be developed to cover a wide spectrum of autoantibody specificities.

Although their importance in diagnosis has been established, the role of autoantibodies in disease pathogenesis is unclear. Convincingly, only antibody to DNA has a documented role in pathogenesis, via the formation of circulating or *in situ* immune complexes with DNA antigen (25, 59). This pathogenetic mechanism is dependent on the exteriorization of antigenically competent DNA which can then react with antibody to trigger the liberation of inflammatory mediators. There are currently no convincing reports that other intracellular antigens have been detected in extracellular sites so that they might be able to react with their cognate autoantibodies.

Are there other mechanisms which might implicate autoantibodies in pathogenesis? It has been reported by some but refuted by others that autoantibodies can penetrate cell membranes and thus react with intracellular antigens. Discussion on the issue of cellular penetration by autoantibody is moot because no reliable data are available. Examination of the information on Table VIII should bring into focus another important issue. Many antigens are involved in essential or important functions of cells, such as pre-mRNA splicing, DNA replication, transcription, and protein synthesis, and autoantibodies have been shown to inhibit such functions. It would be difficult to imagine autoantibodies interfering with or inhibiting these universal and essential cellular activities without some overt evidence of their action. It would also be difficult to understand how, say, inhibiting DNA topoisomerase I activity results in the tissue lesions of scleroderma or how inhibiting certain tRNA synthetases results in the muscle inflammation of polymyositis. It seems appropriate to state at the present time that most autoantibodies to intracellular antigens are not known to be involved in pathogenesis of tissue lesions.

Although the new biology of autoantigens and autoantibodies has not led us to a better understanding of tissue pathogenesis, it has provided us with new insights and understanding into the nature of autoantigens

and perhaps into what we should be looking for in terms of inciting events. Analysis of the data point strongly toward active or functioning subcellular particles as immunogens. Already, attention can be focused on particles which are involved in certain specific activities such as pre-mRNA splicing, polymerase δ-mediated DNA replication, polymerase I-mediated transcription, and certain tRNA synthetase-regulated amino acylation, and there are others. The evidence that such functioning particles are the immunogens needs to be further explored. There is evidence that these immunogenic particles are dynamic subcellular oganelles. Is it possible that these organelles might be induced into activation and multiplication by certain stimuli, such as external biological agents, chemicals, drugs, and environmental factors? In the case of scleroderma, the nucleolus may be the compartment from which such immunogenic particles are generated. All the identified autoantigens can be associated with the nucleolus or nucleolar anlage at one phase or another of the cell cycle (231). And finally, how do they become immunogenic?

Spontaneously occurring autoantibodies in human disease have already been useful as powerful tools to dissect and clarify important biological processes and will obviously continue to be valuable resources for understanding other processes regulating cell function. The evidence that autoantibodies recognize active sites or functioning regions might not be surprising if one considers the growing acceptance of the notion that antigenic regions of proteins are surface exposed, hydrophilic, and relatively mobile (232), which would be the property of active sites or catalytic centers. Autoantibodies might be regarded as "reporter molecules" for the active sites of intracellular functioning particles and the use of autoantibodies might therefore be a direct approach to the identification of such active sites.

The clinical science of autoimmunity has gained much from the contributions of molecular and cell biology and the benefits have been returned in some measure. In this area of research, there has already been a stimulating interaction between the clinical biologist and molecular and cell biologists to advance our knowledge in many fields.

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Interleukin-1 and Its Biologically Related Cytokines

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I. Introduction

Interleukin-1 (IL-1) is the term for two polypeptides (IL-1α and IL-1β) that possess a wide spectrum of immunologic and nonimmunologic activities. Although both forms of IL-1 are distinct gene products, they recognize the same receptor and share the same biological properties. IL-1 is produced in response to infection, microbial toxins, inflammatory agents, products of activated lymphocytes, complement, and clotting components. Its name as an interleukin, which means "between" leukocytes, is somewhat inappropriate because IL-1 is synthesized by both leukocytic as well as nonleukocytic cells; furthermore, IL-1 effects are not restricted to leukocytes but rather are manifested in nearly every tissue. Nevertheless, the term "interleukin" is often used for lack of a better system of nomenclature. To date, the primary amino acid sequences for eight molecules of human origin have been reported (IL-1α, IL-1β, and IL-2 through IL-7); most of these molecules share some biological activities.

There are other polypeptides for which the primary human amino acid sequences are known, but they are not called interleukins. IL-1 is the prototype of a group of biologically potent polypeptides with molecular weights between 10,000 and 30,000. Because these substances are produced by a variety of cells and act on many different cell types, there is a growing acceptance of the terminology "cytokines," rather than "lymphokines" or "monokines." Of the various "cytokines," several share the ability to stimulate or augment cell proliferation, initiate the synthesis of new proteins in a variety of cells, and induce the production of inflammatory metabolites. IL-1 is biologically similar to tumor necrosis factor (TNF), lymphotoxin, IL-6, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β). Although monocytes and macrophages are, in fact, sources of these cytokines, some are prominent products of platelets, fibroblasts, keratinocytes, and endothelial cells. In general, the various cytokine polypeptides possess diverse biological properties, most of which are

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associated with either host responses to various disease states or participate as part of a pathological process.

IL-1 was originally described in the 1940s as a heat-labile protein found in acute leukocytic exudate fluid, which, when injected into animals or humans, produced fever. This material was a small protein (10–20 kDa) and was called endogenous pyrogen (Atkins, 1960). In the 1970s it was shown that 30–50 ng/kg of homogeneous endogenous pyrogen produced monophasic fever in rabbits (Murphy *et al.*, 1974; Dinarello *et al.*, 1977). No amino acid sequence for endogenous pyrogen was known, but studies demonstrated that endogenous pyrogen did more than cause fever. When injected into animals it induced hepatic acute-phase protein synthesis, caused decreases in plasma iron and zinc levels, produced neutrophilia, stimulated serum amyloid A protein synthesis, and augmented T cell responses to mitogens and antigens *in vitro* (Kampschmidt, 1981; Dinarello, 1984). The multiple biological activities of endogenous pyrogen, particularly its ability to affect immunocompetent cells, resulted in changing its name to IL-1. The name IL-1 now includes several substances originally named for their biological activities, but according to data generated with recombinant IL-1 or compared to N-terminal amino acid sequences, these are identical to IL-1. These are leukocytic endogenous mediator (Kampschmidt, 1981), lymphocyte-activating factor (Gery and Waksman, 1972), mononuclear cell factor (Krane *et al.*, 1985), catabolin (Saklatvala *et al.*, 1985), osteoclast-activating factor (Dewhirst *et al.*, 1985), and hemopoietin-1 (Moore and Warren, 1987; Mochizuki *et al.*, 1987).

The various cytokines listed above are for the most part structurally distinct, with the exception of IL-1α/IL-1β and fibroblast growth factor. IL-1α and IL-1β, despite distinct primary amino acids sequences, are structurally related as shown by molecular modeling, crystallographic analysis, and receptor recognition. A similar case exists for TNF and lymphotoxin. In addition, IL-1β is structurally related to fibroblast growth factors (acidic forms) and shares the growth-promoting properties of these molecules (Thomas *et al.*, 1985). TNF and IL-1 induce some nearly identical biological effects, particularly those associated with systemic and local inflammatory as well as destructive joint disease (Dinarello, 1986; Beutler and Cerami, 1986; Krane *et al.*, 1985; Dayer *et al.*, 1985), but share only 3% primary amino acid structure. Receptors for IL-1 and TNF are distinct.

Considerable interest has focused on IL-1, TNF, and IL-6 as mediators of systemic "acute-phase" responses. Injecting experimental animals with either IL-1 or TNF results in fever, hypozincemia, hypoferremia, increased hepatic acute-phase proteins synthesis, and other manifestations of the

response. IL-6 induces fever and hepatic acute-phase protein synthesis. Recent evidence suggests that IL-6, like IL-1 and TNF, is present in human body fluids associated with inflammatory and febrile diseases. This chapter focuses on IL-1, but the related cytokines, TNF and IL-6, are also discussed, particularly as these molecules share biological properties with IL-1.

II. Interleukin-1 Structure

Two forms of IL-1 have been cloned; IL-1β was cloned from human blood monocytes (Auron *et al.*, 1984) and IL-1α was cloned from the mouse macrophage line P388D (Lomedico *et al.*, 1984). Subsequent to the description of cDNAs to these two forms, IL-1β has been cloned in the cow, rabbit, rat, and mouse and IL-1α has been cloned in the human, rat, and rabbit. It is unclear whether more than these two gene products exist for IL-1. IL-1β is the prominent form of IL-1 and the amount of IL-1β mRNA found in activated cells is usually 10- to 50-fold greater than the α form. In addition, culture supernates and various human body fluids contain more IL-1β than the α form. However, several studies have shown that IL-1β is readily secreted from activated cells whereas IL-1α remains cell associated.

Originally identified as a p*I* 7 (IL-1β) and p*I* 5 (IL-1α) species on isoelectric focusing, the two forms of IL-1 are initially synthesized as 31-kDa precursor polypeptides and share only small stretches of amino acid homology (26% in the case of the two human IL-1 forms). Neither form contains a signal peptide sequence which would indicate a cleavage site for the N-terminus. This fact makes IL-1 a highly unique substance. Other cytokines such as TGF and TNF have clearly identifiable signal peptide sequences. Lacking a clear signal peptide, a considerable amount of the IL-1 that is synthesized remains cell associated (Lepe-Zuniga and Gery, 1984). In fact, membrane-associated IL-1 is biologically active and may be the form which participates in activating lymphocytes, particularly in lymphoid tissue where lymphocytes form rosettes around macrophages (Kurt-Jones *et al.*, 1985). "Membrane-bound" IL-1 is also active on nonlymphocyte target cells. The steps involved in transcription, translation, and "processing" of IL-1 are discussed below.

Within the various animal species of IL-1β, the primary amino acid sequences are conserved in the range of 75–78% whereas the α sequences are in the range of 60–70%; between the β and α IL-1 within each species, conserved amino acid homologies are only 25%. The entire human genes for each IL-1 form have also been cloned (Clark *et al.*, 1986; Furutani *et al.*, 1986). Each gene contains seven exons coding for the processed

IL-1 mRNA and raising the possibility of alternate RNA processing. The gene for human IL-1β is located on chromosome 2 (Webb *et al.*, 1986); the gene for mouse IL-1α is also on chromosome 2. The existence of other IL-1 forms (as separate gene products or the result of processed mRNA) has recently been introduced in studies on IL-1 from Epstein–Barr virus-infected human B cells (Rimsky *et al.*, 1986). However, it remains to be shown whether these latter cells produce a different IL-1 gene product or that posttranslational processing results in IL-1 products of different molecular weights and charges. The N-terminal amino acid sequence reported for B cell IL-1 (Rimsky *et al.*, 1986) is unrelated to the N-termini of IL-1β or IL-1α. Recently, a T cell factor with physical characteristics similar to those of the human B cell IL-1 has been shown to possess the identical N-terminal amino acids (Tagaya *et al.*, 1988). This T cell factor is biologically related to IL-1 in that it induces the p55/TAC antigen on T cells. The factor is derived from human adult T cell lymphotropic virus-1-transformed T cell lines. It appears that the IL-2R-inducing lymphokine is the same as B cell IL-1.

When the amino acid sequences of the two IL-1 forms are compared, only four small regions of amino acid homologies exist. These have been identified and called regions A–E (Auron *et al.*, 1985) (see Fig. 1); since regions A and B are contained in the precursor sequence, which is missing

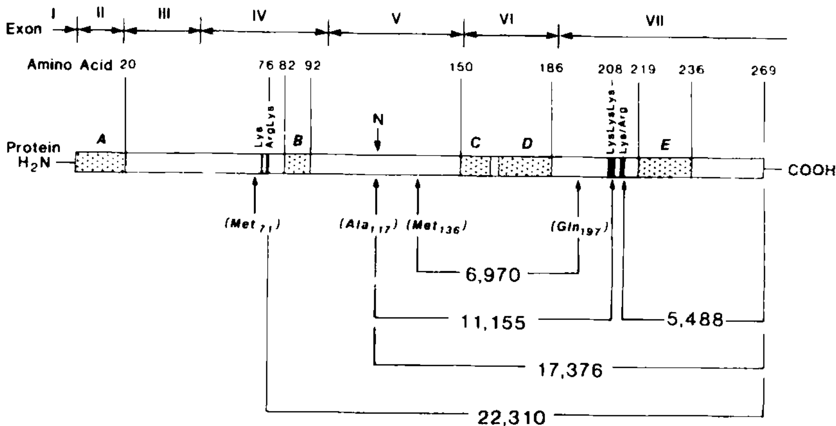

FIG. 1. Structure of IL-1β. The top line depicts the seven exons of the human IL-1β gene (adapted from Clark *et al.*, 1986). The bar represents the IL-1β precursor protein, with the stippled areas showing the amino acid regions homologous with IL-1α (adapted from Auron *et al.*, 1985). The amino acid numbers are shown as likely trypsinlike cleavage sites. The predicted size of each IL-1 fragment is given (adapted from Auron *et al.*, 1987).

in the mature IL-1 form, the important regions of homology for the two mature IL-1 forms are located in the carboxyl C–D and E regions. These regions may represent a putative "active site" of the IL-1 molecule which would explain the observation that although the two forms are structurally distinct, they share the same spectrum of multiple biological properties and recognize the same receptor. Of interest to the evolution of IL-1 is that the C–D region of IL-1β/α homology is coded by the entire VIth exon and this region also contains some limited amino acid homology with interferon-α2 and interferon-β1. There is also a small (3%) region of amino acid homology with TNF but this region does not correspond to the IL-1-β/α homologous regions.

Since both mature forms of IL-1 recognize the same cell receptor and both forms possess the same biological properties, attention has focused on the concept that the C–D region contains the minimal structural requirements for receptor activation. Several peptides have been produced either by synthetic (Antoni *et al.*, 1986) or recombinant DNA methods (Rosenwasser *et al.*, 1986). Although these IL-1 peptides have some biological activity, the specific activities are low and they do not block the receptor binding of the mature peptide. Antibodies to this C–D region do not block the activity of the mature IL-1β peptide on a variety of target cells whereas antibodies to synthetic C-terminal and N-terminal peptides do reduce biological activity (Massone *et al.*, 1988). These data support other studies (see below) suggesting that both the N-terminal as well as C-terminal amino acids are involved in receptor-binding events.

Small-molecular-weight peptides, at 4 and 2 kDa, with IL-1 immunoreactive and biological activity, have been consistently isolated from human body fluids (Cannon and Dinarello, 1985; Kimball *et al.*, 1984; Dinarello *et al.*, 1984b). These small-molecular-weight peptides have been observed in preparations of recombinant IL-1 and appear to be generated by trypsin-sensitive sites. The amino acid sequence of human IL-1β contains several cleavage sites for serine proteases which would generate peptides of various molecular weights. These are illustrated in Fig. 1. There is a 6970-Da peptide which contains the C–D region and is generated by serine proteases. There is also a 5488-Da C-terminal peptide, generated at the lysine-lysine-lysine site, which could represent the active C-terminal fragment (Palaszynski, 1987). Inhibitors of serine proteases prevent the appearance of this and other small-molecular-weight immunoreactive IL-1 peptides in supernates of human monocytes (Auron *et al.*, 1987). Recently, an endopeptidase that is membrane bound and is involved with degradation of neuropeptides has been shown to destroy the biological activity of IL-1β (Pierart *et al.*, 1988).

Human IL-1β has recently been crystallized and its tertiary structure analyzed at a resolution of 3.0 Å (Priestle *et al.*, 1988). The molecule that was crystallized had an N-terminus at alanine 117 and hence represents a 17.5-kDa processed form. This is the dominant form found in extracellular fluids. The three-dimensional structure revealed 12 β strands forming a complex of hydrogen bonds. Computerized molecular modeling of IL-1 from the primary sequence also revealed a similar structure (Cohen and Dinarello, 1987). The basic structure is similar to a tetrahedron, the interior of which is filled with hydrophobic side chains. The overall folding of the 12 β strands is similar to that found in the soybean trypsin inhibitor. The interior of IL-1β is strongly hydrophobic with no charged amino acids. The histidine at position 147 is on the surface of the molecule, and when this amino acid is substituted by site-specific mutation, a corresponding loss of biological and receptor-binding activity takes place (MacDonald *et al.*, 1986). The N-terminal mutations have also yielded altered biological and receptor-binding data (Horuk *et al.*, 1987) suggesting that N-terminal amino acids, seemingly the result of limited proteolysis by serine proteases (Dinarello *et al.*, 1986a), play an important role in either stabilizing the tertiary structure or by direct interaction with receptor-binding domains. Studies on the active site of IL-1 have been carried out using antibodies directed against different synthetic peptides and assessing their ability to neutralize the immunostimulatory as well as inflammatory properties of the whole IL-1 (Riveau *et al.*, 1988; Massone *et al.*, 1988).

Fibroblast growth factors have been shown to stimulate fibroblast and endothelial cell proliferation, smooth muscle cell proliferation, and angiogenesis, properties similar to those of IL-1. Like IL-1, these molecules exist in two forms, acidic (p*I* 5) and basic (p*I* 8). Bovine brain-derived acidic and basic FGFs have significant amino acid homologies with the IL-1β (Thomas *et al.*, 1985) and to a lesser degree with IL-1α. The stretches of IL-1 and FGF amino acid homologies are distributed throughout the sequences. However, comparison of the IL-1 sequence in the C–D region (see above) with that of the FGFs does not reveal any particular homology. Nevertheless, the amino acid sequence similarities between the two IL-1 forms and the two forms of FGF support the observation that some biological properties are shared, particularly their ability to induce cell proliferation. It is unknown whether IL-1 binding to fibroblasts is displaced by FGFs. Other growth factors such as TGF (β and α) and PDGF do not have any structural homologies to IL-1 or TNF at the level of amino acid sequences. In addition, the receptors to these cytokines appear distinct. It seems likely that these different cytokines bring about similar biological changes because they induce similar postreceptor cellular signals.

III. Gene Expression, Synthesis, and Processing of IL-1

A critical aspect of understanding IL-1 gene expression in a variety of cells is the exquisite sensitivity of some IL-1-producing cells to the effects of endotoxins (bacterial lipopolysaccharides). This is particularly the case with human blood monocytes, which will produce IL-1 when stimulated by concentrations of endotoxin as low as 5–10 pg/ml. Routine tissue culture media contain orders of magnitude of greater amounts of endotoxin. It is often difficult to assess gene expression in some experiments since IL-1 transcription can easily be stimulated by routine laboratory culture media. In order to demonstrate increases in IL-1 mRNA, some investigators have used 10 μg/ml of endotoxin to show stimulation of IL-1 transcription over that of the "unstimulated" control (Kern *et al.*, 1988). Therefore, in many studies, IL-1β transcription has already taken place during the preparation and early culture of monocyte/macrophages. Adherence to glass and some plastic surfaces can serve as a stimulus of RNA synthesis. If one carefully separates human blood mononuclear cells on endotoxin-free Ficoll–Hypaque and avoids activating cells by adherence or endotoxin-contaminated culture media, there is no IL-1 mRNA present in unstimulated cells and no IL-1 protein is translated, in both intracellular and extracellular compartments (Schindler and Dinarello, 1988).

Transcription of IL-1 mRNA is rapid in stimulated cells: in both human macrophage cell lines as well as in human blood mononuclear cells, endotoxin-stimulated IL-1β RNA transcription can be observed within 15 minutes (Fenton *et al.*, 1987, 1988). In human cultured endothelial and smooth muscle cells, a similar rapid increase has been reported (Libby *et al.*, 1986a,b). Transcription increases and reaches peak levels in 3–4 hours and then levels off for several hours before decreasing. Transcription of IL-1α appears to be under tighter control in that inhibitors of protein synthesis is sometimes required to observe IL-1α RNA (Libby *et al.*, 1986b). In fact, the total amount of IL-1β mRNA increases and is maintained at higher levels when inhibitors of protein synthesis are used (Fenton *et al.*, 1987). During endotoxin stimulation, transcriptional repressors are translated (or activated by phosphorylation) and these either suppress further transcription or increase mRNA degradation.

In addition to a tight control over transcription, IL-1 is translated by a mechanism which is poorly understood. For example, human monocytes can be stimulated by adherence to glass surfaces and yet not translate any of the mRNA into IL-1 protein. In fact, the level of mRNA following adherence to glass or cellulosic membranes can be as high as that following endotoxin stimulation and yet no IL-1 protein is produced

in the absence of another stimulant, in most cases endotoxin. This requirement for a second signal for translation is similar to the case for ferritin biosynthesis in which cells contain high levels of mRNA but require iron to stimulate translation. Of course, endotoxin and similar stimulants serve the dual purpose of initiating transcription as well as translation. IL-1 serves to stimulate both transcription and translation of more IL-1 (Dinarello *et al.*, 1987; Warner *et al.*, 1987). Corticosteroids, when added to cells before they are stimulated, prevent transcription as well as translation (Knudsen *et al.*, 1987). However, if corticosteroids are added after IL-1 mRNA is present, there is no evidence of decreased transcription and most of the effect is on translation (Kern *et al.*, 1988).

Prostaglandins and prostacyclins have no effect on transcription but reduce translation (Knudsen *et al.*, 1986; Kunkel *et al.*, 1985). Blocking cyclooxygenase results in increased production of IL-1 protein, particularly when cells are stimulated by agents which increase PGE synthesis. In these situations, reduction of PGE and PGI by cyclooxygenase inhibitors removes the suppressive effect of the arachidonate metabolites, but this effect may represent the artifact of *in vitro*-cultured cells since PGE accumulates under these conditions, whereas, *in vivo*, efficient mechanisms exist to rapidly remove PGE metabolites. The mechanism of PGE-induced suppression of IL-1 translation appears to be via the induction of cAMP (Knudsen *et al.*, 1986). The addition of PGE and dibutyl cAMP or PGE and theophylline augments the suppression. It is unclear exactly how increased cAMP affects the translation of IL-1 protein, but studies show no effect on IL-1β mRNA when cAMP degradation is prevented by inhibitors of phosphodiesterase.

Despite the lack of a signal peptide, IL-1 is found in the supernates of stimulated monocytes and macrophages. Other cells producing IL-1—for example, endothelial cells, keratinocytes, smooth muscle cells, and renal mesangial cells—transcribe large amounts of IL-1 mRNA and translate IL-1 protein, but a considerable amount of the IL-1 remains intracellular as the precursor molecule (31 kDa) (Auron *et al.*, 1987; Matsushima *et al.*, 1986a). The amount of IL-1 that is "secreted" depends upon the cell type and the conditions of stimulation. The monocyte/macrophage appears to be the cell best equipped to "secrete" IL-1. These cells contain polyadenylated RNA coding for IL-1β at concentrations as high as 2-5% of the total poly(A) after adherence and stimulation by endotoxin. Most of the IL-1β mRNA transcribed is translated under these conditions. Using radioimmunoassays or enzyme-linked assays, studies indicate that as much as 100 fg of IL-1 is synthesized per human monocyte (or 100 ng/10^6 monocytes) during the 24 hours following stimulation (Lisi *et al.*, 1987; Endres *et al.*, 1988a). Although the amount

of mRNA coding for IL-1α is approximately 20–50 times less than IL-1β in these cells (Demczuk *et al.*, 1987), there is more total (cell associated plus extracellular) IL-1α protein produced following endotoxin stimulation (Endres *et al.*, 1988a–c).

The reason for the discrepancy between the amount of IL-1α mRNA and the amount of IL-1α protein translated remains unclear. One possibility is that a considerable amount of the IL-1β mRNA is never translated whereas translation of the IL-1α RNA is highly efficient. An alternative explanation is that the mRNA for IL-1β is more rapidly degraded than that for IL-1α. Control of IL-1 translation is affected by other cytokines. IL-1-induced IL-1 production (either IL-1β-induced IL-1α, or IL-α-induced IL-1β) is suppressed by IFN-γ whereas IFN-γ augments the amount of IL-1 synthesized following endotoxin or TNF stimulation (Ghezzi and Dinarello, 1988). The suppression of IL-1-induced IL-1 by IFN-γ is posttranscriptional. Posttranscriptional suppression of IL-1 synthesis is also observed in cells treated with PGE_2 and is a cAMP-dependent mechanism (Knudsen *et al.*, 1986); corticosteroids can suppress IL-1 synthesis when added before transcription (Knudsen *et al.*, 1987) as well as after transcription (Kern *et al.*, 1988).

As depicted in Fig. 2, activators of cells for IL-1 synthesis also trigger the events leading to increased prostaglandins and leukotrienes. As mentioned above, prostaglandins suppress IL-1 translation; however, leukotrienes appear to augment IL-1 production. This has been shown by adding LTB-4 to human monocytes and stimulating IL-1 production (Rola-Plenszczynski and LeMaire, 1985). Agents that block the lipoxygenase pathway of arachidonate metabolism leading to formation of leukotrienes also reduce IL-1 production (Dinarello *et al.*, 1984a; Kunkel and Chensue, 1985). Similar studies demonstrate that this series of events also occurs in macrophages producing TNF. Recent evidence supports the importance of lipoxygenase products in the production of IL-1. In human volunteers taking eicosapentaenoic acid fatty acid dietary supplements, there is a 70% reduction in the ability of their mononuclear cells to synthesize IL-1β and IL-1α *in vitro* (Endres *et al.*, 1988c). A similar observation was made for TNF. The mechanism probably involves the ability of these ω-3 fatty acid precursors to be metabolized to LTB-5 rather than LTB-4. LTB-5 competes with LTB-4 for receptor occupancy. It is unclear at which stage the lipoxygenase metabolites act on IL-1 production. Since one can add a lipoxygenase inhibitor 1–2 hours after cell stimulation without affecting the amount of IL-1 synthesized, it appears that lipoxygenase metabolites are involved with early events such as transcription.

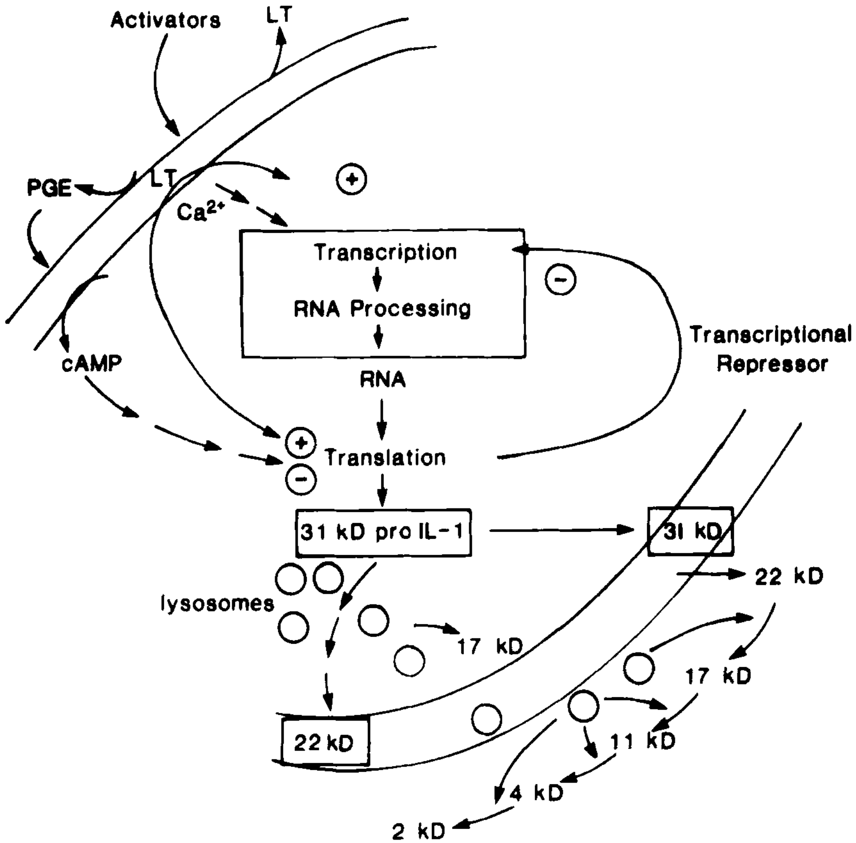

FIG. 2. Activation of a monocyte leading to the transcription, translation, processing, and secretion of IL-1. Activators stimulate several changes in cell membrane lipids and intracellular signals. PGE, leukotrienes (LT), calcium, and cAMP are shown with their respective roles as enhancers or suppressors of IL-1 production. The effect of a transcriptional repressor is shown (adapted from Fenton *et al.*, 1987). The 31-kDa IL-1 precursor is primarily found intracellularly in the cytosol, but also occurs in the plasma membrane (IL-1α) and associated with lysosomes. A 22-kDa peptide (see Fig. 1) is also associated with the three compartments. The extracellular generation of the mature 17.5-kDa IL-1 peptide and its fragments is depicted.

The first translation product of IL-1 is the 31-kDa precursor. This can be found mostly in the intracellular pool. The intracellular pool also contains other molecular-weight fragments of IL-1 at 22 and 17 kDa, but it is unclear whether these occur as a result of artifactual proteolysis during specimen preparation (Auron *et al.*, 1987). The localization of cell-associated IL-1 (as compared to extracellular IL-1) is almost entirely

cytoplasmic. Using antibody staining or radioimmunoassays, the data consistently indicate that cell-associated IL-1β is primarily in the cytosol, not in the endoplasmic reticulum, Golgi, or plasma membrane fraction (Singer *et al.*, 1988; Sisson and Dinarello, 1988). Despite the failure to measure IL-1 in the plasma membrane fractions, several studies have shown IL-1β staining on the cell surface (Folks *et al.*, 1987). Lysosomal localization appears to be important for the processing and secretion of IL-1 (Bakouche *et al.*, 1987; Singer *et al.*, 1988). Studies on the localization of IL-1α also indicate a similar predominance in the cytosol (Sisson and Dinarello, 1988), but others have demonstrated IL-1α associated with the plasma membrane (Beuscher *et al.*, 1987). IL-1α is also phosphorylated and it is unclear how this contributes to its cellular localization (Kobayashi *et al.*, 1988).

There is a 22-kDa form which can be isolated from the intracellular pool and the extracellular fluid. The 22-kDa IL-1 is also thought to be an intermediate form which may be located transiently in the membrane. The 31-kDa IL-1 precursor is immunoprecipitated by anti-IL-1α where it is biologically active as an inducer of hepatic acute-phase protein synthesis (Beuscher *et al.*, 1987). Most of the IL-1 found in the extracellular fluid, however, is the "mature" 17.5-kDa peptide with an N-terminal at position 117 (alanine) for the IL-1β precursor (Van Damme *et al.*, 1985) and at position 113 (serine) for the human IL-1α. Smaller peptide fragments of these mature peptides have been found in monocyte supernates and the appearance of these peptides can be reduced in the presence of serine protease inhibitors (Auron *et al.*, 1987). Elastase and plasmin have been implicated as monocyte proteases which cleave IL-1 into its 17.5-kDa mature fragment (Matsushima *et al.*, 1986a). This and other various subfragments are biologically active and are routinely found in human plasma, urine, and peritoneal, pleural, and joint fluids. As shown in Fig. 2, smaller peptides at 22, 17, 11, 6, 4, and 2 kDa are found in the extracellular fluid of stimulated human monocytes and may correspond to the potential cleavage sites depicted in Fig. 1.

The mechanism of IL-1 cleavage is thought to be via lysosomal enzymes (Bakouche *et al.*, 1987). There is a correlation between the amount of processed 17.5-kDa mature IL-1 found in the extracellular fluid and the type of stimulus used to activate the monocyte or macrophage. Particles such as *Staphylococcus epidermidis* or zymosan induce large amounts of IL-1 that are mostly mature and extracellular. These stimulators are also potent inducers of lysosomal exocytosis. Other stimulators such as low-dose (50–100 pg/ml) bacterial endotoxins induce approximately equal amounts of intracellular and extracellular IL-1β whereas IL-1α is nearly entirely cytosolic. Adherence to plastic or glass surfaces in the absence of endotoxins induces mRNA for IL-1β but no detectable IL-1 protein.

Small amounts of endotoxin rapidly induce translation of this mRNA (Schindler and Dinarello, 1988). However, most commercial tissue culture media or sera contain endotoxin concentrations (10–50 pg/ml) sufficient to stimulate cell-associated IL-1, including membrane-bound IL-1 which is biologically active (Kurt-Jones *et al.*, 1985). Thus, it is likely that most cultures containing monocyte/macrophages have active cell-associated IL-1 despite a failure to demonstrate extracellular IL-1.

IV. The Biological Effects of IL-1

The expression of recombinant IL-1 has been accomplished and, in general, there does not seem to be any difference in the spectrum of biological activities of either form. IL-1β is vulnerable to oxidation, and thus biological specific activities of IL-1β can be lower than those of IL-1α, which is highly stable. If IL-1β is purified under nondenaturing conditions, it is equipotent with the α form in a variety of *in vivo* and *in vitro* assays. The binding of either IL-1β or IL-1α to various cells in receptors binding assays is blocked by each form (Kilian *et al.*, 1986). Both recombinant human IL-1β and IL-1α augment T, B, and natural killer cell responses. The ability of IL-1 to activate immunocompetent cells seems unique to the group of cytokines which affect cellular growth and proliferation; FGF, PDGF, and TGF have either no effect on immunocompetent cells, or in the case of TGF-β, are potent immunosuppressive agents (Kehrl *et al.*, 1987). Both forms of IL-1 induce sleep and systemic acute-phase responses including fever, hepatic acute-phase protein synthesis, neutrophilia, hypoferremia, hypozincemia, and increased levels of hormones. At higher doses, IL-1 induces hypotension and a shocklike state (Okusawa *et al.*, 1988). Some biological properties reported for natural IL-1 have not been confirmed with either recombinant form. These include the ability of IL-1 to cause neutrophil superoxide production and degranulation *in vitro* (Georgilis *et al.*, 1987) and to induce muscle proteolysis *in vitro* (Goldberg *et al.*, 1988; Moldawer *et al.*, 1987). Others, however, do show an effect of recombinant IL-1β on muscle proteolysis (Clowes *et al.*, 1987), particularly a fragment of IL-1 generated from the recombinant IL-β form. It appears that the IL-1 effects on neutrophils are due to the ability to augment the biological effects of other neutrophil activators, such as the chemotactic peptides. There are receptors for IL-1 on neutrophils (Rhyne *et al.*, 1987), and IL-1 acts as a cofactor or permissive factor for the activity of fMet-Leu-Phe. Recombinant human IL-1, however, directly stimulates basophil (Subramanian and Bray, 1987; Haak-Frendscho *et al.*, 1988) and eosinophil degranulation of histamine and arylsulfatase (Pincus *et al.*, 1986), respectively.

The multiple biological activities of IL-1 have been studied in terms of *in vivo* and *in vitro* effects. In patients with bacterial infection, injury, or chronic inflammatory disease, IL-1 may account for a majority of observed acute-phase changes. It is difficult to study such subjective symptoms as headache, myalgias, arthralgias, and lassitude in animal models, but the potency (10^{-12}-10^{-15} *M*) of IL-1 in inducing release of prostaglandin E_2 (PGE_2) from fibroblast, synovial, and other cells suggests that these symptoms are likely mediated by increased levels of IL-1.

A. Effects on Hepatic Protein Synthesis

Hepatic proteins which change during acute-phase responses include clotting factors, complement components, fibrinogen, haptoglobin, ceruloplasmin, and others. In addition, there are increases in hepatic proteins generally not synthesized in health but in association with infection, injury, or other pathological processes. IL-1, TNF, and hepatocyte-stimulating factor (now identified as the same molecule as interferon-β2 and B-cell-stimulating factor 2, or IL-6) play important roles in regulating the synthesis of hepatic proteins. The cytokine-induced increases in normal hepatic proteins is usually in the range of 2-fold to 3-fold, but the synthesis of pathological proteins can increase 100-fold to 1000-fold. Two such proteins, serum amyloid A (SAA) protein and C-reactive protein, are classical "acute-phase reactants," and serve as markers of disease. SAA contributes to the development of secondary amyloidosis.

IL-1 induces hepatocytes to synthesize a spectrum of acute-phase proteins; these include SAA (Ramadori *et al.*, 1985), fibrinogen (Ghezzi *et al.*, 1986), complement components, and various clotting factors. At the same time, albumin levels decrease. Studies have shown that IL-1 regulates the synthesis of these and other hepatic acute-phase proteins at the level of mRNA transcription. In isolated hepatocytes, IL-1 decreases the transcription of RNA coding for albumin, increases transcription of factor B, and initiates SAA mRNA synthesis, but has no effect on gene expression of a control protein, actin. Other proteins have been studied in hepatic cell line cultures (HepG2 and Hep3B), and, in picomolar ranges, IL-1 stimulates the biosynthesis of complement protein C3 and α_1-antichymotrypsin (Perlmutter *et al.*, 1986a). There is also a modest stimulation of α_1-acid glycoprotein and inter-α_1-trypsin inhibitor synthesis. In addition to decreased albumin transcription, hepatic cells exposed to IL-1 synthesize less transferrin. In hepatic cell lines, IL-1 does not increase the expression of C-reactive protein (CRP), although the intravenous injection of recombinant IL-1 does result in elevated CRP levels after 24 hours. In murine fibroblasts transfected with cosmid DNA bearing the genes for C2 and factor B,

IL-1 stimulated the expression of factor B but did not affect synthesis of C2 (Perlmutter *et al.*, 1986b).

IL-1 has other effects on liver metabolism. It depresses the activity of liver cytochrome P_{450}-dependent drug metabolism in mice (Ghezzi *et al.*, 1986), and this observation may explain the impaired drug clearance and excretion in patients with infections and fever. The liver's response to IL-1 also includes the synthesis of metalloproteins which bind serum iron and zinc and account for the hypozincemia and hypoferremia induced by IL-1. Bacteria and tumor cells require large amounts of iron for cell growth, particularly at elevated temperatures, and the ability of the host to remove iron from tissue fluids seems to be a fundamental host defense mechanism.

B. Effects of IL-1 on Endothelial Cells

Of its many biological properties, IL-1-induced changes in endothelial cells relate directly to the initiation and progression of pathological lesions in vascular tissue. From a physiological viewpoint, IL-1 activates human endothelial cells *in vitro* to synthesize and release PGI_2, PGE_2, and platelet-activating factor (Rossi *et al.*, 1985; Dejana *et al.*, 1987). A 10-fold increase in PGI_2 release is observed with concentrations of IL-1 in the femptomolar range. Although arachidonate metabolites increase blood flow, IL-1 also orchestrates a cascade of cellular and biochemical events that lead to vascular congestion, clot formation, and cellular infiltration. One of these initiating steps involves the ability of IL-1 to alter endothelial cell plasma membranes so that neutrophils, monocytes, and lymphocytes adhere advidly (Bevilacqua *et al.*, 1985). Endothelial cells need be exposed to IL-1 for 1 hour or less in order to increase their adhesiveness. The action of IL-1 in this process appears to be related to the interaction of the leukocyte glycoprotein complex called "leukocyte function antigen" with a fibroblast and endothelial cell surface molecule called "intercellular adhesion molecule-1." Within 1 hour following IL-1 exposure, endothelial cells increase their expression of intercellular adhesion molecule-1 (Dustin *et al.*, 1986). Patients with defective leukocyte function with respect to antigen expression have repeated bouts of bacterial infection. In addition to activating endothelial cell–leukocyte adhesion, IL-1 also increases the binding and lysis by natural killer cells by a variety of tumor targets and is chemotactic for monocytes and lymphocytes. Consistent with the effects of IL-1 on leukocyte chemotaxis and adherence to endothelial cells, IL-1 injected intradermally causes the accumulation of neutrophils and IL-1 can substitute for endotoxin in either limb of the local Schwartzman reaction. This latter property is most apparent using the combination of IL-1 and TNF (Movat *et al.*, 1987).

IL-1 increases endothelial cell surface procoagulant activity (Bevilacqua *et al.*, 1984) and production of a plasminogen activator inhibitor (Nachman *et al.*, 1986). These events lead to activation of thrombin in the initiation of clotting. Taken together, these effects would decrease blood flow in vessels and increase the accumulation of leukocytes and platelets. Since IL-1 stimulates neutrophil thromboxane release (Conti *et al.*, 1986), activated neutrophils adhering to endothelial cells may increase platelet aggregation. Finally, IL-1 has angiogenic properties in the rabbit eye anterior chamber model (Prendergast and Dinarello, unpublished observations) and following brain injury (Giulian *et al.*, 1988); this may be related to the fact that IL-1 and the related fibroblast growth factors share significant amino acid homologies. In general, the effects of IL-1 on endothelial cells represent a well-coordinated effort to localize tissue inflammation and contribute to the initiation of pathological lesions leading to vasculitic-like changes. Figure 3 illustrates the activity of IL-1 (and TNF; see below) on vascular tissue.

The effects of IL-1 stimulation of endothelial cell functions should be considered in light of the fact that endothelial cells produce their own IL-1. Nanogram/milliliter concentrations of bacterial endotoxins or TNF induce cultured endothelial cells to release IL-1 (Libby *et al.*, 1986a). In addition, thrombin stimulates endothelial cell IL-1 production. Northern hybridization of endothelial cell mRNA supports the close relationship between the predominant IL-1β and endothelial cell-derived IL-1. Thus, the induction of endothelial cell IL-1 by two clinically relevant stimulators (endotoxin and thrombin) may initiate a cascade of events leading to further development of vasculitic processes. Although immune complexes stimulate monocyte IL-1 production, there are no reported studies as yet demonstrating that immune complexes stimulate endothelial cell IL-1 synthesis. Recent studies demonstrated that arterial smooth muscle produces IL-1 (Libby *et al.*, 1986b), that IL-1 is a growth

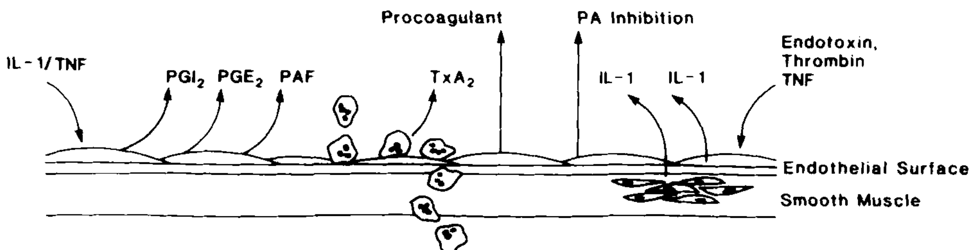

FIG. 3. IL-1 and TNF effects on vascular tissue. PA indicates plasminogen activator inhibition.

factor for smooth muscle cells (Libby *et al.*, 1988), and IL-1 induces IL-1 in these cells (Warner *et al.*, 1987).

C. Catabolic Effects of IL-1

The catabolic properties of IL-1 are usually considered in terms of its local effects. For example, IL-1 produced locally acts in a paracrine like fashion in destructive joint and bone disease and local tumor invasion. On the other hand, IL-1 in the systemic circulation exerts its catabolic effects indirectly by affecting the metabolism of lipoproteins by liver and fat. IL-1 is a potent inducer of collagenase production in synovial cells (Krane *et al.*, 1985; Dayer *et al.*, 1986); in addition, IL-1 induces release of metalloproteinases and proteoglycanases from chondrocytes (Saklatvala *et al.*, 1985; Schnyder *et al.*, 1987). In fact, because of its local catabolic biological activities, pig IL-1 was previously known as "catabolin." Recombinant IL-1s added to bone cultures *in vitro* induce dramatic resorptive processes and shrinkage of bone matrix. Just as pig IL-1 was known previously as catabolin, osteoclast-activating factor (OAF) is now identified as having the same amino acid sequence as the human IL-1β (Dewhirst *et al.*, 1985). Thus, it is presently considered that the catabolic properties of IL-1 in cartilage and bone contribute to the tissue destruction and matrix loss in a variety of joint diseases (Krane *et al.*, 1985; Pettipher *et al.*, 1986). As discussed below, TNF can also act as an osteoclast-activating factor. IL-1 and the related FGF synergize in the induction of proteases from chondrocytes (Phadke, 1987).

D. Effects of IL-1 on Fibroblasts and Fibrosis

In contrast to its catabolic activities, IL-1 increases fibroblast proliferation (Schmidt *et al.*, 1984) and collagen synthesis (Canalis, 1986). IL-1 is mitogenic for fibroblasts and is thought to play a major role in pannus formation. In fact, cellular infiltration and early pannus formation has been observed in rabbit joints injected with recombinant IL-1 (Pettipher *et al.*, 1986). *In vitro*, however, IL-1-induced fibroblast and smooth muscle cell proliferation is often difficult to observe unless cyclooxygenase inhibitors are present (Libby *et al.*, 1986a,b). This seems to be related to the PGE_2 levels induced by IL-1. This is not the case with other fibroblast growth factors such as PDGF, epidermal growth factor, and TGF. In comparison to epidermal growth factor and TGF-β, numbers of receptors for IL-1 on fibroblasts are low (2000/cell) (Bird and Saklatvala, 1986; Chin *et al.*, 1987), but compared to IL-1 receptors on other cells, the fibroblast ranks as one of the tissues with high numbers of receptors. One mechanism for IL-1-induced fibroblast growth is IL-1 via increased production of receptors for endogenous growth factors such

as epidermal growth factor. In terms of the role of IL-1 in fibrosis, recombinant IL-1 directly increases the transcription of Type I and Type III collagen (Canalis, 1986) and Type IV (basement membrane) collagen (Matsushima *et al.*, 1985a).

E. Other Cytokines Affecting Fibroblasts and Fibrosis

Other cytokines share with IL-1 the ability to stimulate fibroblast proliferation. These include TNF (Kohase *et al.*, 1987), FGF (Gimenez-Gallego *et al.*, 1985), PDGF (Shimokado *et al.*, 1985), and TGF-β. In addition, another growth factor from macrophages, called macrophage-derived growth factor, seems to act as a competence factor for PDGF-induced fibroblast growth (Martinet *et al.*, 1987); however, this factor now seems to be PDGF itself. The mechanism of action of PDGF on cell proliferation includes a requirement for insulin whereas IL-1 and TNF effects are seen in the absence of insulin. Despite the fact that these various cytokines have distinct receptors, their effects on cell growth appear similar. These cytokine growth factors also share other biological properties. For example, like IL-1, PDGF is chemotactic for neutrophils and monocytes *in vivo*, induces cell aggregation and adhesiveness, and induces degranulation (Duel *et al.*, 1982; Tzeng *et al.*, 1984). IL-1, TNF, TGF, and PDGF induce the synthesis of collagen and collagenases. At present, the multiple biological properties of IL-1 are clearly shared, to some extent, with these growth factors, raising issues such as synergism and antagonism between the cytokines on mesenchymal tissue. Recent data demonstrate that considerable synergism between IL-1 and TNF exist in a variety of biological effects; little is known about the synergism or antagonism between the non-IL-1 growth factors, except that TGF-β is immunosuppressive (Kehrl *et al.*, 1987).

V. Systemic versus Local Effects of IL-1

A. Systemic Effects

Table I lists the systemic effects of recombinant IL-1. These studies were carried out by injecting IL-1 into experimental animals either intravenously or intraperitoneally. In many cases, the IL-1-induced changes mimic an animal's responses to an injection of microbial toxins or antigen-antibody complexes. When administered intravenously into rabbits at 200 ng/kg, human recombinant IL-1 results in a decrease in circulating neutrophils within 5 minutes, and this is due to rapid margination on the endothelial surfaces; approximately 10 minutes after an intravenous injection, rectal temperature begins to rise and reaches

TABLE I
SYSTEMIC EFFECTS OF RECOMBINANT IL-1[a]

Central nervous system	Metabolic
Fever	Hypozincemia, hypoferremia
Brain PGE_2 synthesis	Decreased cytochrome P_{450} enzyme
Increased ACTH	Increased acute-phase proteins
Decreases REM sleep	Decreased albumin synthesis
Increased slow-wave sleep	Increased insulin production
Decreased appetite	Inhibition of lipoprotein lipase
	Increased sodium excretion
	Increased corticosteroid synthesis
Hematologic	**Vascular wall**
Neutrophilia	Hypotension
Nonspecific resistance	Increased leukocyte adherence
Increased GM-CSF	Increased PGE synthesis
Radioprotection	Decreased systemic vascular resistance
Bone marrow stimulation	Decreased central venous pressure
Tumor necrosis	Increased cardiac output
	Increased heart rate
	Decreased blood pH
	Lactic acidosis
	Chemoattractant

[a]These effects have been demonstrated by administration of recombinant IL-1 to animals.

peak elevation between 45 and 55 minutes after the injection (Dinarello *et al.*, 1986a). Increases in slow wave sleep parallel the fever course but the sleep-inducing property of IL-1 is not linked to its ability to increase body temperature. After 4–6 hours, temperature and sleep patterns return to preinjection levels but there is an increase in circulating neutrophils, particularly new forms released from the marrow. After 12 hours, serum albumin, iron, zinc, and cytochrome P_{450} enzyme activities are decreased (Ghezzi *et al.*, 1986). When approximately 10 times the amount of IL-1 is injected, a decrease in systemic arterial pressure, systemic vascular resistance, and central venous pressure can be observed within 10 minutes (Okusawa *et al.*, 1988). Although these hemodynamic changes are similar to the changes observed in animals given TNF, tissue damage is not characteristic of IL-1, at least at doses less than 5 μg/kg, whereas TNF is associated with death in some organs (Tracey *et al.*, 1986). Large doses of recombinant IL-1 injected into mice has been given without death or tissue damage unless the mice have been previously adrenalectomized (P. Ghezzi, personal communication).

Information derived from the *in vitro* effects of IL-1 may relate to some of the biological properties of IL-1 observed *in vivo*. For example, IL-1

stimulates somatostatin release from cultured pituitary cells (Scarborough *et al.*, 1987) and also increases steroid synthesis (Roh *et al.*, 1987) from perfused adrenal glands. Therefore it is not surprising that other neuropeptides are stimulated when IL-1 is given systemically. The effects of IL-1 on endothelial cell arachidonic acid metabolism, platelet-activating factor generation, and procoagulant activity *in vitro* likely explain the shocklike state that IL-1 produces *in vivo* (Okusawa *et al.*, 1988). In addition, the ability of IL-1 to induce various lymphokines *in vitro*—for example, interferon-β1, interferon-γ, IL-2, IL-3, and IL-6—likely occurs *in vivo*, but it is difficult to demonstrate circulating levels of interferons and interleukins unless large doses of IL-1 are administered. One approach to interpreting the large body of evidence for the multiple biological effects of IL-1 is to view local production and action as the "autocrine/paracrine" action of IL-1. These are listed in Table II. By contrast, the systemic effects of IL-1 can be viewed as the "hormonal" property such as fever and decreased appetite (McCarthy *et al.*, 1987). At present, it seems that autocrine/paracrine effects of IL-1 predominate in some diseases such as type I diabetes mellitus (Bendtzen *et al.*, 1986), whereas systemic effects are characteristic of IL-1 produced as a result of toxemia, septicemia, widespread tissue damage, or intravenous antigen challenge.

B. Local Effects

It is increasingly clear that tissues producing IL-1 are either themselves targets of IL-1 or are capable of acting on adjacent tissues. For example, IL-1 produced by macrophages in a lymph node acts on macrophages as well as on lymphocytes inducing IL-2, IL-2 receptors, IL-3, IL-6, and interferon-γ. IL-1 produced by microglia and astrocytes in the brain has its effect on local gliosis (Giulian and Lachman, 1985), but may induce no systemic responses. A similar case can be made for the ability of IL-1 to stimulate production of granulocyte-macrophage colony-stimulating factor (Zucali *et al.*, 1986; Bagby *et al.*, 1986), interferon-β1 (van Damme *et al.*, 1985), and IL-6 (van Damme *et al.*, 1987a,b) in bone marrow. The local production and biological activity of IL-1 in the joint space has attracted considerable attention because of a potential role for IL-1 in the pathogenesis of various joint diseases. However, in the joint space, IL-1 likely exerts its autocrine/paracrine effects in the absence of major systemic responses. A similar case can be made for the IL-1 that is found in the nerve fibers of the human hypothalamus (Breder *et al.*, 1988). Locally produced IL-1 in the brain may exert various neuroendocrinologic changes with no detectable systemic changes other than what is detected as normal physiologic changes. The ability of membrane- or cell-associated IL-1 to be biologically active underscores the importance of local effects of IL-1 in the absence of systemic signs and symptoms.

TABLE II
LOCAL EFFECTS OF RECOMBINANT INTERLEUKIN-1[a]

Nonimmunological
Chemoattractant (*in vivo*)
Basophil histamine release
Eosinophil degranulation
Increased collagenase production
Chrondrocyte protease release
Bone resorption
Induction of fibroblast and endothelial CSF activity
Production of PGE_2 in dermal and synovial fibroblasts
Increased neutrophil and monocyte thromboxane synthesis
Cytotoxic for human melanoma cells
Cytotoxic for human beta islet cells
Cytotoxic for thyrocytes
Keratinocyte proliferation
Proliferation of dermal fibroblasts
Increased collagen synthesis
Mesangial cell proliferation
Gliosis
Immunological
T cell activation
IL-2 production
Increased IL-2 receptors
B cell activation
Synergism with IL-4
Induction and synergism with IL-6
Activation of natural killer cells
Synergism with IL-2, interferons, on NK cells
Increased Lymphokine production (IL-3, IL-6, IFN-γ)
Macrophage Cytotoxicity
Growth factor for B cells
Increased IL-1 production

[a]These effects are derived from *in vitro* studies.

VI. Effect of IL-1 on Immunocompetent Cells

A. IS IL-1 REQUIRED FOR T CELL ACTIVATION?

Recombinant IL-1 has been used to confirm a role for IL-1 in the mechanism of T and B cell activation, eliminating previous doubts about the purity of natural IL-1 preparations and whether these were capable of participating in immune responses. This is a particularly important issue because recent data on the ability of IL-6, IL-4 (Ho *et al.*, 1987), and GM-CSF to act as "lymphocyte-activating factors" have been used

to cast doubts on the role of IL-1 as an activator of lymphocytes. Clearly, some preparations of natural IL-1 could have been contaminated with other lymphokines, thus clouding the issue. However, recombinant IL-1 and antibodies to recombinant IL-1 or antibodies to synthetic IL-1 peptides have clarified the presence of IL-1 receptors on lymphocytes and the behavior of IL-1 as an activator of lymphocytes. Recombinant IL-1 has not, however, helped settle the issue of whether IL-1 is an absolute requirement for a primary immune response. Certain aspects of the biology of IL-1 have confounded the issue of whether soluble IL-1, like IL-2 and other T cell growth factors, is required for T cell activation; for example, evidence exists that (1) some T cells synthesize IL-1 (Tartakovsky *et al.*, 1986), (2) IL-1 induces other cytokines (such as IL-6 and IFN-γ), and (3) IL-1 is biologically active as a cell-associated protein, presumably as a cell membrane surface protein (Kurt-Jones *et al.*, 1985).

There is little question that, in many models of T and B cell activation, IL-1 amplifies the response to antigens or mitogens. This is particularly the case when suboptimal concentrations (concentrations that actually may be close to the *in vivo* situation) are used and when some attempt at macrophage depletion has been employed. In fact, the original immunologic effect of IL-1 was its ability to act in a costimulator assay, the so-called "lymphocyte-activating factor" assay using murine thymocytes and suboptimal amounts of mitogens. It soon became clear that, given the proper conditions, IL-1 would amplify the activation and induce IL-2 gene expression and IL-2 receptors (Smith *et al.*, 1980). What remains to be resolved since those initial observations is whether mature, resting T cells have an absolute requirement for IL-1 during primary activation. The present data suggest that in the strict absence of macrophage membranes, soluble, recombinant IL-1 does not restore the macrophage requirement for activation leading to proliferation. In examining the role for IL-1 in T cell activation, it appears best to divide the discussion into three areas: (1) the effect of IL-1 on thymocytes, (2) the effect of IL-1 on T cell lines, and (3) the ability of IL-1 to activate mature, resting T cells.

B. Effect of IL-1 on Thymocyte Responses to Mitogens

From the initial experiments of Gery and Waksman (1972), IL-1 had been "defined" as a comitogen for murine thymocytes. Further work demonstrated that, when depleted of macrophages, rat thymocytes (or lymph node cells) will not proliferate when incubated with concanavalin A (Con A) and are unresponsive to IL-2 but require IL-1. Alone, IL-1 does not lead to proliferation of thymocytes, an observation repeatedly made by many investigators. Although IL-1 treatment does not affect

the ability of thymocytes to bind Con A, the addition of soluble, recombinant IL-1 renders the thymocytes and lymph node cells capable of responding to Con A by producing both IL-2 and IL-2 receptors (Simic and Stosic-Grujicic, 1985). In a similar fashion, peanut agglutinin-negative murine thymocytes proliferated when IL-1 was added to sub-optimal concentrations of IL-2. The combination of IL-1 and IL-2 induced the IL-2 receptors (Maennel *et al.*, 1985). The monoclonal antibody directed against the thymocyte receptor L3T4 blocks the ability of IL-1 to act as a comitogen and it has been speculated that the L3T4 molecule is functionally involved with IL-1-induced thymocyte proliferation (Loegdberg *et al.*, 1985). Other data show that the Ly-1 antigen may serve as an IL-1 receptor (Loegdberg and Shevach, 1985).

C. Effect of IL-1 on IL-2 Production from T Cell Lines

The D10.G4.1 murine T-helper cell line (Kaye *et al.*, 1984) responds to IL-1 in the femtomolar range and may be an ideal cell to study the T cell response to IL-1. Initially this cell line entered a proliferative phase when IL-1 was added to mitogen-primed cells; in the absence of IL-1, there was no proliferative signal. Subsequently, it was shown that the effect of IL-1 on these cells was not through increased production of IL-2 but rather that IL-4 was providing the proliferative signal. Several laboratories have now demonstrated the exquisite sensitivity of these cells to IL-1 (subfemtomolar concentrations) and that these cells will proliferate to IL-1 in the absence of mitogens and antigens (Orencole *et al.*, 1987; Lacey *et al.*, 1987). The IL-1 receptors for these cells have been demonstrated to be composed of both a high- and a low-affinity class, and the number of high-affinity receptors is in excess of 20,000/cell (Savage *et al.*, 1988). At present it remains unclear whether these cells are inducing a secondary cytokine (which provides the proliferative signal) such as IL-4 (Kupper *et al.*, 1987a) or colony-stimulating factor (Kupper *et al.*, 1987b).

There is growing body of evidence that the largest number of high-affinity receptors for IL-1 exists on T cell lines. These include the EL-4 mouse thymoma cell line, the LBRM33 mouse thymoma cell line, the NOB-1 line (a subclone of the EL4 cell), and the D10S subclone of the murine T-helper cell line, D10.G4.1. With the exception of the NOB-1 and the D10S cells, cell lines produce little or no IL-2 in response to IL-1. However, when costimulated with mitogens, antibodies to the CD3 complex, ionophores, or phorbol esters, the cells produce large amounts of IL-2. For example, the human Jurkat cell line produces no IL-2 in response to IL-1, but, in the presence of mitogens and phorbol esters, there is increased transcription of IL-2 mRNA (Arya

and Gallo, 1984). Similar observations have been established in the EL-4 and LBRM33 cell lines, i.e., IL-1 does not induce IL-2 secretion but requires a second signal, usually one which increases cytosolic calcium (Simon, 1984; Lowenthal and MacDonald, 1986; Lowenthal *et al.*, 1986; Abraham *et al.*, 1987).

These data are consistent with the well-established synergistic effect of IL-1 with antigens, mitogens, ionophores, or activators of protein kinase C and support the existence of a two-signal hypothesis for the induction of IL-2 in T cells and T cell lines (Abraham *et al.*, 1987). The first signal is provided by agents which increase cytosolic calcium whereas increases in protein kinase C is a secondary signal event. Thus IL-2 synthesis takes place when cells are stimulated with ionophore and phorbol esters comparable to mitogen and IL-1, respectively. Examining the events in the LBRM33 cell line, mitogen induces rapid hydrolysis of phosphatidylinositol 4,5-bis(phosphate) (PI), and increased cytosolic calcium. Phorbol esters induced a protracted association of cellular protein kinase C with the plasma membrane. IL-1, however, although providing the signal which leads to increased IL-2 production when cells are stimulated with mitogen, did not activate protein kinase C nor mobilize calcium. Clearly IL-1 in combination with mitogen-induced intracellular events was able to trigger IL-2 production by a non-phosphatidylinositol, noncalcium mechanism.

D. IL-1-Induced Diacylglycerol in T Cells through Phosphatidylcholine Turnover

In recent studies by Rosoff and co-workers (Rosoff *et al.*, 1988), similar findings were reported using the Jurkat cell line. As noted above, IL-1 amplifies the gene expression for mitogen- or ionophore-primed Jurkat cells (Arya and Gallo, 1984). These cells produce increased IL-2 when stimulated by a combination of phorbol esters and IL-1 or anti-CD3 and IL-1. However, despite this activation, IL-1 does not increase cytosolic calcium in these cells or in neutrophils (Georgilis *et al.*, 1987). On the other hand, IL-1 causes a rapid rise in diacylglycerol production. If this were due to hydrolysis of phosphatidylinositol 4,5-bis(phosphate), generation of inositol triphosphate would have occurred. But similar to the results of others (Abraham *et al.*, 1987), this did not occur. These results suggest that IL-1 induces phospholipid hydrolysis through a non-phosphatidylinositol turnover pathway, for example, phosphatidylcholine.

When the Jurkat cells were labeled with [^{3}H]choline and then stimulated with IL-1, phosphorylcholine increased dramatically (within 5 seconds) in the supernatant whereas the concentration of phosphatidylcholine (PC) decreased in the organic phase of the cells. The

increase in supernatant phosphorylcholine was not due to PC turnover via protein kinase C-stimulated diacylglycerol PI turnover, because that pathway leads to increased cytosolic PC hydrolysis, and the IL-1-stimulated PC turnover is primarily observed in the extracellular compartment. The concentration of IL-1 which induced the PC turnover was 30 f*M*, reaching a maximum at 100 f*M*, the concentration of IL-1 that stimulates T cell proliferation and other biological effects *in vitro*. The IL-1-induced PC turnover was also observed in the D10.G4.1 T-helper cell line, the EL4 cell line, and in nylon wool-purified human peripheral blood T cells.

When the Jurkat, EL-4, and D10.G4.1 cells were examined for IL-1 receptor affinity and numbers, we observed that Jurkat cells have no high-affinity (see below) IL-1 receptors whereas the EL-4 and D10.G4.1 cells studied with the same labeled IL-1α and under the same equilibrium conditions clearly contained the typical high-affinity (5-50 p*M*) 80-kDa IL-1 receptor. These studies suggest that the high-affinity receptors for IL-1 are probably unrelated to the initiation of PC turnover and that this may be due to activation of a second, very low-affinity receptor. This may explain the discrepancies in the biological response of many cells to IL-1 and the number of high-affinity receptors.

Regardless of the proposed existence of a very low IL-1 receptor–IL-1 binding being the mechanism by which IL-1 stimulates PC turnover, the fact that there are at least two pathways by which diacyglycerol becomes elevated in T cells may explain the comitogen effect of IL-1 in a variety of immunocompetent cells (and even some nonimmunocompetent cells). The T cells are rich in protein kinases, and several isoenzymes with different tissue distribution exist. It has been proposed that the different protein kinases differ in the binding sites of the diacylglycerols (DAGs) and that this may be determined by the type of fatty acid side chain. For example, mitogen which stimulates DAGs through a PI turnover pathway results in activation of one type of protein kinase C, whereas IL-1 which stimulates DAGs through PC turnover stimulates another isoenzyme. In some cells, such as the Jurkat, there is a requirement for both (synergism?) protein kinases before IL-2 gene expression is initiated. The mitogen (in this case anti-CD3) is not sufficient without phorbol esters or IL-1 to provide the second signal, in this case another protein kinase through a PC-specific DAG. This may be one explanation for the amplifying effect of IL-1 in a variety of immunocompetent cells. In comparing the response of EL-4 cells to IL-1, ionophores, and phorbol esters, Truneh *et al.* (1986) concluded that IL-1 and TPA induce protein kinase C activation by two different mechanisms. It is thus probable that these two mechanisms reflect the two different pathways by which IL-1 and mitogens stimulate DAGs.

E. Effect of IL-1 on Peripheral Blood T Cells

Employing human peripheral blood T cells activated by immobilized antibodies directed against the CD3 cell surface protein complex, soluble IL-1 serves a cofactor for activation in the absence of macrophages (Williams *et al.*, 1985). Under these conditions, the addition of soluble recombinant IL-1 partially restores the role of the macrophage and one observes increased synthesis of IL-2 and expression of the IL-2 receptor. Increased DNA synthesis follows these events as an indicator of cell proliferation. However, when the cells are diluted, the ability of soluble IL-1 to replace the macrophage function is lost suggesting that total macrophage depletion was not accomplished. IL-2 will, on the other hand, drive these cells to proliferate.

F. Effect of IL-1 on B Cell Activation

Many investigators have shown that B cells and other cells serve as accessory cells in antigen recognition but have failed to demonstrate a role for IL-1 because IL-1 cannot be detected in B cell supernatants. This issue may have been resolved by studies which demonstrate that B cells produce IL-1 (Matsushima *et al.*, 1985b; Scala *et al.*, 1984a) and that B cells express membrane-bound IL-1 (Kurt-Jones *et al.*, 1985). In fact, nearly all cells which can act as accessory cells produce IL-1. These include astrocytes, mesangial cells, keratinocytes, and endothelial cells. Effects of IL-1 on B cells and immunoglobulin production continue to be made (Muraguchi *et al.*, 1984; Lipsky, 1985). The function of IL-1 in B cell activation seems to be similar to that shown in T cells, that is, IL-1 acts as a helper or cofactor during the activation process, particularly together with IL-4, also known as B-cell-stimulating factor-1. IL-1 activates B cells and contributes to formation of antibody. The first experiments to show the critical role for IL-1 in B cell activation were performed with anti-IL-1 (Lipsky *et al.*, 1983). Adding antibody early to human peripheral blood mononuclear cells stimulated with pokeweed mitogen completely prevented B cell activation and subsequent antibody formation. Other studies demonstrated that IL-1 synergized with various B cell growth and differentiation factors, leading to increased proliferation and antibody formation (Falkoff *et al.*, 1983, 1984). Some of the biological activity of the natural B cell growth and differentiation factors may have been due to the presence of IL-6 (see below). The ability of IL-1 to synergize with IL-6 on T cell activation probably extends to B cell activation. The ability of IL-1 to induce other B-cell-stimulating factors, including IL-6, interferon-γ, and IL-2, must also be considered because these substances activate B cells.

The recent evidence demonstrating that IL-1 induces the production

of B-cell-stimulating factor-2/hybridoma growth factor should be considered as a role for IL-1 in B cell and immunoglobulin synthesis. The hybridoma growth factor protein also has the ability to stimulate plasmacytoma cell growth. The N-terminus of the plasmacytoma/hybridoma growth factor matches that of an interferon-β2 (van Damme *et al.*, 1987a,b). Others have also reported a B-cell-stimulating factor (BSF-2) (Hirano *et al.*, 1986) which has the same sequence as the 26-kDa interferon-β2. Interferon-β2 has also been identified as the factor previously known as hepatocyte-stimulating factor, which caused an increase in C-reactive protein as well as other hepatic acute-phase proteins (Gauldie *et al.*, 1987). Interferon-β2, with its activity on B cells and liver cells, is now named IL-6. It seems that the role of IL-1 in augmenting B cell function and ultimately leading to the production of protective antibodies may be through its ability to induce the synthesis of B-cell-stimulating factors and/or upregulating their receptors.

G. IL-1 and Natural Killer Cells

IL-1 also plays a role in host defense against tumor cells. There is evidence that IL-1 increases the binding of natural killer cells to tumor targets and that tumor cells induce synthesis of IL-1 by natural killer cells (Herman *et al.*, 1985b). In addition, natural killer cells from patients with large tumor burdens produce significantly less IL-1 and have decreased killing ability compared to cells from healthy individuals. When incubated with exogenous IL-1, impaired natural killer function is restored. IL-1 also augments the binding of natural killer cells from healthy donors to tumor targets. Since IL-1 induces interferon and since interferon synergizes with IL-1 with respect to its actions on natural killer cells (Dempsey *et al.*, 1982), one could view both mechanisms as an efficient aspect of host defense against tumors. However, unlike augmentation of T and B cell responses by IL-1, which are enhanced at febrile temperatures (Duff and Durum, 1982; Hanson and Murphy, 1985), the effect of IL-1 on natural killer cells is reduced by febrile temperatures (Dinarello *et al.*, 1986c).

NK cells also produce IL-1 (Scala *et al.*, 1984b). Recent studies suggest that, in addition to endotoxin, high-dose IL-2 is a stimulant for IL-1 and TNF production by NK cells (Numerof *et al.*, 1988). The ability of IL-2 to induce IL-1 and TNF production appears to be due to the CD4 receptor on monocytes. This is upregulated by IFN-γ, which also increases the responsiveness of NK cells to IL-1-induced cytokine production.

VII. The IL-1 Receptor

The initial studies on the binding of radiolabeled IL-1 were carried out using a variety of cells (Dower *et al.*, 1985); nearly all subsequent

studies have focused on two cell types: murine thymoma cells and fibroblasts (reviewed in Savage and Dinarello, 1988). From these early studies, there appeared to be a single class of intermediate-affinity receptor (K_d ranging from 400 p*M* to 1 n*M*) and relatively few receptors (200/cell). Subsequent studies showed that the receptor number and affinity could be higher on thymoma cell lines and fibroblasts. Although the IL-1 receptor was specific in that it did not recognize other cytokines, the IL-1 receptor did not distinguish between IL-1α or IL-1β (Kilian *et al.*, 1986; Matsushima *et al.*, 1986b, c; Lowenthal and MacDonald, 1986; Lowenthal *et al.*, 1986; Chin *et al.*, 1987). In general, the binding correlated with the capacity of the cells to respond to IL-1. However, it soon became clear that there were two classes of IL-1 receptors (Lowenthal and MacDonald, 1986; Bird and Saklatvala, 1986). There is little question that an IL-1 receptor has been recognized by all groups and has a variable dissociation constant of 200–600 p*M*. From cross-linking experiments, this binding protein is probably what is often called the IL-1 receptor: an 80-kDa glycosylated peptide whose unglycosylated form is approximately 55 kDa. This molecule has been solubilized (Paganelli *et al.*, 1987) and cloned; the sequence reveals that the IL-1 receptor belongs to the immunoglobulin superfamily (Sims *et al.*, 1988).

A recent report depicts the molecular structure of the IL-1 receptor belonging to the immunoglobulin superfamily (Sims *et al.*, 1988). In fact, it appears that there are three regions linked by S–S bonds which show striking similarities to the structure of the immunoglobulin light chain domains. These three domains are extracellular; there is a transmembrane portion of approximately 21 amino acids and a cytosolic region. This cytosolic region has no homology with any known protein kinase but may be a substrate for protein kinase C. The possibility exists that the IL-1 receptor may be polymeric, with rearrangements in the three domains. It is also possible that lower molecular-weight (25-kDa) IL-1-binding protein (low affinity) is composed of only two domains and that the nonglycosylated 50-kDa form represents further molecular polymerization. The higher molecular-weight forms of IL-1-binding proteins would also fit into this scheme. Further experimentation will clarify this issue. However, one of the overriding aspects of the IL-1 receptor is that cells respond to subpicomolar concentrations of IL-1 without possessing demonstrable IL-1 binding. One explanation is that these cells have another set of very low-affinity receptors or that the high-affinity receptors are low in number but that occupancy is sufficient to trigger intracellular events. Alternatively, there may be large numbers of low-affinity receptors that cannot be easily demonstrated. However, binding presumably takes place with sufficient interaction that signal transduction takes place. To date, the current IL-1 receptor that has been cloned

appears to be the high-affinity receptor, and there exists the possibility of a very low-affinity receptor of different molecular structure.

The cloned molecule likely represents the high-affinity receptor described by Lowenthal and MacDonald (1986) and Bird and Saklatvala (1986). These groups described binding sites with an affinity of 5-10 p*M*, considerably higher affinity than that previously reported. Furthermore, it has also been reported that both high- and low-affinity receptors exist on a wide variety of T cell lines and normal cells. We have also observed two classes of IL-1 receptors on the D10.G4.1 murine T-helper cell line. In the case of our own studies (Savage *et al.*, 1988), receptors of both high and low affinity were detected simultaneously on a subclone (D10S) of these cells. The failure to detect both classes of receptors in some studies may be due to the low specific activity of the radiolabeled IL-1 or that the low affinity receptor is not easily detectable.

In addition to the conflicting data on the IL-1 receptor affinity, there are also low numbers of receptors. However, Lowenthal and MacDonald (1986) and Savage *et al.* (1988) have reported much higher numbers of IL-1 receptors on murine EL-4 cells and murine T helper cells, respectively. However, the high numbers (about 20,000/cell) are not in the same range as those observed for other growth factor receptors, which can be of the order of 100,000-200,000/cell.

A. Comparison of the IL-1 and IL-2 Receptor

In evaluating the significance of high- and low-affinity receptors for IL-1 on different cells, some investigators have compared the situation with that of the receptors for IL-2. IL-2 receptors also exist as two classes distinguished by their ligand-binding affinities (Lowenthal *et al.*, 1985; Robb *et al.*, 1986). The IL-2 receptor is made up of two distinct polypeptide chains (the so-called "TAC" 55-kDa antigen and a lower affinity polypeptide chain of 75 kDa), each of which may bind to IL-2. One of the components binds with a lower affinity than the other; the two may both interact and combine with IL-2 to produce the high-affinity receptor. The binding of IL-2 to its 75-kDa intermediate affinity receptor is sufficient to activate Na^+/H^+ exchange in YT 202 (Mills and May, 1987), a cell line expressing only the 75-kDa IL-2-binding component. The same situation has been proposed for the IL-1 receptor (Bird *et al.*, 1987). Our own studies on D10S cells suggests that the IL-1 receptor comprises more than one polypeptide chain (Savage *et al.*, 1988) and others have also observed the existence of a second chain (Bird *et al.*, 1987).

Studies on the IL-2 receptor have revealed that high- and low-affinity receptors differ functionally and that a similar case can be made for the two classes of IL-1 receptors (Lowenthal and MacDonald, 1986). It

has been proposed that the high-affinity IL-1 receptor is incorporated into the cell and the number of IL-1 molecules endocytosed by various T cell lines correlates with the number of high-affinity IL-1 receptors expressed by these cells. This has been demonstrated for IL-2, where ligand internalization is believed to be mediated only by high-affinity receptors (Weissman *et al.*, 1986). In contrast, the internalization of IL-1 appears to be via a receptor with an intermediate affinity of 150 p*M* (Bird *et al.*, 1987). Internalization of IL-1 by a large granular lymphocyte cell line was demonstrated by receptors with a K_d of about 100 p*M* (Matsushima *et al.*, 1986b,c), which is lower than the high-affinity receptors of 5-10 p*M*. One explanation for this discrepancy is that two different cell types have been studied, the fibroblast and the T cell, respectively.

B. Both IL-1α and IL-1β Bind to the Same Receptor

Most studies have demonstrated that in using either form of IL-1 (α or β) in receptor binding experiments, the other IL-1 form effectively competes with the binding. However, some studies suggest that although human and murine IL-1α may bind to human endothelial cells with an equal affinity, there is an unequal ability to induce a biological response (Thieme *et al.*, 1987). Recombinant murine IL-1β was found to be 250- to 1250-fold less active than recombinant human IL-1α in inducing endothelial cell adherence of human lymphocytes. Furthermore, Bird *et al.* (1987) have shown that the concentration of porcine IL-1β required to elicit half-maximal IL-2 production from NOB-1, a subline of murine thymoma IL-4, was 100-fold greater than that for porcine IL-1α. However, both forms of IL-1 were equally active at similar doses when acting on BALB/c 3T3 fibroblasts to increase lactate production.

These results and those of other investigators suggest that, contrary to what was first believed, the receptor binding site on IL-1α and IL-1β may not be recognizing the same receptor loci in the different cells. Some attempts have been made to localize the receptor binding site or specific recognition peptides on IL-1. MacDonald *et al.* (1986) produced various mutations of the histidine residue at position 147 in IL-1β and showed that this resulted in up to a 100-fold reduction in receptor binding affinity. Short synthetic peptide fragments of human IL-1β with immunostimulatory activity (Antoni *et al.*, 1986; Rosenwasser *et al.*, 1986) have been produced. These peptides code for hydrophobic regions of the IL-1β molecule which share significant amino acid homologies to those regions in the IL-1α molecule. Although they are biologically active, large amounts are required. Furthermore, there are no data suggesting that these peptides block the binding of full-length IL-1β to cells. Recently,

attention has focused on the carboxyl terminal of the IL-1β structure. X-Ray crystallographic studies of IL-1β have revealed that the histidine at position 147 as well as the N-terminus and carboxyl terminus are exposed and available for membrane interaction (Priestle *et al.*, 1988). In one study, antibodies produced to the C-terminal (amino acids 247–269) blocked IL-1 biological activities whereas antibodies produced to the N-terminal amino acids (117–134) had no effect (Riveau *et al.*, 1988). On the other hand, mutations in the IL-1β N-terminus have dramatically affected IL-1 binding (Horuk *et al.*, 1987). It appears that both the N- and C-termini interact with the receptor.

C. Regulation of IL-1 Receptor Expression

Both up regulation and down regulation of the IL-1 receptor may occur. Up regulation of the IL-1 receptor on T cells has been reported on human peripheral T cells or murine splenic T cells stimulated with concanavalin A (Shirakawa *et al.*, 1987; Dower and Urdal, 1987), and human mononuclear cells treated with corticosteroids increase receptor number. EL-4 cells treated with *trans*-retinoic acid increase their binding of IL-1 (Kilian *et al.*, 1986). Down regulation by IL-1 of the IL-1 receptor has been reported (Matsushima *et al.*, 1986c; Mizel *et al.*, 1987; Savage *et al.*, 1988).

D. Physical Structure of the IL-1 Receptor

Cross-linking experiments in a variety of cells identify a major binding protein of 80 kDa on SDS-PAGE. A second cross-linked species has been observed at 116 kDa. It has been suggested that the higher molecular mass species could either represent a protein of 116 kDa or a tertiary complex of the 80-kDa receptor, a 25-kDa binding protein, and IL-1. Cross-linking studies in rat brain indicate that the rat brain IL-1 receptor also has a molecular mass of 80 kDa (Farrar *et al.*, 1987). It may be significant that Farrar and co-workers also found a second IL-1-binding protein in rat brain at 58 kDa. The homogeneous IL-1 receptor, purified from EL-4 6.1 cells, has been found to be a protein of 80 kDa, which correlates with the results found for the affinity cross-linking experiments.

Most experimental evidence to date has confirmed the existence of a plasma membrane protein of molecular size ranging from 70–80 kDa for the IL-1 receptor. The Raji B-lymphoma cells have a lower binding affinity but much higher receptor density (K_d = 2.1 n*M*, 7709 sites/cell) (Horuk *et al.*, 1987) than the murine T cells (K_d = 400 p*M*, 241 sites/cell). Cross-linking studies showed that the IL-1 receptor in the B cells had a lower molecular mass than that in the T cells (68 compared to 80 kDa). We, however, have found that the D10S subclone of the murine

T-helper cell D10.G4.1 possesses a high number of IL-1 receptors which would imply that the cells do not differ from the B cells in this regard (Savage *et al.*, 1988). It is likely that variations in size of the IL-1-binding components in the different cells are caused by glycosylation. In fact, treatment of EL-4 as well as fibroblast-derived IL-1 receptors with glycanases reduces the molecule size to about 50 kDa (T. A. Bird, J. Saklatvala, and H. R. MacDonald, personal communications).

In our laboratory we have digested radioactive IL-1 cross-linked proteins from El-4 and D10S cells with glycanase, generating bands 10–15 kDa lower in mass than the major species (Savage *et al.*, unpublished observations). Most authors have also reported on the presence of minor radioactive bands of either higher or lower molecular mass than the major species. We have observed a 25-kDa IL-1-binding protein on D10S cells which was not observed with EL-4 cells. It is possible that the 116-kDa protein is a combination of the 80- and 25-kDa proteins. The significance of these lower molecular-weight IL-1-binding components is not clear; however, a recent report on the structure of the IL-1 receptor may explain the molecular-weight differences (see below).

Alternatively, random lateral association is occurring with neighboring membrane proteins or additional proteins may be involved in the signal transduction mechanism, both leading to cross-linked IL-1 proteins with various molecular weights. Again, as in the case of the IL-2 receptor, there may be more than one polypeptide comprising the IL-1 receptor complex. Indeed, there exists some evidence to support the latter possibility (Bird *et al.*, 1987). A recent report depicts the molecular structure of the IL-1 receptor belonging to the immunoglobulin class (Sims *et al.*, 1988). In fact, it appears that there is a repeating sequence of light-chained immunoglobulin molecules with molecular weights of about 12.5 kDa. This would implicate IL-1 receptors as being polymeric. It would explain the lower molecular-weight 25-kDa IL-1-binding protein (low affinity) as being a dimer and the deglycosylated form of 50 kDa as representing further molecular polymerization. The higher forms of IL-1 binding proteins would also fit into this scheme. Further experimentation will clarify this issue. Of similar importance is the likelihood that the IL-1 receptor, being part of the immunoglobulin class, would be rearranged on some cells. The significance is unclear. However, one of the overriding aspects of the IL-1 receptor is that cells respond to subpicomolar concentrations of IL-1 without possessing demonstrable IL-1 binding. One explanation is that these cells have very low-affinity receptors and the receptor occupancy is slow, short, and of such low affinity that binding cannot be easily demonstrated. However, binding presumably takes place with sufficient interaction that signal transduction takes place.

E. Post-Receptor-Binding Events

Information concerning the intracellular events following the IL-1-1/IL-1 receptor complex has only recently been studied. No clear picture has emerged. Of importance when considering the postreceptor events of IL-1 action is the fact that in addition to the growth-promoting properties of IL-1, the cytokine has a wide variety of other biological activities. These properties can only be explained if there are different receptors on the various cells, or alternatively if the receptors are identical but the postreceptor machinery differs in the various cells in which the biological events occur. When considering the cellular proliferation effects, some evidence suggest that, like other growth factors such as EGF and insulin, IL-1 may activate a tyrosine kinase, although it is not known whether the kinase is part of the IL-1 receptor. However, the same authors have also observed plasma membrane protein phosphorylation as a result of IL-1 action on membranes from K562 cells. Surprisingly the K562 cells have been shown to possess very small numbers of receptors (<10 molecules bound per cell) and the effect of IL-1 in these cells is not to produce proliferation, but rather to induce killing mechanisms. Do the two phosphorylation events induced by IL-1 differ? Results from our laboratory (G. von Bulow, N. Savage, and C. A. Dinarello, unpublished) show that recombinant IL-1 acts on the plasma membrane isolated from EL-4 cells and can also induce the phosphorylation of certain membrane proteins, although the site of phosphorylation is unknown at present. On the other hand, Matsushima *et al.* (1987) have observed the phosphorylation of a cytosolic 65-kDa protein when IL-1 acts on normal human peripheral blood mononuclear leukocytes pretreated with glucocorticoids. Phosphorylation was observed in serine residues of the protein and not on tyrosine residues, as described by Martin *et al.* (1986) for K562 cells.

In addition to the phosphorylation effects of IL-1, internalization of the ligand occurs. Internalization of IL-1 has been shown by several groups (Bird and Saklatvala, 1987; Matsushima *et al.*, 1986b,c; Mizel *et al.*, 1987). Some studies show subsequent lysosomal trafficking of ligand and reutilization of receptors. After 3 hours at 37°C, the ligand was located in the lysosomal fraction and there was an increase in the TCA-soluble fraction at 6 hours. In addition, some studies show electron microscopic evidence, using autoradiographic detection techniques, for the appearance of radioactive IL-1 within the nucleus of the cells. There was little evidence for the degradation of IL-1 by the cells, at least up to 4–6 hours. Lowenthal and MacDonald (1987) have also observed the uptake of IL-1 in EL-4 cells and noted that a significant fraction internalized IL-1 was found in the nucleus.

Another postreceptor event that has been described for IL-1 is the ion flux of both Na^+ and Ca^{2+} across the plasma membrane of a murine pre-B cell line (Stanton *et al.*, 1986). Major questions require clarification of the nature of the signal initiated by the formation of the cell surface IL-1 complex and the role of internalization of the IL-1 ligand in the transfer of information. The finding that IL-1 will rapidly increase phosphatidylcholine turnover in Jurkat cells that do not manifest a detectable IL-1 receptor underscores the complexity of IL-1 effects on cells and the postexposure events. It is possible that there are both receptor (high-affinity) events leading to PI turnover and the generation of DAG in some cells, and low-affinity receptor interaction resulting in the generation of another DAG from PC turnover. Moreover, both systems may work in some cells. The end result would perhaps provide a number of activated protein kinases.

VIII. Comparison of IL-1, TNF, and IL-6

A. IL-1 AND TNF

TNF was initially identified in the circulation of animals following the injection of endotoxin. It was also discovered in the supernates from stimulated macrophage cell lines, where its property as an inhibitor of lipoprotein lipase led to its being named "cachectin," because it produced a wasting syndrome when chronically administered to mice. The amino acid sequences of TNF (Pennica *et al.*, 1984) are identical to cachectin (Beutler and Cerami, 1986). TNF, a product of stimulated monocytes and macrophages, is also produced by lymphocytes (Cuturi *et al.*, 1987), endothelial cells, and keratinocytes. A structurally related polypeptide, initially isolated from activated T cells, is lymphotoxin. Lymphotoxin and TNF produce similar biological changes in a variety of cells. The amino sequence of TNF and lymphotoxin are closely related (Pennica *et al.*, 1984) and both molecules are recognized by the same cell membrane receptor. Like IL-1β and IL-1α, TNF and lymphotoxin are sufficiently structurally distinct molecules that antibodies produced to each cytokine do not cross-react with the other cytokine. Although originally studies for its ability to kill tumor cells *in vitro* as well as when injected into tumor-bearing mice, the widespread biological effects of TNF on mesenchymal and other cells have been the focus of studies related to its inflammatory properties, particularly in mediating synovial cell activity and cartilage and bone degradation. Moreover, recombinant human TNF has been injected into human subjects and many of its systemic effects, such as fever, leukopenia, and hypotension, which

were studied in animals, have now been observed in humans (Chapman *et al.*, 1987).

The biological properties of TNF share remarkable similarities to those of IL-1, particularly the nonimmunological effects of IL-1. Some lymphocyte-activating properties of IL-1 or IL-6 are shared with TNF, but these require considerably higher concentrations of TNF than of IL-1. There are recent reports on the ability of TNF to activate T cells (Ranges *et al.*, 1988), including the expression of IL-2 receptors (Plaetinck *et al.*, 1987). The B cells are also stimulated by TNF (Kehrl *et al.*, 1987; Jelinek and Lipsky, 1987). Compared to IL-1, the molar concentration of TNF required to stimulate immunocompetent cells is one or two orders of magnitude greater than IL-1 (Ranges *et al.*, 1988; Kehrl *et al.*, 1987). Since TNF induces the synthesis and release of immunostimulatory polypeptides such as IL-1 and IL-6 from monocytes, fibroblasts, and endothelial cells, it is possible that these cytokines augment the action of TNF on lymphocytes. Some investigators have attempted to separate a direct action of TNF on lymphocytes from that secondary to the induction of IL-1 or IL-6 (Ranges *et al.*, 1988). It also appears that unlike IL-1, the immunostimulatory effects of TNF are species specific.

Nearly every nonimmunological biological property of IL-1 has also been observed with TNF. These include fever (Dinarello *et al.*, 1986b), the induction of PGE_2 and collagenase synthesis in a variety of tissues (Dayer *et al.*, 1986), bone and cartilage resorption (Saklatvala, 1986), inhibition of lipoprotein lipase (Beutler and Cerami, 1985), increases in hepatic acute-phase proteins and complement components, and a decrease in albumin synthesis (Perlmutter *et al.*, 1986a). Slow-wave sleep and appetite suppression are also observed following the injection of TNF (Shoham *et al.*, 1987). As discussed above, both molecules induce fibroblast proliferation and collagen synthesis (Kohase *et al.*, 1987). The cytotoxic activity of TNF differs from that of IL-1 in that IL-1 is inactive on a variety of tumor targets for which TNF is a potent cytotoxin. However, IL-1 exhibits cytotoxic effects on melanoma cells which are unaffected by TNF (Onozaki *et al.*, 1985). Another difference between IL-1 and TNF is that IL-1 can function as a cofactor for stem cell activation (hemopoietin-1 activity) (Stanley *et al.*, 1986; Moore and Warren, 1987; Mochizuchi *et al.*, 1987), whereas TNF suppresses bone marrow colony formation (Zucali *et al.*, 1988). Both IL-1 and TNF induce the synthesis of colony-stimulating factors (Bagby *et al.*, 1986; Zucali *et al.*, 1986).

Similar to IL-1, TNF induces fever by its direct ability to stimulate hypothalamic PGE_2 synthesis (Dinarello *et al.*, 1986b). Levels of circulating TNF rise rapidly in human subjects injected with endotoxin

(Hesse *et al.*, 1988; Michie *et al.*, 1988) and are associated with the symptoms of the prodrome and chill period of the fever. In addition to fever, TNF produces hypotension, leukopenia, and local tissue necrosis (Tracey *et al.*, 1986). On a weight basis in rabbits, TNF is more potent than IL-1 in producing a shock (Okusawa *et al.*, 1988). Administration of anti-TNF antibodies to rabbits prevents the shock induced by endotoxin (Mathison *et al.*, 1988). The shocklike responses to TNF likely reflect the effects on the vascular endothelium. TNF stimulates PGI_2, PGE_2, and platelet-activating factor production by cultured endothelium. In addition, like IL-1, TNF stimulates procoagulant activity, leukocyte adherence, and plasminogen activator inhibitor on these cells. TNF also induces a capillary-leak syndrome. Despite the similarities, receptors for TNF and IL-1 are distinct and specific and receptor binding to the respective ligand is only displaced by the specific cytokine. Furthermore, IL-1 down regulates its own receptor as well as that of TNF. The most likely explanation is that TNF and IL-1 stimulate similar intracellular messages by different pathways and alter the same cascade of intracellular metabolites. Table III lists the biological similarities of IL-1 and TNF. Of note is the fact that TNF stimulates human neutrophil oxidative metabolism (Klebanoff *et al.*, 1986), whereas IL-1 does not (Georgilis *et al.*, 1987); on the other hand, IL-1 induces histamine and arylsulfatase release from human basophils (Subramanian and Bray, 1987) and

TABLE III
COMPARISON OF BIOLOGICAL PROPERTIES OF IL-1 AND TNF

Biological property	IL-1	TNF
Endogenous pyrogen fever	+	+
Slow-wave sleep	+	+
Hemodynamic shock	+	+
Increased hepatic acute-phase protein synthesis	+	+
Decreased albumin synthesis	+	+
Activation of endothelium	+	+
Decreased lipoprotein lipase	+	+
Decreased cytochrome P_{450}	+	+
Decreased plasma Fe/Zn	+	+
Increased fibroblast proliferation	+	+
Increased synovial cell collagenase and PGE_2	+	+
Induction of IL-1	+	+
T/B cell activation	+	±
Hemopoietin-1 activity	−	+

eosinophils, respectively (Pincus *et al.*, 1986), and TNF does not. There is another macrophage product with a molecular weight of 8 kDa that is chemotactic for neutrophils and is clearly not IL-1 nor TNF (Wolpe *et al.*, 1988; Yoshimura *et al.*, 1987). This factor may have been present in some preparations of natural IL-1 and TNF and could have accounted for their effects on neutrophils. The biological properties of this macrophage product in comparison to IL-1 and TNF remain to be ascertained.

B. Synergism between IL-1 and TNF

The effects of IL-1 or TNF on a variety of cells *in vitro* as well as systemic effects *in vivo* are often biologically indistinguishable. When the two cytokines are used together in experimental studies, the net effect often exceeds the additive effect of each cytokine. Potentiation or frank synergism between these two molecules has been demonstrated in several studies. On fibroblasts, IL-1 and TNF act synergistically in the production of PGE_2 (Elias *et al.*, 1987). The cytotoxic effect of TNF and IL-1 on certain tumor cells is also synergistic *in vitro,* and when administered to tumor-bearing mice *in vivo,* both molecules act synergistically to eliminate the tumor (M. Palladino, personal communication). IL-1 combined with TNF protects rats exposed to lethal hyperoxia (White *et al.*, 1987). IL-1 and TNF act synergistically in the induction of radioprotection (Neta *et al.*, 1987, 1988). IL-1 induces cytotoxic effects on the insulin-producing beta cells of the islets of Langerhans; this effect is dramatically augmented by TNF (Mandrup-Poulsen *et al.*, 1986, 1987). IL-1 and TNF induce neutrophilic infiltration when injected intradermally into experimental animals; when injected together, these cytokines act synergistically and can replace endotoxin in the generation of the local Schwartzman reaction (Movat *et al.*, 1987). Rats receiving intravenous infusions of IL-1 or TNF manifest metabolic changes reflected in plasma amino acid levels, but, when given together, negative nitrogen balance and muscle proteolysis can be demonstrated (Pomoselli *et al.*, 1987). Although high doses (10–20 μg/kg) of TNF produce a shocklike state with tissue damage (Tracey *et al.*, 1986), IL-1 and TNF act synergistically to produce hemodynamic shock and pulmonary hemorrhage at doses of only 1 μg/kg when given together (Okusawa *et al.*, 1988). The two cytokines also act synergistically in the aggregation of neutrophils and the synthesis of thromboxanes (Conti *et al.*, 1986). Considering the fact that IL-1 and TNF are often present together in human body fluids, including inflammatory joint fluid, the synergism between the two cytokines cannot be considered a laboratory observation. The synergism between these two cytokines seems to be due to second message molecules

rather than up regulation of cell receptors; in fact, IL-1 reduces TNF receptors (Holtmann and Wallach, 1987).

C. IL-1 AND IL-6

The recent cloning and expression of B-cell-stimulating factor-2 (B cell growth factor-2) (Hirano *et al.*, 1986) and hybridoma growth factor (Brakenhoff *et al.*, 1987) have supported the observation that these molecules are identical to interferon-β2 (van Damme *et al.*, 1987a,b). Interferon-β2 was cloned and expressed before its identification as B-cell-stimulating factor-2. In addition, interferon-β2 appears to be the same molecule previously termed "hepatocyte-stimulating factor" (Gauldie *et al.*, 1987), inducing a variety of hepatic acute-phase proteins in cultured liver cells. The molecule interferon-β2, hybridoma growth factor, plasmacytoma growth factor, B-cell-stimulating factor-2, and hepatocyte-stimulating factor is now termed IL-6. Like IL-1 and TNF, IL-6 is an endogenous pyrogen and an inducer of acute-phase responses. In a clinical study, serum levels of IL-6 correlated with the amount of fever present in patients with burn injuries (Nijsten *et al.*, 1987). IL-6 levels have also been reported elevated in patients undergoing renal rejection (van Oers *et al.*, 1988) and in the cerebrospinal fluid of patients with central nervous system infections (Houssiau *et al.*, 1988). Although IL-6 is an inducer of fibrinogen synthesis in hepatic cell lines, these cultured cells require the presence of corticosteroids to observe a response (Andus *et al.*, 1987). In mice and rats, IL-6 does not induce fibrinogen unless corticosteroids are administered at the same time (Marinkovic *et al.*, 1988); IL-1, on the other hand, induces large amounts of fibrinogen without the requirement of such cofactors (Bertini *et al.*, 1988).

The effects of IL-6 on lymphocytes and bone marrow stem cells are broadly based. Attention has focused on the ability of natural or recombinant IL-6 to act as a "lymphocyte-activating factor" in the typical murine thymocyte comitogenesis assay. Similar to recombinant IL-1α or IL-β, recombinant IL-6, in the presence of concanavalin A, induces the production of IL-2 from cytotoxic T cell lines (CTLL) (Garman *et al.*, 1987). A similar response was observed when recombinant IL-6 was added with mitogen or antibody to the T cell antigen receptor-stimulated peripheral blood $CD4^+$ T cell depleted of accessory cells. IL-2 was released and acted as the proliferative signal for these cells. Using high concentrations (100 units) of recombinant IL-1, production of IL-2 was not observed (Garman *et al.*, 1987), leading these authors to the conclusion that IL-6 rather than IL-1 was providing the "first signal" along with mitogen or anti-T cell receptor activation in mature circulating

T cells. The molar concentration of the recombinant IL-6 used in these studies is difficult to ascertain and limiting dilutions of the purified T-cell targets were not carried out. Therefore, like IL-1, it is difficult to ascertain whether IL-6 activates lymphocytes in the absence of macrophages, particularly since IL-6 is a product of activated macrophages; moreover, hemopoietic growth factors (Yang *et al.*, 1988) as well as IL-6 are inducible by IL-1 (van Damme *et al.*, 1987a,b; Schindler *et al.*, 1988).

IL-6 will act as a comitogen for human thymocytes and macrophage-depleted human T cells (Lotz *et al.*, 1988). However, unlike IL-1, the comitogenesis effect of IL-6 was not inhibited by antibodies to the IL-2 receptor (Lotz *et al.*, 1988). Similar studies have been reported for thymic and peripheral blood T cells of the $L3T4^+$ and $Lyt\text{-}2^+$ subsets (Uyttenhove *et al.*, 1988). Recombinant IL-6 acts as an autocrine growth factor for human myeloma cells *in vitro* (Kawano *et al.*, 1988), and IL-1 has also been shown to act as an autocrine growth factor for EBV-transformed human B cells (Scala *et al.*, 1987). In addition, recombinant IL-6 induces the production of immunoglobulin from activated B cells in the absence of growth (Muraguchi *et al.*, 1988).

We have compared recombinant IL-1 and IL-6 in a variety of assays. In the murine thymocyte comitogenesis assay, human recombinant IL-1 stimulates proliferation at 1–10 pg/mℓ, whereas 1–10 ng/mℓ of recombinant IL-6 is required for the same amount of proliferation. This may be due to species specificity of IL-6. Using a highly IL-1-sensitive murine T-helper cell line (D10.G4.1), five to six orders of magnitude greater concentrations of IL-6 compared to IL-1 are required for a proliferative signal. In rabbits, IL-6 behaves as an endogenous pyrogen, producing a rapid-onset monophasic fever; however, 20- to 50-fold greater amounts of IL-6 are required to produce the same elevation in body temperature as that following IL-1 (Dinarello *et al.*, 1988). Once again, this may be due to species specificity. However, when recombinant *human* IL-1 was compared to recombinant human IL-6 on *human* monocyte PGE_2 production, 50- to 100-fold more IL-6 than IL-1 was required. Similar dose-response differences have been observed using human synovial cells or fibroblasts as targets (J.-M. Dayer, personal communication). Unlike IL-1 and TNF, IL-6 does not induce IL-1 or TNF; in fact, IL-6 suppresses endotoxin- and TNF-induced IL-1 production (Dinarello *et al.*, 1988). IL-6 does not activate endothelial cells *in vitro* (Sironi *et al.*, 1988; Lapierre *et al.*, 1988). In general, IL-6 appears to be a weak inflammatory peptide. Of considerable importance is the observation that IL-1 and IL-6 both act as hemopoietin-1 on bone marrow cultures (Moore and Warren, 1987; Mochizuki *et al.*, 1987; Ikebuchi *et al.*, 1987). In

addition, IL-6 protects granulocytopenic mice against lethal Gram-negative infection (J. W. M. van der Meer, personal communication) similar to the protection afforded by IL-1 (van der Meer *et al.*, 1988). IL-1 and IL-6 act synergistically in protecting mice given lethal irradiation (R. Neta, personal communication). The lack of inflammatory properties and positive effects on B and T cell functions as well as bone marrow and nonspecific host defense mechanisms makes IL-6 potentially useful in treating some diseases, especially bone marrow transplantation. Table IV lists the biological activities of IL-1, TNF, and IL-6.

IX. IL-1 and Related Cytokines in Human Disease

A. IL-1 and TNF in Human Joint Fluid

IL-1 and TNF have been detected in human joint fluids from patients with a variety of joint diseases, including rheumatoid arthritis, osteoarthritis, traumatic arthritis, and psoriatic arthritis (Nouri *et al.*, 1984; Fontana *et al.*, 1982; Wood *et al.*, 1983; DiGiovine *et al.*, 1988). Recently, IL-6 has been detected in the fluid from patients with rheumatoid arthritis (T. Kishimoto, personal communication). Although substances that inhibit the bioassays for IL-1, TNF, and IL-6 have made detection difficult, specific assays to detect immunoreactive levels of the cytokines are presently available (Lisi *et al.*, 1987). Because of joint fluid proteases, it is not surprising to find IL-1 in small-molecular-weight fragments in the 10- and 4000-Da range. These fragments exhibit biological activity

TABLE IV
COMPARISON OF IL-1, TNF, AND IL-6

Biological property	IL-1	TNF	IL-6
Endogenous pyrogen fever	+	+	+
Hepatic acute-phase proteins	+	+	+
T cell activation	+	±	+
B cell activation	+	±	+
B cell Ig synthesis	±	−	+
Fibroblast proliferation	+	+	−
Stem cell activation (hemopoietin-1)	+	−	+
Nonspecific resistance to infection	+	+	+
Radioprotection	+	+	±
Synovial cell activation	+	+	−
Endothelial cell activation	+	+	−
Induction of IL-1 and TNF from monocytes	+	+	−
Induction of IL-6	+	+	−

for T and B lymphocytes (Miossec *et al.*, 1986). It is unclear whether TNF has biological activity after limited proteolysis. The sources of the IL-1, TNF, and IL-6 in human joint fluid includes the joint macrophage and other cells such as B cells and the synovial dendritic cells (Duff *et al.*, 1985). Large granular lymphocytes (also known as natural killer cells) release IL-1 and TNF upon stimulation with endotoxin (Scala *et al.*, 1984b) or high-dose IL-2 (Numerof *et al.*, 1988). The two major blood vessel cells, endothelium and smooth muscle, both produce IL-1 and may be a source of cytokines (Libby *et al.*, 1986a,b). In disease processes in which antigen-antibody complexes mediate tissue injury and vessel disruption, endothelial and/or smooth muscle IL-1 likely act as an autocoid and contribute to the progression of the lesion. In addition to the fixed cell pool in the synovium, IL-1 production may also take place in the joint space from B lymphocytes (Matsushima *et al.*, 1985b) and neutrophils (Tiku *et al.*, 1986). Of importance is the finding that Epstein-Barr virus infection in B cells leads to IL-1 production (Scala *et al.*, 1984a); some recent studies suggest that T cells may also be a source of IL-1, but these studies have been carried out only in transformed T cell lines (Durum *et al.*, 1987). On the other hand, human blood T cells stimulated with mitogens produce TNF (Cuturi *et al.*, 1987).

B. Circulating IL-1, TNF, and IL-6 in Human Disease States

In general, the levels of these cytokines are low and most studies to date are based on bioassays. These include elevated IL-1 levels in humans following strenuous exercise and ultraviolet light for the treatment of psoriasis, in women following ovulation (Cannon and Dinarello, 1985), and in patients with renal allograft rejection. IL-6 levels have been reported to be elevated in patients with burn injuries (Nijsten *et al.*, 1987) and undergoing organ transplant rejection. TNF levels are elevated in patients with acute meningitis due to *Neisseria*. Recently, TNF levels as measured by specific enzyme-linked immunoassay were elevated in the plasma of human volunteers injected with endotoxin (Michie *et al.*, 1988). IL-1 levels using a radioimmunoassay with a detection limit of 250 pg/ml did not show any statistical change. There are several unresolved issues of measuring cytokines in the circulation during various disease states; these include technical aspects of extracting cytokines that may bind to plasma proteins, specific plasma proteins that inhibit the assays, rapid degradation by plasma proteases, rapid clearance of the cytokines by cell receptors or by excretion into the urine, and transiently elevated cytokine levels. There has been considerable research effort focused on measuring circulating cytokine in various disease states but few convincing reports.

C. Production of IL-1 *in Vitro* by Blood Monocytes of Patients with Diseases

The amount of IL-1 produced from human blood monocytes *in vitro* has been examined in the setting of various disease states. There have been consistent results which indicate that IL-1 production is decreased in monocytes from patients with large tumor burdens (Herman *et al.*, 1985a) or metastatic disease (Pollack *et al.*, 1983; Santos *et al.*, 1985). It is unclear, however, whether this decreased production of IL-1 represents a mechanism relevant to cancer or reflects the state of nutrition of the individual. Malnourishment seems to reduce IL-1 production and this has been shown in experimental animal models (Kauffman *et al.*, 1986) as well as in human studies (Bhaskaram and Sivakumar, 1986; Helyar and Sherman, 1987). On the other hand, there are several studies which suggest that blood monocytes from patients with active rheumatoid arthritis produce *more* IL-1 than do blood monocytes from normal individuals (Nouri *et al.*, 1985; Martini *et al.*, 1986; Shore *et al.*, 1986). Patients with ankylosing spondylitis produce the same amount of IL-1 as that produced by monocytes from healthy control subjects (Keystone *et al.*, 1986), whereas *decreased* IL-1 production has been consistently demonstrated from monocytes of patients with scleroderma (Sandborg *et al.*, 1985) or systemic lupus erythematosus (Alcocer-Varela *et al.*, 1984; Linker-Israeli *et al.*, 1983; Horwitz *et al.*, 1987).

These and other data on IL-1 levels are based upon bioassays which are affected by a variety of substances present in the supernatant medium of cultured mononuclear cells. It is, however, unlikely that the "increase" in IL-1 production observed with monocytes of patients with rheumatoid arthritis is due to IL-2, since the amount of IL-2 produced from the cells of these patients is, in fact, decreased. On the other hand, the demonstration that IL-1 production by monocytes of patients with lupus erythematosus or scleroderma is decreased may be due to the presence of inhibitory factors which suppress the IL-1 bioassay. These inhibitory substances include prostaglandins as well as polypeptides. In one study, the decreased production of IL-1 from monocytes *in vitro* from patients with lupus erythematosus was only partially reversed by the addition of indomethacin (Horwitz *et al.*, 1987).

In general, the supernatant media of cultured human mononuclear cells contain polypeptides that specifically suppress T cell bioassays for IL-1 but not IL-2. These nondialyzable suppressor factors have been demonstrated in the supernatant media of stimulated human mononuclear cells from apparently healthy individuals under a variety of conditions including incubation with immune complexes and viruses (Arend *et al.*, 1985; Rogers *et al.*, 1985). There has been partial characterization

of the IL-1 inhibitory protein found in the supernatants of cytomegalovirus-infected human mononuclear cells (Rogers *et al.*, 1985). Cultured human mononuclear cells from the joint fluid of patients with rheumatoid arthritis contain and/or produce an IL-1-specific inhibitor (Lotz *et al.*, 1986), which has been implicated as the cause of the depressed response of these cells to mitogens (Lotz *et al.*, 1986). There is evidence that the IL-1 inhibitory protein produced by a human glioma cell line is transforming growth factor-β (Fontana *et al.*, 1987). Clearly, these IL-1 inhibitory molecules are biologically functional molecules but their presence obscures the bioassays for IL-1. Hence, the data demonstrating decreased IL-1 production from the mononuclear cells of patients with autoimmune diseases may reflect excess production of inhibitory substances. Measurement of immunoreactive IL-1 will clarify whether a production defect for IL-1 (either increased or decreased amounts) exists in autoimmune diseases.

D. Natural Inhibitors of IL-1 Activity

Polypeptides which specifically inhibit IL-1 biological activity have also been detected in the serum of human volunteers injected with bacterial endotoxin (Dinarello *et al.*, 1982) and isolated in the urine of febrile patients (Liao *et al.*, 1984), in the urine of patients with myelomonocytic leukemia (Balavoine *et al.*, 1986), and in the urine of pregnant women. The urinary inhibitor isolated from pregnant women has been identified as uromodulin (Muchmore *et al.*, 1987), which is a glycosylated form of the Tamm-Horsfall protein (Pennica *et al.*, 1987). The carbohydrate portion of the uromodulin molecule binds IL-1 and TNF and this binding accounts for the inhibition of IL-1 activity in bioassays. In addition, the binding of IL-1 and TNF to uromodulin may be one mechanism in which biologically potent molecules are removed from the glomerular filtrate. An inhibitory molecule isolated from the urine of patients with myelomonocytic leukemia has been shown to block the binding of IL-1 to its receptor on thymoma cells (Balavoine *et al.*, 1986). In general, the urinary inhibitors from febrile patients are biochemically different from uromodulin.

E. Cytokine Self-Augmentation Network

IL-1 and TNF participate in self-augmentation induction mechanisms. Recombinant human IL-1 and TNF are each capable of inducing the production of their respective molecules as well as each other (Dinarello *et al.*, 1986b, 1987; Philip and Epstein, 1986; Ikejima *et al.*, 1987). IL-1 and TNF both induce IL-6. The target cells include monocytes, endothelial cells, smooth muscle cells, and B cells. The concentrations

of IL-1 and TNF that stimulate their own production in this self-amplification cycle are within the range (1-10 ng/ml) of what has been measured in the supernatant media of cultured cells stimulated with viruses, bacterial toxins, and active complement components, and of immune complex (Okusawa *et al.*, 1987). In addition, the induction of circulating IL-1 by either TNF or by IL-1 can be demonstrated in experimental animals. In tissues such as joint spaces or lymph nodes, this self-amplification network may play an important role in sustaining a pathological process. As a result of exogenous activators or IL-1, increased PGE_2 occurs in macrophages and endothelial cells, serving to suppress further production of IL-1. The suppressive effect of PGE_2 on IL-1 production is, however, not at the transcriptional levels, but rather at the translation of new IL-1, and this seems to be via PGE_2-induced cyclic AMP (Knudsen *et al.*, 1986). The B-cell-derived IL-1 also has an autocrine effect for B cell functions involved in antibody formation (Scala *et al.*, 1987).

In addition to PGE_2 providing a negative feedback signal for IL-1-induced IL-1 production, interferon-γ also suppresses IL-1-induced IL-1 production (Ghezzi and Dinarello, 1988). This effect of interferon-γ on IL-1 action has also been observed for IL-1 effects on osteoclasts and fibroblasts (Gowen *et al.*, 1985; Granstein *et al.*, 1987). The ability of interferon-γ to reduce fibroblast collagen synthesis and IL-1-induced osteoclast activation is in contrast to the well-established ability of interferon-γ to augment IL-1 production by a variety of cell activators, including endotoxins, staphylococcal entertoxins, complement components, and synthetic adjuvants. The ability of interferon-γ to suppress IL-1-induced IL-1 production takes place in the presence of cyclooxygenase inhibition. Similarly, the ability of corticosteroids to reduce the transcription of IL-1 mRNA also takes place in the presence of cyclooxygenase inhibition. One possible explanation for the clinical efficacy of disease-modifying drugs on the progression of rheumatoid arthritis may relate to the ability of these drugs to reduce IL-1 and related cytokine production and arrest the self-amplification interactions.

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ADVANCES IN IMMUNOLOGY, VOL. 44

Molecular and Cellular Events of T Cell Development

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I. Introduction

T cell development has long been one of the most complex and enigmatic processes in immunology. In the past few years, however, there have been major advances in our understanding of the molecular and cellular events which lead to the generation of the mature T cell pool. Although some T cell differentiation can occur outside the thymus, by far the majority of T cells develop within the thymus, and this review will concentrate on intrathymic differentiation. A major turning point in this field came with the identification of the surface receptors used by T cells to recognize and respond to foreign antigens in association with products of the major histocompatibility complex (MHC). For the majority of T cells (>95%), this consists of a clonally distributed disulfide-linked $\alpha\beta$ heterodimeric T cell receptor (TCR$\alpha\beta$) whose subunits are encoded by sets of rearranging gene segments. The remaining 2-5% of T cells express a second heterodimeric T cell receptor, termed TCR$\gamma\delta$, whose clonal diversity is also generated via gene rearrangement.

Understanding the molecular basis of T cell recognition allows us to divide T cell development into three stages:

1. *Early genetic events leading to TCR expression:* This first stage encompasses the rearrangement of TCR gene segments, resulting in the synthesis of productive mRNA transcripts and surface TCR expression. As will be discussed, the developmental regulation of TCR gene rearrangement and expression appears to be intimately related to the mechanism of lineage commitment to either TCR$\gamma\delta^+$ or TCR$\alpha\beta^+$ T cells. Most of the intrathymic proliferation occurs at this stage.

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2. *Cellular selection:* Once a developing T cell expresses a TCR on its surface, it is able to pass through the selection stage. We use the term cellular selection to distinguish the events of this stage from the potential molecular selection (e.g., preferential V gene rearrangements) that would occur during the first developmental stage prior to surface TCR expression. Thymocytes must pass through two selection filters, based on the TCR they express. Negative selection eliminates thymocytes that express self-reactive TCRs and which could, therefore, cause autoimmunity. Positive selection favors the development of thymocytes expressing TCRs that preferentially recognize foreign antigens in association with self-MHC molecules.

3. *Acquisition of mature effector function:* The eventual acquisition of effector function appears to occur after cellular selection. Fewer than 10% of thymocytes are fully mature by the standard criterion of helper or cytolytic function.

The dissection and investigation of these developmental events has been quite a challenge, given the extreme heterogeneity among thymocytes. One approach that has proved valuable, particularly in studying the early stages of development, is the analysis of fetal thymic ontogeny. Early fetal thymuses are highly enriched in T cell precursors. In addition, because fetal thymocytes proceed through the early developmental stages in a relatively synchronized wave, their ontogeny provides clues to the ordering of differentiation sequences; however, because developing thymocytes can potentially split off into multiple separate lineages, the ontogenic appearance of population A (defined, for example, by antigen A) prior to population B (defined by antigen B) does not prove that population A is a precursor to population B. The rigorous establishment of precursor–product relationships generally requires the demonstration that a significant proportion of cells from a purified precursor population differentiates either *in vitro* or after transfer into a thymic environment (e.g., organ culture or intrathymic injection). It is also important to prove that the apparent increase in product population is not due to selective outgrowth or survival of small numbers of product cells contaminating the initial precursor cell population.

Analysis of T cell development in the adult thymus has been greatly facilitated by the fact that thymocyte subsets can be separated, on the basis of surface markers, into relatively homogeneous populations that represent defined developmental stages. Some of these populations correspond closely to early fetal thymocytes but are present in much smaller proportion. There is an astounding evolutionary conservation in thymocyte ontogeny and subset distribution ranging from chicken to human. Whereas the majority of developmental work has been done in mice, we will also discuss significant studies in other species, including the chicken, rat, frog, sheep, and human.

II. T Cell Surface Molecules Involved in Antigen Recognition

Any current review of T cell development must begin with a discussion of the T cell surface structures involved in antigen recognition. Recent studies indicate that, in addition to the TCR, the accessory molecules CD4 and CD8 are also intimately involved in this process. As there are a number of in-depth reviews on the structure and function of these molecules, we will present a brief overview, designed primarily for the uninitiated reader.

A. The $\alpha\beta$ T Cell Receptor

Beginning with the studies of Zinkernagel and Doherty (1974, 1979), evidence has accumulated supporting the concept that T cells recognize a complex of peptide antigen and MHC product on antigen-presenting cells (APCs) (Schwartz, 1985; Babbit *et al.*, 1985; Buus *et al.*, 1987). Recent X-ray crystallographic studies of a human class I MHC antigen (HLA-A2) demonstrate that a β-pleated sheet and two α helices form the walls of a single peptide binding site (Bjorkman *et al.*, 1987a,b). The TCR structure on ~95% of T cells was first identified with "anticlonotypic" monoclonal antibodies (mAbs) raised against T lineage tumor lines or T cell clones (Allison *et al.*, 1982; Haskins *et al.*, 1983; Meuer *et al.*, 1983; Kaye *et al.*, 1983; Samelson *et al.*, 1983). The fact that these mAbs bound only to the clones against which they were raised was the first indication that this structure was clonally distributed in a manner analogous to surface immunoglobulin on B cells. In most cases, the anticlonotypic mAb stimulated (generally when cross-linked) or inhibited the stimulation of (generally when not cross-linked) only the clones to which they were raised. Each of these mAbs recognized a disulfide-linked heterodimer α (acidic isoelectric point) and β (basic isoelectric point) (Fig. 1). Proteolytic cleavage of α and β chains from different T cell clones defined variable and constant segments (McIntyre and Allison, 1983; Kappler *et al.*, 1983; Acuto *et al.*, 1983). Cloning of the β and α genes revealed that generation of diversity among TCR$\alpha\beta$ is quite similar to immunoglobulins—namely, rearrangement of variable (V) segments to diversity (D) and joining (J) regions associated with one or two constant region segments (Hedrick *et al.*, 1984; Yanagi *et al.*, 1984; Chien *et al.*, 1984; Saito *et al.*, 1984b). As shown in Fig. 2, the α locus has a larger number of V and many more J segments than does the β locus, but, as yet, no Dα has been described. The combinatorial diversity potential for the $\alpha\beta$ receptor (including possible N-region nucleotides) is ~10^{18} (reviewed by R. K. Wilson *et al.*, 1988).

In addition to providing structural information, the cloning of the TCR α and β genes led to the ultimate resolution of the long-standing question of whether the dual recognition of antigen + MHC by T cells

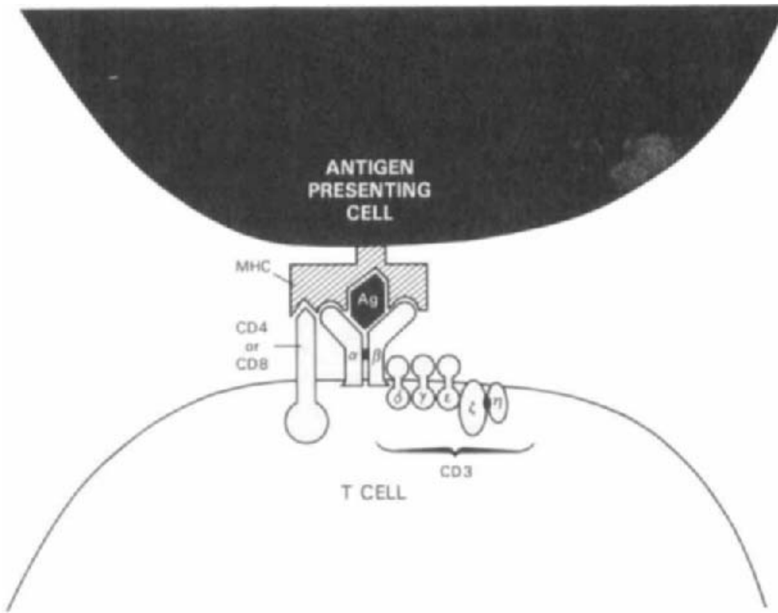

FIG. 1. Model for interaction of the murine T cell receptor complex with an antigenic peptide imbedded in a polymorphic groove of the major histocompatibility complex product, expressed by an antigen presenting cell (APC). The clonally distributed heterodimeric $\alpha\beta$ receptor is disulfide linked and is noncovalently associated on the cell surface with five invariant chains collectively referred to as CD3. Some CD3 complexes possess a disulfide-linked ζ-ζ homodimer and no η component. Featured as an integral part of the TCR-ligand interaction is an accessory molecule, either CD4 or CD8.

was mediated by one or two receptors. This was accomplished by demonstrating that transfection of solely the TCR α and β genes, derived from a fluorescein-specific, H2-D^d-restricted T cell clone, transferred the specificity for both fluorescein antigen and class I (D^d) MHC (Dembic *et al.*, 1986). An analogous result was obtained for a cytochrome *c*-specific, class II (I-E^k)-restricted TCR (Saito *et al.*, 1987).

The TCR$\alpha\beta$ heterodimer is noncovalently associated with an invariant membrane complex termed CD3. To date, five CD3 proteins have been identified (CD3-γ, CD3-δ, CD3-ϵ, CD3-ζ, CD3-η) (Samelson *et al.*, 1985a). Because CD3 cross-linking can mimic antigen–MHC stimulation of T cells (resulting in protein kinase C activation, elevation in intracellular Ca^{2+}, and transcription of activation-specific genes such as interleukin-2 and interferon-γ), and because all of the CD3 proteins have large intracytoplasmic portions (as opposed to only a few intracytoplasmic amino acids for the TCR α and β chains), it is generally

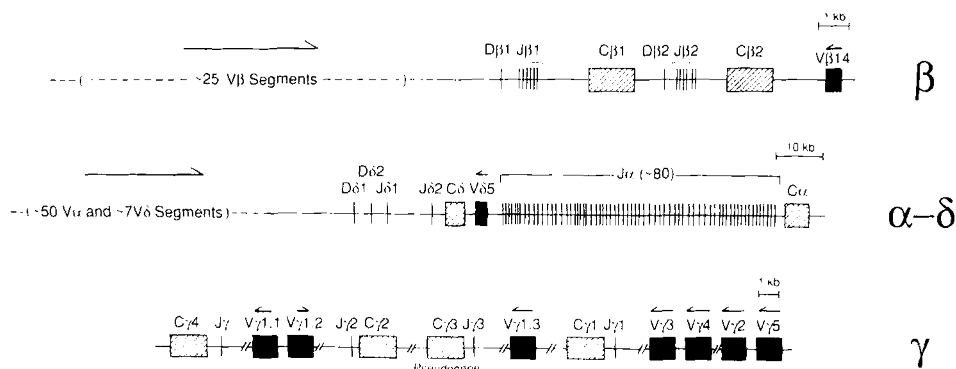

FIG. 2. Genomic organization of the murine T cell receptor loci. Arrows depict transcriptional orientation.

accepted that the CD3 complex is involved in signal transduction that is initiated when TCR$\alpha\beta$ is engaged by the antigen–MHC complex (reviewed by Weiss *et al.*, 1986; Clevers *et al.*, 1988).

B. THE ACCESSORY MOLECULES: CD4 AND CD8

Virtually all mature $\alpha\beta$-bearing T cells express either CD4 or CD8. The association of CD8 expression with MHC class I restriction, and CD4 expression with MHC class II restriction, suggested that the CD8 and CD4 molecules were involved in class I and class II recognition, respectively (Reinherz *et al.*, 1983; Swain, 1983). While quantitative binding studies with solubilized protein have yet to be performed, a number of investigations have provided convincing evidence that this interaction is important for T cell activation. The ability to block most class I- or class II-specific responses with anti-CD8 or anti-CD4 antibodies, respectively, represented the first suggestion that such interactions were functionally relevant (Reinherz and Schlossman, 1980; Swain, 1981; Nakayama *et al.*, 1979; Engleman *et al.*, 1981a; Spitz *et al.*, 1982; Swain *et al.*, 1983; Meuer *et al.*, 1982; Marrack *et al.*, 1983; Dialynas *et al.*, 1983). The most compelling evidence has again involved gene transfection experiments. When the fluorescein + H-2D^d-specific TCR α and β chains (referred to above) were transfected into a CD8$^-$ cytolytic (CTL) recipient, only fluoresceinated cells that expressed high levels of H-2D^d were targets for specific cytolysis; however, when the CD8 gene was subsequently introduced into this TCR α + β transfectant, then fluoresceinated cells with low levels of H-2D^d also became targets (Dembic *et al.*, 1987). Similar results with CD8 transfections were also obtained for an anti-K^k-specific TCR (Gabert *et al.*, 1987).

A different type of transfection experiment has been reported for CD4 (Gay *et al.*, 1987). The human CD4 gene was first transfected into a murine class I-specific T cell hybridoma. Recognition of a murine class II^- target by this CD4 transfectant was only enhanced when the target was transfected with genes encoding human class II antigen (HLA-DR). At face value, this experiment suggests that CD4–class II interactions (between T cell and antigen-presenting cell) can enhance functional TCR recognition even when completely independent of the TCR complex or the MHC antigen being recognized by TCR (since human CD4 does not recognize murine class I MHC). If such were the case, one might expect that many $CD4^+8^-$ T cells might be class I restricted as long as the APC coexpressed class II molecules for interaction with CD4. The symmetric reasoning would predict many class II-restricted $CD4^-8^+$ T cells, as well. Examples of such "cross-over" specificities are, in fact, rare. A number of investigations support the alternative concept that the coaggregation of accessory molecules with TCR complexes is extremely important for T cell activation. Experimentally induced coaggregation of accessory molecules with TCRs, using heteroconjugates of anti-CD8 or anti-CD4 mAbs with anti-CD3 or anti-TCR, stimulated T cells between 10 and 100 times more effectively than anti-CD3 or anti-TCR alone (Emmrich *et al.*, 1987, 1988; Ledbetter *et al.*, 1988). Presumably, natural coaggregation of accessory molecules and TCRs occurs because both would focus on the same MHC molecule. Comodulation of CD4 has been directly observed using certain anti-TCR mAbs (Saizawa *et al.*, 1987). It is, at this point, unclear whether the contribution of accessory molecule–MHC interactions to T cell activation is due mainly to direct avidity effects or to signaling effects related to coaggregation with TCRs. Whichever the case may be, it is clear that the CD4 and CD8 molecules coparticipate with TCR$\alpha\beta$ in antigen-specific T cell responses.

C. THE $\gamma\delta$ T CELL RECEPTOR

More recently, a second TCR, consisting of a (usually) disulfide-linked heterodimer, has been identified (reviewed by Brenner and Krangel, 1989; Raulet, 1989). The two chains of this receptor have been termed γ and δ. As opposed to TCR$\alpha\beta$, whose existence and ligand recognition characteristics had been predicted in the 1970s based on cellular experiments, the first evidence for the second TCR came from the unexpected cloning of a novel T cell-specific rearranging gene that was distinct from the α and β genes (Saito *et al.*, 1984a). This gene was termed γ. More recently, the δ gene locus has been cloned and found to reside within the α locus, between Vα and Jα (Chien *et al.*, 1987a) (Fig. 2). The initial identification of the $\gamma\delta$ receptor was on cell lines

derived from the peripheral blood of certain immunodeficiency patients (Brenner *et al.*, 1986). Shortly thereafter, this receptor was identified on a small subset of thymocytes and peripheral blood lymphocytes from normal humans, mice, and chickens (Bank *et al.*, 1986; Lew *at al.*, 1986; Borst *et al.*, 1987; Brenner *et al.*, 1987; Nakanishi *et al.*, 1987; Pardoll *et al.*, 1987a; Lanier *et al.*, 1987; Moingeon *et al.*, 1987). The many similarities between TCR$\gamma\delta$ and TCR$\alpha\beta$ can be briefly summarized as follows:

1. Both receptors display clonal diversity generated by the developmentally regulated rearrangement of variable and diversity (β and δ) gene segments to one or a few joining and constant region segments (see below).
2. The cells that express these receptors develop predominantly within the thymus (Pardoll *et al.*, 1988).
3. Both receptors are associated with a very similar CD3 complex (Krangel *et al.*, 1987; Marusic-Galesic *et al.*, 1988a).
4. Signaling through both receptors appears to involve phosphoinositol metabolism with associated protein kinase C activation and increased $[Ca^{+2}]_i$ (Krangel *et al.*, 1987). *In vitro* stimulation of both TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells results in similar effector function, e.g., cytolysis or lymphokine production.

There are two major distinctions between TCR$\alpha\beta$- and TCR$\gamma\delta$-bearing cells. First, TCR$\gamma\delta^+$ cells usually express neither the CD4 nor the CD8 accessory molecules, although a small proportion appear to express CD8 *in vivo* (Borst *et al.*, 1988) or after stimulation *in vitro* (Brenner *et al.*, 1986). Probably the most striking difference between TCR$\gamma\delta$ and TCR$\alpha\beta$ is the diversity of their respective V region repertoires. In the murine germ line, while there are roughly 25 Vβ and 50 Vα genes (reviewed in R. K. Wilson *et al.*, 1988), there have been identified only 7 Vγ and 7 Vδ genes (Garman *et al.*, 1986; Pelkonen *et al.*, 1987; Elliot *et al.*, 1988). Thus, the repertoire diversity afforded by the Vγ and Vδ combinations is roughly two orders of magnitude less than TCR$\alpha\beta$. The number of available Jγ (3) and Jδ (2) segments relative to Jβ (12) and Jα (roughly 80) segments indicates yet two orders of magnitude further limitation on the TCR$\gamma\delta$ repertoire relative to TCR$\alpha\beta$. On the other hand, the δ locus possesses two D regions that can be utilized individually or in tandem, each of which has been found to display extensive N-region diversity (Chien *et al.*, 1987b). Thus, the TCR$\gamma\delta$ repertoire could potentially be expanded a number of logs, based solely on the diversity of the D-J joint of the δ chain. While it appears that diversity in the $\alpha\beta$ receptor is distributed relatively evenly among the variable and joint

regions of both chains, the vast majority of the diversity of the $\gamma\delta$ receptor is concentrated at the δ joint region. Presumably, this unique pattern of diversity generation relates to the spectrum of ligand recognition by the $\gamma\delta$ receptor. At this point, the nature of the TCR$\gamma\delta$ ligand is unknown, although the recent report of TCR$\gamma\delta^+$ clones with specificity for MHC class I and MHC class II alloantigens suggests that MHC antigens are an important component of the TCR$\gamma\delta$ ligand (Matis *et al.*, 1987; L. Matis, personal communication).

III. T Cell Ontogeny Studies

As pointed out in Section I, ontogeny studies have provided many important insights into the early events of T cell development. It is therefore appropriate to begin with an overview of thymocyte ontogeny. Not unexpectedly, a number of groups have recently focused on the developmentally regulated expression of the cell surface molecules, described in the previous section, which are directly involved in antigen recognition. While the majority of these studies have been performed in the mouse, the order at which these critical events occur appears to be identical in species ranging from the chicken to the human.

A. Early Events in Thymocyte Ontogeny: Rearrangement and Expression of T Cell Receptor Genes

The murine embryonic thymus is first colonized by hematopoietic cells on the eleventh day of gestation (Moore and Owen, 1967). The earliest fetal thymocytes express neither CD4, CD8, nor surface T cell receptors (Bluestone *et al.*, 1987). Fetal thymus organ cultures have been used to demonstrate that the $CD4^-8^-$ thymocytes are precursors to the more mature cell types, since effector T cells bearing CD4 or CD8 develop in the absence of any further influx of cells (Ceredig *et al.*, 1982b, 1983b; Kisielow *et al.*, 1984). From these studies, it was apparent that the $CD4^-8^-3^-$ population defines the earliest intrathymic developmental stage. It is now evident that the majority of T cell receptor gene rearrangements occurs during this $CD4^-8^-3^-$ stage (see Fig. 3). This information has come from Southern blot analysis of both whole populations of murine fetal thymocytes from different days of gestation as well as panels of cloned hybridomas produced by fusion of early-stage thymocytes with the BW5147 thymoma (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985; Born *et al.*, 1985, 1986; Haars *et al.*, 1986; Lindsten *et al.*, 1987; Chien *et al.*, 1987a,b). The analyses on whole populations of early fetal thymocytes reveal that the majority of all of the TCR loci are in

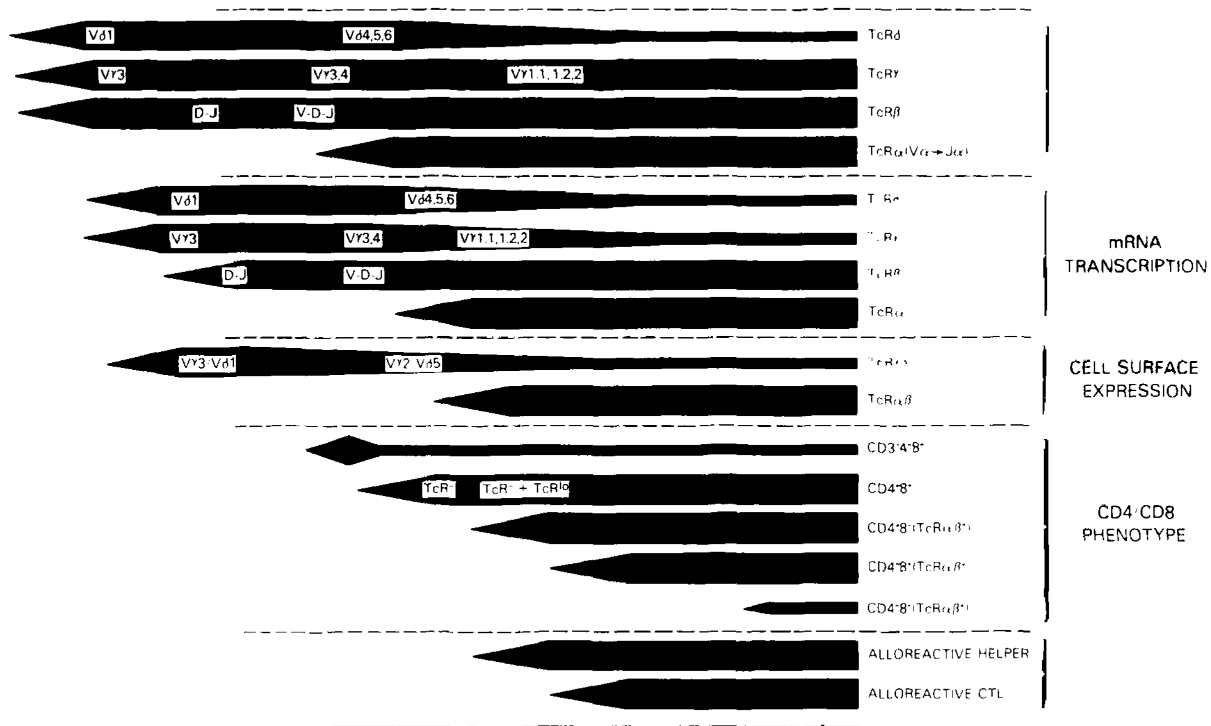

FIG. 3. Time line depicting the onset of the major ontogenic events occurring in the murine thymus during development. Widths of the bars are drawn to reflect relative proportions as a function of time. Designations within the bars depict predominating V gene rearrangements and expression on different days of gestation (see text).

germ line configuration on days 13 and 14 of gestation. Thereafter, progressive rearrangement of all TCR loci occurs. A more quantitative picture of this progressive rearrangement was obtained from analyses of the cloned fetal thymocyte hybridomas. Among the panel of day 14 thymocyte hybridomas, only 15% had rearrangements of the γ locus, 15% had rearrangements of the β locus, and 26% had rearrangements of the δ locus. Similar analyses of hybridoma panels from subsequent days of fetal thymus ontogeny revealed a progressive increase in the proportion of hybridomas bearing rearrangements such that, by day 17, 80% had at least one rearrangement of the γ locus, 90% had at least one rearrangement of the β locus, and virtually all had at least one rearrangement of the δ locus. Furthermore, there was a significant concordance in the rearrangement status of the different TCR loci such that thymocytes from all gestational days which had rearrangements of one TCR locus tended to have rearrangements of the other two TCR loci. Because the δ locus is nested within the α locus—between the Jα and Vα regions—it is reasonable to consider δ locus rearrangements as prerearrangements of the α locus, since they indeed bring the Vα segments closer to the Jα segments. Therefore, it appears that during the early stages of thymocyte development there is progressive and synchronous rearrangement of all the TCR loci within a given cell.

The majority of TCR gene rearrangements occur within $CD4^-8^-3^-$ precursors that are in a stage of rapid division. It is therefore likely that each individual prothymocyte which enters the thymus has the capability of giving rise to progeny with potentially diverse TCR expression. Formal proof of this concept has come from experiments in which deoxyguanosine-treated fetal thymuses (depleted of all endogenous thymocytes) have been recolonized with a single Thy-1 congenically marked day 14 thymocyte using the hanging-drop method. After 12 days of residence in the host thymus, the progeny of that single day 14 thymocyte were shown to display significant diversity of TCRβ locus rearrangements (Williams *et al.*, 1986).

While all of the TCR loci appear to begin rearranging at roughly the same time, complete rearrangements capable of encoding productive cell surface structures do not occur simultaneously. Many thymocytes which have complete V-D-J rearrangements of their δ locus as well as V-J rearrangements of their γ locus have only partial D-J rearrangements of their β locus (Born *et al.*, 1986). (For unknown reasons, V-to-D rearrangements appear to precede D-to-J rearrangements in the δ locus in contrast to the β locus, in which D-to-J rearrangements always precede V-to-D rearrangements.) The kinetics of TCR rearrangement are reflected in studies of TCR mRNA transcription (Raulet *et al.*, 1985; Haars

et al., 1986; Chien *et al.*, 1987a,b). While full-length γ (V-J-C) and δ (V-D-J-C) mRNA are detected on day 14 of gestation, most of the β mRNA on day 14 and 15 is 1.0 kb (representing transcripts of incomplete D-J rearrangements). Full-length 1.3-kb (V-D-J-C) β mRNA begins to be detected at significant levels on day 16 of gestation and is present at high levels in adult $CD4^-8^-3^-$ thymocytes (Samelson *et al.*, 1985b). Full-length α mRNA does not appear until day 17 of gestation, a time at which Vα-to-Jα rearrangements have begun to occur associated with deletion of the δ locus. The Vα-to-Jα recombination is the last TCR rearrangement event and probably occurs shortly after CD8 and CD4 begin to be expressed.

In situ hybridization studies have demonstrated that there is significant transcription of the γ and β constant region segments extremely early in development (day 12 to 13), prior to rearrangement of these loci (Pardoll *et al.*, 1987b). It is likely that this RNA represents germ line transcripts, reflecting the "opening" of chromatin in the TCR loci in preparation for recombinase-mediated rearrangements.

Another interesting feature of the developmentally regulated rearrangement and expression pattern of the murine γ and δ loci is that different V-J segments are rearranged and transcribed on different days of fetal development. The earliest Vγ segment to be rearranged and expressed (day 13 to 14) is Vγ3. Shortly thereafter, Vγ4 also appears at significant frequency. Between days 17 and 20, the frequency of Vγ3 and Vγ4 rearrangements decreases significantly, and rearrangements of Vγ2, Vγ1.2, and Vγ1.1 increase (Garman *et al.*, 1986). It is noteworthy that the order of rearrangements of the Vγ segments, particularly early in development, corresponds exactly to the physical ordering with regard to the Cγ1 segment. The preferential rearrangement of C-proximal Vγ segments early in development is reminiscent of the preponderance of rearrangements of the C-proximal immunoglobulin V_H segment (V_H81X) seen in murine pre-B cells (Yancopoulos *et al.*, 1984). The predominant rearrangement and expression of a particular Vδ segment (Vδ1) is also observed early in fetal development. Just as with the γ locus, rearrangements of Vδ1 become infrequent on later days of development, while other rearrangements of other Vδ segments (Vδ4, Vδ5, and Vδ6) become more frequent (Chien *et al.*, 1987b; Elliot *et al.*, 1988). An interesting feature of early TCRδ rearrangements is the absence of N-region diversity, probably because terminal deoxyribonucleotidyl transferase (TdT) is not produced until a few days later in thymic development. As will be discussed below, this rearrangement pattern is reflected in differential γ and δ chain expression on the surface of early versus late fetal thymocytes. It is unclear at this time whether prevalent temporal

patterns of Vα and Vβ rearrangement are also seen; however, *in situ* hybridization studies with a limited number of Vβ segments indicate that there may be preferential transcription of Cβ-proximal Vβ segments early in ontogeny (Pardoll *et al.*, 1987b).

B. Developmentally Regulated Surface Expression of T Cell Receptors

The recent production of both monoclonal antibodies and antisera reactive with either CD3 components or various subsets of $\gamma\delta$ or $\alpha\beta$ TCRs has facilitated the analysis of developmentally related surface TCR expression, the final event of the early differentiation stage as defined in Section I. In the mouse, surface CD3 was first detected roughly on day 14 of development (Bluestone *et al.*, 1987); however, using a monoclonal antibody reactive with roughly 20% of $\alpha\beta$ TCRs (KJ16, anti-Vβ8.1 + Vβ8.2), surface staining was not seen until day 17 of fetal development (Roehm *et al.*, 1984). By performing immunoprecipitations with an anti-CD3 mAb and anti-γ peptide antibodies (raised against the carboxyl terminal heptapeptide of Cγ1 and Cγ2) on lysates of radioiodinated populations of murine fetal thymocytes from different days of development, it was found that the first CD3-associated TCR to appear in thymocyte development is, in fact, TCR$\gamma\delta$ (Pardoll *et al.*, 1987a). This receptor is first detected on day 14 to 15 of gestation and precedes the expression of TCR$\alpha\beta$ by 2 to 3 days. Subsequent to day 17, there is a progressive increase in the proportion of cells that express TCR$\alpha\beta$ until, by day 20 (time of birth), TCR$\alpha\beta$-bearing cells represent the major CD3$^+$ thymocyte population. The kinetics of surface expression of TCR$\gamma\delta$ relative to TCR$\alpha\beta$ are consistent with the finding that complete rearrangements of the γ and δ loci precede complete rearrangements of the α and β loci. Furthermore, analysis of TCR$\gamma\delta^+$ hybridomas produced from early fetal thymocytes as well as staining of early fetal thymocyte populations with a new mAb specific for the Vγ3 product reveal that, as predicted from the rearrangement pattern, early fetal $\gamma\delta^+$ thymocytes predominantly express products of Vγ3 and Vδ1 on their surface (Havran and Allison, 1988; S. Tonegawa, personal communication). By day 18 of fetal development, there has been a tremendous shift in surface expression such that the majority of TCR$\gamma\delta^+$ thymocytes express the products of other V segments (predominantly Vγ2 and Vδ5) (Raulet, 1989). While the purpose for a specific early fetal $\gamma\delta$ receptor is unclear, it is possible that this receptor defines a specific functional class of fetal thymocytes whose role is to somehow prepare the thymic environment for the subsequent wave of differentiation. It has been postulated that early TCR$\gamma\delta^+$ thymocytes may

perform this function via the production of lymphokines such as interleukins (IL-2, IL-4, IL-6), and interferon-γ (Pardoll *et al.*, 1987c). Of note, the early Vγ3-bearing fetal thymocytes appear to be the only CD3$^+$ thymocyte subset which expresses IL-2 receptors *in vivo*, indeed suggesting that they are active during early fetal development (J. Allison, personal communication). Also, treatment of day 14 fetal organ cultures with anti-CD3 (at a time when most CD3$^+$ thymocytes are Vγ3$^+$) was found to inhibit subsequent rearrangement and expression of TCRβ (though not TCRα), suggesting that early TCR$\gamma\delta$ cell function may be required for development of TCR$\alpha\beta^+$ cells (J. J. T. Owen *et al.*, 1988).

C. Developmentally Regulated Expression of CD4 and CD8

Given that the accessory molecules, CD4 and CD8, are now recognized to be critical participants in antigen recognition, it is not surprising that their expression on developing thymocytes has been found to be important not only in the definition of functionally distinct subpopulations but also in the shaping of the TCR$\alpha\beta$ repertoire. In the mouse, the accessory molecules are first expressed on days 15 to 16 of development (Ceredig *et al.*, 1983a) (Fig. 3). Initially, there is a short period in which a fraction of thymocytes begin to express CD8 (Ceredig *et al.*, 1983a; Kisielow *et al.*, 1984). By day 17 of ontogeny, virtually all of the accessory molecule-bearing thymocytes coexpress both CD4 and CD8 (frequently referred to as double positive). CD4$^+$8$^+$ subset increases in both absolute number and proportion over the ensuing days until it represents 75–80% of the total thymocyte population a few days after birth. Between days 17 and 18 of gestation, the first CD4$^+$8$^-$ thymocytes appear, followed shortly thereafter by an increase in the number of CD4$^-$8$^+$ thymocytes (CD4$^+$8$^-$ and CD4$^-$8$^+$ cells are frequently referred to as single positive).

Recently, a number of important studies have defined a correlation between TCR expression and accessory molecule expression. Within the developing fetal thymus, TCR$\alpha\beta$ is always coexpressed with accessory molecules (CD4 and/or CD8). Thus, TCR$\gamma\delta$ appears to be expressed exclusively on thymocytes which are CD4$^-$8$^-$, whereas TCR$\alpha\beta$ is expressed almost exclusively on thymocytes which express CD4 and/or CD8 (Pardoll *et al.*, 1987a; Bluestone *et al.*, 1987). The one exception to this rule is a recently discovered population of CD4$^-$8$^-$ thymocytes expressing a skewed repertoire of $\alpha\beta$ TCRs; however, this CD4$^-$8$^-$ TCR$\alpha\beta^+$ subset is not detected until 2 to 5 days after birth, significantly later than all of the other subpopulations (Budd *et al.*, 1987b; Fowlkes *et al.*, 1987; Crispe *et al.*, 1987a). Interestingly, the "transient" CD4$^-$8$^+$ thymocytes present on day 16 are CD3$^-$ (TCR$^-$). This

population, which is also seen in the adult thymus, appears to represent thymocytes on their way to becoming $CD4^+8^+$ but have not yet expressed CD4 (see Section IV,C). The first $CD4^+8^+$ thymocytes to appear between days 16 and 17 are also $CD3^-$. By day 18, a proportion of $CD4^+8^+$ thymocytes (roughly 5%) have begun to express low levels of TCR$\alpha\beta$. In contrast, the $CD4^+8^-$ thymocytes and the $CD4^-8^+$ thymocytes which appear on days 18 and 19, respectively, express high levels of TCR$\alpha\beta$. By including the $CD4^-8^-$ TCR$\alpha\beta^+$ subset that develops later, there are a total of eight phenotypically distinct subsets of thymocytes defined by the four surface molecules, CD4, CD8, TCR$\gamma\delta$, and TCR$\alpha\beta$ (Fig. 4). All of these thymocyte subsets can also be found in the adult thymus (Lanier and Weiss, 1986; Lanier *et al.*, 1986; Bluestone *et al.*, 1987); Fig. 4 gives a diagrammatic representation of their relative proportions. As will become evident in the subsequent sections, the thymocyte subsets defined by these markers generally define functionally relevant stages of development, and some represent functionally distinct lineage branches. With the development of monoclonal antibodies recognizing many additional surface antigens, it has become popular to subset these subpopulations even further. For completeness, we have compiled a list of additional commonly studied surface antigens, together with the monoclonal antibodies used to detect these antigens, and the subset distribution relative to CD4 and CD8 (Tables I and II). In the ensuing discussion, however, we will concentrate mainly on surface antigens whose analysis has contributed to the

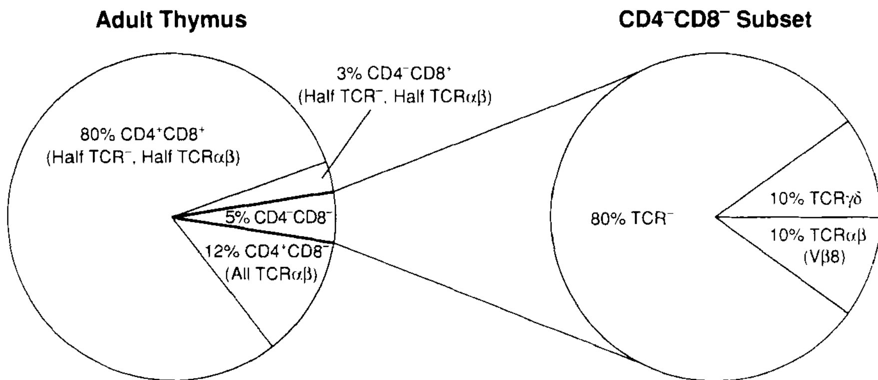

FIG. 4. Diagrammatic representation of relative proportions of the major thymocyte subpopulations in the mouse as defined by CD4, CD8, and T cell receptor. Percentages given are general averages. Significant strain and age variation is observed.

general understanding of lineage relationships and functional aspects of T cell differentiation.

D. Ontogeny of T Cell Function

The earliest and most straightforward correlation between thymocyte phenotype and function came from investigations into the developmental kinetics of T cell growth factor (TCGF/interleukin-2) precursors and cytolytic (CTL) precursors (Ceredig *et al.*, 1983a). Limiting-dilution analyses using allogeneic stimulators indicated that cells capable of producing TCGF in response to alloantigen first appeared on day 18 of murine fetal thymus development, whereas allospecific CTL precursors first appeared on day 19 of fetal thymus development. It was further shown that, just as in the periphery, TCGF-producing precursors had a $CD4^+8^-$ phenotype, whereas the CTL precursors had a $CD4^-8^+$ phenotype. Using an adoptive transfer into irradiated hosts, it has been recently demonstrated that MHC-restricted helper cells for antibody production also develop at about day 18 of murine fetal thymic development (Marrack *et al.*, 1988a). As all of these functions tend to be associated with mature $TCR\alpha\beta^+$ T cells, it is not surprising that they begin to be detected concomitant with the appearance of T cells with phenotype similar to mature $TCR\alpha\beta^+$ peripheral T cells; however, the first cells capable of producing lymphokine (IL-2 and IL-4) in response to mitogenic anti-CD3 or anti-Thy-1 antibodies are $TCR\gamma\delta^+$ cells. Anti-CD3-responsive $\gamma\delta$-bearing cells have been detected as early as days 13 to 14 of murine fetal thymocyte development, roughly 4 days prior to the appearance of alloresponsive ($TCR\alpha\beta$) $CD4^+8^-$ thymocytes (Tentori *et al.*, 1988). By the same token, using a retargeted cytotoxic assay in which anti-CD3 antibody bridges a $CD3^+$ cytolytic T cell to an Fc-bearing target, $TCR\gamma\delta^+$ thymocytes with cytolytic capacity have been detected on day 16 of fetal thymocyte development, 3 days prior to the appearance of alloreactive ($TCR\alpha\beta$) $CD4^-8^+$ thymocytes (Bluestone *et al.*, 1987). Given the success in growing alloreactive $TCR\gamma\delta^+$ cells from nude mice (Matis *et al.*, 1987), it is perhaps surprising that alloreactive $TCR\gamma\delta^+$ thymocytes were not detected on earlier gestational days (days 14 to 17); however, in the former case very different conditions were used to elicit the alloreactive responses. Responses of $TCR\gamma\delta^+$ cells may be quantitatively and qualitatively very different from the responses of $TCR\alpha\beta^+$ T cells.

E. T Cell Ontogeny from Species Other Than the Mouse

The review of ontogeny in the preceding sections has focused on the murine system, partially to maintain consistency and partially because

TABLE I
SELECTED MARKERS USED TO DELINEATE SUBPOPULATIONS AND DEVELOPMENTAL STAGES OF T CELLS[a]

Antigen	Antibodies[b]	References
CD1 (T6; Leu-6)	*SK9; NA1/34; OKT6*	McMichael *et al.* (1979)
TL	18/20; TL.m4-9	Lemke *et al.* (1979); Shen *et al.* (1982)
CD2 (T11; Leu-5)	*ATM 1.1*; *95-5-49*; *OKT11*	Howard *et al.* (1981); Verbi *et al.* (1982); Quinones and Gress (1988)
CD5 (T1; Leu-1; Ly1)	*OKT1*; *L17F12*; *T101*; C3P0; 53.7.3	Engleman *et al.* (1981b); Royston *et al.* (1980); Mark *et al.* (1982); Ledbetter and Herzenberg (1979)
CD7 (Tp-40)	*3A1*	Haynes *et al.* (1979)
CD25 (IL-2R; TAC)	*anti-TAC*; *2A3*; *33B7.3*; 7D4; PC61	Leonard *et al.* (1982); Olive *et al.* (1986); Malek *et al.* (1983); Ceredig *et al.* (1985)
CDw29	*4B4*	Morimoto *et al.* (1985b)
CD45 (T200; HLe-1; Ly5)	*UCHL1*; *2D1*; 13/23; 30-F11	Smith *et al.* (1986); Beverly (1980); Trowbridge (1978); Ledbetter and Herzenberg (1979)

CD45R (Lp220; Leu-18)	*L48*; *2H4*; *3AC5*; *14.8*; RA3-3A1 or 2C2; OX-22[c]	Morimoto *et al.* (1985a); Kincade *et al.* (1981); Coffman (1982); Spickett *et al.* (1983)
HSA	M169; B2A2; J11d	Springer *et al.* (1978); Scollay *et al.* (1984); Bruce *et al.* (1981)
Thy-1.2	F7D5; 30-H12	Lake *et al.* (1979); Ledbetter and Herzenberg (1979)
Pgp-1 (Ly-24)	IM7.8.1	Trowbridge *et al.* (1982)
ThB	53-9.1	Ledbetter and Herzenberg (1979)
B14	anti-B14	Sidman *et al.* (1983)
PNA receptor		Reisner *et al.* (1976)
Lymphocyte homing receptor	Mel 14	Gallatin *et al.* (1983)
RL73	Anti-RL73	MacDonald *et al.* (1985)
Qa-2	H-1-9-9; D3.262	Forman *et al.* (1982)
OX-44	MRC OX-44[c]	Patterson *et al.* (1987)

[a]Markers used to distinguish TCR or TCR-associated structures (CD3), accessory molecules (CD4 and CD8), MHC, and many activation antigens are omitted.

[b]Only a partial listing of frequently used antibodies is cited. Antibodies in italics are against human antigens.

[c]Rat-specific antibodies.

TABLE II
MAJOR SUBPOPULATIONS AND/OR DEVELOPMENTAL STAGES OF THYMOCYTES[a]

Thymocyte subpopulations and subsets	Relative percentage of total thymocytes[b]
$CD4^-8^-$	**5.0**
$CD3^-$, Thy-1^{lo}, HSA^-, Pgp-1^+, IL-$2R^-$, $CD5^{lo}$	0.2
$CD3^-$, Thy-1^{hi}, HSA^{hi}, Pgp-1^-, IL-$2R^+$, $CD5^{lo}$	2.3
$CD3^-$, Thy-1^{hi}, HSA^{hi}, Pgp-1^-, IL-$2R^-$, $RL73^+$, $CD5^{lo}$	1.5
$CD3^+$, TCR$\gamma\delta$, $HSA^{+/-}$, $CD5^{lo/hi}$	0.5
$CD3^+$, TCR$\alpha\beta$, HSA^-, Pgp-1^+, IL-$2R^-$, $CD5^{hi}$, Qa-2^+	0.5
$CD4^+8^+$	**80.0**
$CD3^-$, HSA^+, IL-$2R^-$, $CD5^{lo}$	40.0
$CD3^+$, TCR$\alpha\beta$, HSA^+, IL-$2R^-$, $CD5^{lo}$, Qa-2^-	40.0
$CD4^+8^-$	**10.0**
$CD3^+$, TCR$\alpha\beta$, HSA^+, $CD5^{hi}$, Qa-2^-	7.0
$CD3^+$, TCR$\alpha\beta$, HSA^-, $CD5^{hi}$, Qa-2^+	3.0
$CD4^-8^+$	**5.0**
$CD3^-$, HSA^{hi}, Pgp-1^-, IL-$2R^-$, $RL73^+$, $CD5^{lo}$	1.5
$CD3^+$, TCR$\alpha\beta$, HSA^+, $CD5^{hi}$, Qa-2^-	1.2
$CD3^+$, TCR$\alpha\beta$, HSA^-, $CD5^{hi}$, Qa-2^+	2.3

[a]Only a partial listing of phenotypes is given. Not all phenotypes have been determined on all subsets.

[b]Percentages are obtained from references given in the text and vary with age and mouse strain.

the largest body of information has been generated using mice. Nonetheless, a number of important ontogeny studies carried out concomitantly in other species (particularly the chicken and the human) bear mentioning. In the chicken, investigations utilizing staining monoclonal antibodies specific for CD3, TCR$\gamma\delta$, or TCR$\alpha\beta$ have produced a clear ontogenic picture that is strikingly similar to that in the mouse (Sowder *et al.*, 1988; Chen *et al.*, 1988). Between days 10 and 15 of gestation, all of the $CD3^+$ thymocytes stain with the anti-$\gamma\delta$ mAb. Over the next 5 days, the proportion of thymocytes expressing TCR$\gamma\delta$ falls from the peak of 30 to roughly 5%, concomitant with an increase in TCR$\alpha\beta$-bearing thymocytes (first at dull-staining levels on $CD4^+8^+$ cells and then at bright-staining levels on $CD4^+8^-$ and $CD4^-8^+$ cells). These studies clearly demonstrated that, as opposed to TCR$\alpha\beta$, TCR$\gamma\delta$ is immediately expressed at full levels with no evidence of a dull-staining population. In the human, an identical sequence of TCR expression appears to occur. Staining of thymus sections from $9\frac{1}{2}$-week fetuses revealed that roughly 10% of the thymocytes express surface TCR$\gamma\delta$, whereas surface TCR$\alpha\beta$ was not yet detectable. By 10 weeks of gestation,

TCR$\alpha\beta$-expressing thymocytes begin to progressively increase in proportion concomitant with a decrease in the proportion of TCR$\gamma\delta^+$ thymocytes (Haynes *et al.*, 1988). The tremendous evolutionary conservation of ontogenic events suggests that this sequence is critically important for the proper development of the T cell limb of the immune system and further implies that conclusions drawn from studies in one species will probably be applicable for most other species.

IV. Function and Lineage Relations of the Major Thymocyte Subpopulations

As mentioned in the above section, CD4, CD8, and TCR ($\alpha\beta$ and $\gamma\delta$) define functionally relevant subsets in both the fetal and adult thymus. In this section, we will discuss each of the major subsets individually, with emphasis on their role in development and precursor-product relationships with other subsets.

A. CD4$^-$8$^-$3$^-$ Thymocytes

CD4$^-$8$^-$ thymocytes constitute ~5% of the murine adult thymus and can be subdivided into three groups with respect to expression of the TCR: (1) CD4$^-$8$^-$3$^-$, (2) CD4$^-$8$^-$TCR$\gamma\delta^+$, and (3) CD4$^-$8$^-$TCR$\alpha\beta^+$ (de la Hera *et al.*, 1985; Lew *et al.*, 1986; Bluestone *et al.*, 1987; Budd *et al.*, 1987b; Fowlkes *et al.*, 1987; Crispe *et al.*, 1987a). Although it had originally been reported that human CD4$^-$8$^-$ thymocytes are all CD3$^-$ (Reinherz *et al.*, 1980; Royer *et al.*, 1984), subsequent studies indicated that a subset of human CD4$^-$8$^-$ thymocytes is CD3$^+$ as well (de la Hera *et al.*, 1985; Lanier and Weiss, 1986; Lanier *et al.*, 1986), including some that express TCR$\gamma\delta$ and some that express TCR$\alpha\beta$. These three CD4$^-$8$^-$ subpopulations have distinct functional properties and represent different stages of T cell development. Markers used to further characterize and define these subpopulations are summarized in Tables I and II.

1. *Phenotype and Proliferative Properties of CD4$^-$8$^-$3$^-$ Thymocytes*

Most of the murine adult CD4$^-$8$^-$3$^-$ subset are CD5^{lo}, HSA^{hi}, Thy-1^{hi}, and Pgp-1^{lo} (Wilson *et al.*, 1988a; Ewing *et al.*, 1988). The CD5^{lo}/HSA^{hi} double-negative (CD4$^-$8$^-$) cells closely resemble the large cortical (CD4$^+$8$^+$) thymocytes in size, phenotype, high proliferation rate *in vivo* (as assessed by [^{3}H]Tdr uptake), and a poor proliferative response to mitogens *in vitro* (Ewing *et al.*, 1988). At least half of this CD3$^-$4$^-$8$^-$ subset expresses interleukin-2 receptors (IL-2R) (Ceredig

et al., 1985; Raulet, 1985; von Boehmer *et al.*, 1985; Habu *et al.*, 1985; Palacios and von Boehmer, 1986), a marker typically associated with T cell activation.

Using antibodies specific for the α chain of the IL-2R, it has been observed that IL-2R are found on the majority of murine fetal thymocytes at days 14–15 of gestation, only 4 days after the initial colonization of the thymic rudiment by hematopoietic stem cells. By *in situ* hybridization, IL-2R mRNA is detected even earlier, first at day 13 of gestation (18% IL-2R$^+$), peaking at day 15 (80% IL-2R$^+$), and decreasing thereafter (D. Pardoll, unpublished observations). The fraction of IL-2R-bearing cells subsequently declines to a steady state of 1–2% of adult thymocytes, most of it on CD4$^-$8$^-$3$^-$ cells. Obviously, induction of the IL-2R on CD4$^-$8$^-$3$^-$ cells cannot be related to conventional stimulation through the $\alpha\beta$ T cell receptor, raising questions about how it is induced and what role it plays in the proliferation of immature thymocytes.

There is further uncertainty about the significance of the IL-2R on fetal thymocytes, because their *in vitro* proliferative response to IL-2 alone is minimal (Raulet, 1985; Ceredig *et al.*, 1985; von Boehmer *et al.*, 1985; Palacios and von Boehmer, 1986). One possible explanation for the lack of response to IL-2 is that many of the IL-2 receptors, detected with antibodies against the α (*p*55) subunit, may not possess an accompanying β (*p*75) subunit necessary for high-affinity IL-2 binding and internalization (Sharon *et al.*, 1986). In general, the low-affinity binding of the isolated α subunit is insufficient to mediate a proliferative response to IL-2. Scatchard analyses of IL-2 binding to fresh CD4$^-$8$^-$ thymocytes has not clearly distinguished between the one-chain versus two-chain IL-2R structure, in that these analyses reveal an affinity constant (K_m) intermediate between the typical high- and low-affinity K_m seen with mature T cell blasts (Lowenthal *et al.*, 1986). CD4$^-$8$^-$ thymocytes show poor IL-2 internalization indicative of low affinity IL-2 receptors.

Many groups have demonstrated that double-negative adult and/or fetal thymocytes will proliferate to IL-2 *in vitro* when stimulated with the protein kinase C agonist, phorbol myristate acetate (PMA) and calcium ionophore (Palacios and von Boehmer, 1986; Lugo *et al.*, 1986; Ceredig, 1986; Ewing *et al.*, 1988). From subset analyses in the adult, it appears that only the TCR-bearing double-negative cells respond to PMA + ionophore in the absence of IL-2 (Howe and MacDonald, 1988). The implications of these results to growth *in vivo* are unclear but suggest that stimulated CD4$^-$8$^-$3$^-$ thymocytes can use exogenously supplied IL-2, but cannot make it.

Fetal and/or adult double-negative thymocytes can be induced to produce IL-2 *in vitro* using PMA and calcium ionophore (Lugo *et al.*, 1986; Ceredig, 1986; Howe and MacDonald, 1988). Subset analysis of

adult double-negative thymocytes reveals that this response is also confined to a TCR-bearing population, unless IL-1 is also added to the culture, in which case all double-negative subsets respond (Howe and MacDonald, 1988). Studies on the capacity of fetal thymocytes to produce IL-2 *in vitro* have yielded conflicting results: one group has reported that day 15 fetal thymocytes do not produce IL-2 upon stimulation with PMA and a calcium ionophore (Lugo *et al.*, 1986), while a second group, using a similar stimulation protocol, reported low but detectable production of IL-2 by day 14 fetal thymocytes (Ceredig, 1986). The difference between these studies may have been due to the sensitivity of the IL-2 detection assay.

Using *in situ* hybridization, D. Pardoll and J. Fowlkes (unpublished observations) have found that a subset of freshly isolated fetal thymocytes does express IL-2 mRNA with kinetics similar to IL-2R mRNA expression, although IL-2 mRNA expression appeared to occur in a smaller subset of thymocytes than does IL-2R mRNA expression. The fraction of IL-2-producing cells at day 15 ($\sim 58\%$) indicates that at least some of the same cells must simultaneously produce IL-2 and express the IL-2R. Furthermore, the labeling index paralleled the fraction of cells expressing IL-2R mRNA. When day 14 fetal thymocytes were electronically sorted with respect to IL-2R expression and examined for labeling index, it was found that the bulk of the dividing cells were in the IL-2R$^+$ fraction, indicating a relationship between IL-2R and cell division in early fetal development. While it is possible that only the TCR$\gamma\delta$-bearing cells are making IL-2 mRNA in the fetus, this appears unlikely since at day 15, more than 50% of thymocytes are making IL-2 mRNA when less than 15% are CD3$^+$ at this time point (Bluestone *et al.*, 1987; Havran and Allison, 1988).

Further evidence for involvement of the IL-2/IL-2R pathway in early thymocyte proliferation and development has recently been obtained by treatment of fetal organ cultures with anti-IL-2R mAb. This treatment resulted in significantly decreased cell yields and numbers of TCR$\alpha\beta^+$ cells (Jenkinson *et al.*, 1987). While the decrease in TCR$\alpha\beta^+$ cells has suggested that IL-2/IL-2R interactions are necessary for continued differentiation, the results can all be attributed to interference with early thymocyte proliferation. A second report indicates that treatment of fetal organ cultures with IL-2 arrests proliferation and differentiation to CD4$^+$8$^+$ (double positive) (Skinner *et al.*, 1987); however, promiscuous cytotoxic cells developed in these cultures, probably indicating that they are overgrown with responding TCR$\gamma\delta$-bearing thymocytes (see below).

At later stages of fetal development, the fraction of proliferating IL-2R$^-$ cells increases substantially, indicating that an IL-2R-independent pathway (perhaps using other growth factors) accounts for

much of the proliferation seen later in T cell development (D. Pardoll and B. Fowlkes, unpublished observations). This conclusion is supported by studies on adult $CD4^-8^-$ thymocytes using a monoclonal antibody designated RL73 (Howe and MacDonald, 1988). The RL73 mAb detects an antigen whose expression appears to correlate with *in vivo* size and proliferation. The $RL73^+$, $IL\text{-}2R^-$ subset has the highest percentage of blasts and cycling cells, whereas the $RL73^+$, $IL\text{-}2R^+$ subset is reduced in size and numbers of proliferating cells. Likewise, Shortman and Scollay (Ewing *et al.*, 1988; Scollay *et al.*, 1988) find that the HSA^+, $IL\text{-}2R^-$ subset rather than the HSA^+, $IL\text{-}2R^+$ subset contains the highest level of dividing cells by *in vivo* labeling and cell cycle analyses. Although these studies point to a lack of correlation between IL-2R expression and proliferation in late fetal and adult $CD4^-8^-$ thymocytes, the increased labeling index observed in early fetal $IL\text{-}2R^+$ thymocytes raises the possibility that initiation of this proliferation involves the IL-2/IL-R pathway and, once initiated, an IL-2-independent phase ensues.

In the adult thymus, a small subset (<10%) of freshly isolated, unstimulated $CD4^-8^-5^{lo}$ cells do, indeed, possess IL-2 mRNA (Pardoll *et al.*, 1989). Assuming that the presence of IL-2 mRNA *in situ* reflects production of IL-2 *in vivo*, these findings suggest, by analogy to early fetal thymocytes, that some $CD4^-8^-$ cells of the adult thymus may use the IL-2/IL-2R pathway. Of note, it was not determined whether the subset which makes IL-2 mRNA also coexpresses CD3 on the surface. Nevertheless, the functional capacity of IL-2Rs on the bulk of adult $CD4^-8^-3^-$ thymocytes remains questionable, in light of the failure of this population to internalize receptor, to efficiently respond to IL-2 *in vitro*, and the general failure to correlate proliferation with IL-2R expression *in vivo*.

The importance of the IL-2 receptor in $CD4^-8^-3^-$ thymocyte proliferation has also been questioned because of its apparent absence from this subset in the adult thymus of other species such as rat and human (Paterson and Williams, 1987; Lanier and Weiss, 1986). It is possible, however, that a small fraction that are $IL\text{-}2R^+$ escaped detection. In fact, staining of $9\frac{1}{2}$-week human fetal thymus sections (a time when all the thymocytes are $CD4^-8^-$) revealed significant numbers of $IL\text{-}2R^+$ cells (Haynes *et al.*, 1988).

Elucidating the nature of the receptor/ligand interaction (if any) that induces IL-2 and IL-2R production by $CD4^-8^-3^-$ thymocytes is crucial to a complete understanding of this pathway's role in early thymic development. Clearly, the early expansion phase cannot be TCR mediated, as with mature T cells. It has been proposed that the interaction of CD2 (Howard *et al.*, 1981; Kamoun *et al.*, 1981) with a thymic

ligand [probably LFA-3 expressed on thymic epithelial cells (Denning *et al.*, 1988)] promotes early thymic proliferation and induction of IL-2R prior to CD3-TCR expression in humans (Reinherz, 1985; Vollger *et al.*, 1987; Haynes *et al.*, 1988); however, in the mouse, CD2 transcripts have not been detected until 2 days after IL-2R expression (M. J. Owen *et al.*, 1988). Although the expression of CD2 mRNA coincides with the appearance of TCR$\gamma\delta$ cells during fetal development in the mouse (Bluestone *et al.*, 1987), CD2^{+}3^{-} cells have been detected among human fetal and adult CD4^{-}8^{-} thymocytes (Lanier and Weiss, 1986; Haynes *et al.*, 1988). Thus, it will be important to establish the role, if any, of CD2 on CD3^{-} thymocytes.

In efforts to find evidence for an alternate growth factor to IL-2 for early T lymphocytes, other lymphokines have been found to stimulate *in vitro* proliferation of double-negative thymocytes. IL-4 has been shown to mediate proliferation, but only in combination with mitogens or PMA (Palacios *et al.*, 1987; Zlotnik *et al.*, 1987); double-negative thymocytes can also make IL-4 when stimulated with PMA + calcium ionophore (Palacios *et al.*, 1987). An analysis of isolated subsets has revealed that it is primarily TCR^{+} CD4^{-}8^{-} thymocytes which respond to IL-4 + mitogen (Howe and MacDonald, 1988; Ewing *et al.*, 1988). Interestingly, at least some CD4^{-}8^{-} thymocytes appear to express constitutively high-affinity receptors for IL-4 (Lowenthal *et al.*, 1988).

When antibodies to IL-1 have been added to thymic organ cultures, no functional cells were recovered, indicating that IL-1 may also be an important signal either for growth and/or differentiation of T cells (DeLuca and Mizel, 1986). IL-1 has also been shown to augment IL-2 production and IL-2-dependent proliferation of CD4^{-}8^{-} thymocytes in response to mitogens or PMA + calcium ionophore (Howe *et al.*, 1986; Howe and MacDonald, 1988). CD4^{-}8^{-} thymocytes appear to have functional IL-1R, in that freshly isolated thymocytes express high-affinity receptors and are able to internalize the receptor.

2. *Precursor Potential of Adult CD4^{-}8^{-}3^{-} Thymocytes*

Thymocytes from the earliest days of fetal ontogeny (days 11-13 in the mouse) are virtually 100% CD4^{-}8^{-}3^{-}, whereas about 4% of adult thymocytes possess this phenotype in the mouse (Bluestone *et al.*, 1987).

Initial studies on early precursors in the adult thymus focused on the population of CD4^{-}8^{-} thymocytes with low levels of CD5 (Lyl) (Fowlkes *et al.*, 1985). The CD4^{-}8^{-}5^{lo} population (prepared by cytotoxic

elimination with anti-CD4, anti-CD8, and anti-CD5 plus complement) is about 95% $CD3^-$; the small fraction of $CD3^+$ cells within this compartment express TCR$\gamma\delta$ (Lew *et al.*, 1986; see next section). The precursor potential of the $CD4^-8^-5^{lo}$ subset has been demonstrated by intravenous (iv) transfer into congenically marked, irradiated recipients. This population contains cells with the capacity to home back to the recipient thymus and develop into all of the phenotypically defined thymocyte subsets. Control experiments in which $CD8^+$ or $CD4^+$ cells were transferred by iv injection established that all the homing capacity within the thymus resides in the $CD4^-8^-$ subpopulation.

In these transfer studies, the kinetics of differentiation after iv $CD4^-8^-5^{lo}$ thymocyte transfer were very similar to those of fetal thymocyte differentiation; that is, $CD4^+8^+$ cells begin to appear shortly after recolonization, followed by $CD4^+8^-$ cells and, finally, $CD4^-8^+$ cells. As in normal fetal development, IL-2-producing cells and CTL precursors were detected with the appearance of $CD4^+8^-$ and $CD4^-8^+$ (single-positive) thymocytes (B. Fowlkes and A. Kruisbeek, unpublished). The differentiation of this population occurs in a single wave, indicating that $CD4^-8^-3^-$ thymocyte precursors have a limited self-renewal capacity.

Some effort has been made to characterize the earliest precursors within the $CD4^-8^-3^-$ thymocytes. It seems logical that those $CD4^-8^-$ thymocytes which retain the ability to home to the thymus should include the earliest precursors. One group has shown, using irradiated recipients and intravenous injection of transferred cells, that thymocytes with homing and precursor activity are Thy-1^{lo}, Pgp-1^+, and IL-$2R^-$ (Lesley *et al.*, 1985a,b; Hyman *et al.*, 1986). Nakano *et al.* (1987) have confirmed, using electronically separated fractions of $CD4^-8^-$ thymocytes, that it is a Thy-1^{lo}, IL-$2R^-$ subset with all homing and precursor activity. Moreover, the $CD3^+$, Thy-1^{lo} cells have no activity in this assay. Scollay *et al.* (1988) find the most precursor activity after iv injection in the HSA^- subset. Since the bulk of CD5, Thy-1^{lo} double-negative cells are Pgp^+, HSA^-, and IL-$2R^-$ by multiparameter flow cytometry (FC) (A. Wilson *et al.*, 1988a), these studies taken together suggest that the phenotype of the subset of $CD4^-8^-$ thymocytes with both homing and precursor activity *in vivo* is Thy-1^{lo}, Pgp^+, HSA^-, IL-$2R^-$, and $CD3^-$.

Intrathymic injection has been successfully used to detect precursor activity once thymocytes have differentiated to the point that they are no longer able to home to the thymus (Goldschneider *et al.*, 1986; Scollay *et al.*, 1986, 1988; Crispe *et al.*, 1987a; Shimonkevitz *et al.*, 1987). As might be expected, Scollay *et al.* (1988) have found that, once delivered to the thymus, with the exception of the TCR^+ $CD4^-8^-$ thymocytes,

all of the major $CD4^-8^-$ subsets that they analyzed have reconstituting activity *in vivo* (giving rise to both $CD4^+8^+$ and single-positive thymocytes). These results are consistent with the conclusion that the $CD3^+4^-8^-$ cells are probably end-stage cells (see below), while other double-negative subsets are intermediates between the earliest precursors to enter the thymus and the $CD4^+8^+$ (double-positive) stage and/or $CD3^+$ thymocytes.

While intrathymic injection (it) of most double negative subsets into irradiated recipients results in differentiation to mature phenotypes; some are more efficient and faster than others in the rate at which they differentiate. Shimonkevitz *et al.* (1987) find that the IL-$2R^+$ subset has the most efficient and fastest reconstitution potential. Scollay *et al.* (1988) find that HSA^- (which are also IL-$2R^-$) double-negative thymocytes mature more slowly than HSA^+, but do serve as precursors. Crispe *et al.* (1987a), on the other hand, have argued that the HSA^- (detected with the J11d antibody), $CD4^-8^-$ thymocytes have little precursor activity, either in seeded fetal organ cultures or after intrathymic transfer into irradiated recipients. Since the bulk of the HSA^- double negatives bear TCR (60–80%) without demonstrable precursor activity (Scollay *et al.*, 1988), a very minor subset with very slow differentiation potential may not be easily detected.

In vitro studies have also been used to detect precursor activity in the double-negative thymocytes. Ceredig *et al.* (1983c) used fetal thymocytes to demonstrate that differentiation to $CD4^+8^+$ could occur in overnight culture. Later, it was shown that 20–30% of purified adult $CD4^-8^-CD5^{lo}$ thymocytes could differentiate *in vitro* to $CD4^+8^+$ within 20 hours (Fowlkes *et al.*, 1984). Longer term culture resulted in no additional differentiation to more mature phenotypes. Experiments in which deliberately contaminated, congenically marked thymocytes were added to these cultures demonstrated that the double positives could not be the result of selective outgrowth of contaminants. Nakano *et al.* (1987) extended these findings and showed that it is the IL-$2R^-$, Thy-1^{hi} double-negative cells which differentiate to double-positive cells *in vitro*.

Using intrathymic transfer into irradiated recipients, Shimonkevitz *et al.* (1987) find that the IL-$2R^-$ cells differentiate to an IL-$2R^+$ stage before giving rise to the $CD4^+8^+$ cells. Nakano *et al.* (1987), using an *in vitro* differentiation system, observed that IL-$2R^+$ fetal (day 16) thymocytes progress to IL-$2R^-$ prior to the double-positive stage. These data, taken together with the kinetic data discussed earlier on IL-2R mRNA expressed by fetal thymocytes, suggest that $CD4^-8^-3^-$ precursors begin as IL-$2R^-$, pass through an IL-$2R^+$ stage, and then become IL-$2R^-$ again prior to expressing CD4/CD8 accessory molecules.

Indeed, an IL-2R$^-$, HSA$^+$ subset of double negative thymocytes also expresses ThB and TL, and stain with a monoclonal antibody designated B14 (Table I), all markers of CD4$^+$8$^+$ thymocytes (B. Fowlkes, unpublished observations). (In the actual transition stage to CD4$^+$8$^+$, CD8 is expressed prior to CD4. This phenomenon is discussed at length in Section IV,C.)

In summary, most of the data from *in vivo* and *in vitro* studies are consistent with a model in which they earliest thymocytes to enter the thymus are CD3$^-$, Pgp-1$^+$, HSA$^-$, Thy-1^{lo}, IL-2R$^-$, CD5^{lo}. These cells lose Pgp-1, gain HSA, and start to express high levels of Thy-1. Subsequently, these cells transiently express the IL-2R, such that prior to the CD4$^+$8$^+$ stage, thymocytes are again IL-2R$^-$. Presumably, in the last developmental stages before the transition to CD4$^+$8$^+$ thymocytes, the activation marker RL73 is also expressed (see above). There is, however, no strong evidence that these subsets form a straight-through, single lineage.

In spite of the evidence that double-negative thymocytes differentiate first to CD4$^+$8$^+$, some have found differentiation to single positives without evidence of double-positive progeny. It has been reported that differentiation, using human thymocytes *in vitro*, proceeds as CD4$^-$8$^-$3$^-$ → CD4$^-$8$^-$3$^+$ → CD4$^+$8$^-$3$^+$ and CD4$^-$8$^+$3$^+$, acquiring mature phenotype and function (de la Hera *et al.*, 1986). Adkins *et al.* (1987) have proposed that two precursors exist within the CD4$^-$8$^-$ thymocytes, giving rise directly to two mature phenotypes (CD4$^+$8$^-$ and CD4$^-$8$^+$). This proposal was derived from a study in which CD4$^-$8$^-$ gave rise to these phenotypes one day after intrathymic transfer into unirradiated recipients. Whether these progeny expressed TCR and were immunocompetent is not known.

B. TCR$\gamma\delta^+$ THYMOCYTES

As described in Section III, the $\gamma\delta$ receptor is the first to be expressed in development. Subsequently, the proportion of TCR$\gamma\delta^+$ thymocytes progressively decreases concomitantly with the decrease in percentage of total CD4$^-$8$^-$ thymocytes.

1. Biochemical and Molecular Characterization of TCR$\gamma\delta^+$ Thymocytes

The first identification of the γ protein within the murine thymus was in the CD4$^-$8$^-$5^{lo} population of adult thymocytes (Lew *et al.*, 1986). Staining of isolated CD4$^-$8$^-$5^{lo} thymocytes with an anti-CD3 mAb showed that 5-10% of the cells in this subpopulation expressed the CD3 complex. When the isolated CD4$^-$8$^-$5^{lo} population was

cultured for 3-4 days in a combination of interleukin-1, -2, and -3, the majority of cells that grew out expressed CD3, but were still negative for CD4 and CD8. Northern blot analysis of the cultured $CD4^-8^-3^+$ thymocytes showed no evidence of detectable full-length TCRβ or TCRα mRNA transcripts. Southern blot analysis of these $CD4^-8^-3^+$ populations, as well as individual clones, using Dβ1, Cβ1, and Jβ2 probes, confirmed that the TCRβ loci were predominantly in a partially (D-J) rearranged state. Immunoprecipitation of ^{125}I-labeled cell lysates with the anti-CD3 antibody coprecipitated a disulfide-linked heterodimer of 35 and 45 kDa. The identical heterodimer was also immunoprecipitated by an antiserum raised against a synthetic heptapeptide corresponding to the predicted carboxyl terminus of the murine Cγ1 and Cγ2 chains (from DNA sequencing). The 35-kDa subunit was shown to be the γ chain by virtue of its reactivity with the anti-γ peptide antiserum after reduction of the interchain disulfide bond. The 45-kDa partner chain is the product of the TCRδ gene (Born *et al.*, 1987). In BALB/c and C57BL/6 mice, both the γ and δ chains are glycosylated; after *N*-glyconase treatment, the γ chain displays a core molecular mass of roughly 32 kDa, and the δ chain displays a core molecular mass of roughly 37 kDa.

2. *Lineage Relationships between TCRγδ⁺ and TCRαβ⁺ Thymocytes*

An important conclusion was drawn from the biochemical analysis of γ chains expressed in both the fetal and adult thymus regarding the lineage relationship between $TCR\gamma\delta^+$ and $TCR\alpha\beta^+$ T cells. Since virtually all the γ species on thymocytes from BALB/c and C57BL/6 are glycosylated and are recognized by the anti-Cγ1/Cγ2 serum, they must be derived from Cγ1 (neither Cγ2 nor the Vγ1.2 segment to which it rearranges possess any N-glycosylation acceptor sites); however, virtually all Cγ1 rearrangements analyzed in mature αβ-expressing T cells are out of frame (Traunecker *et al.*, 1986). Thus, the $TCR\gamma\delta^+$ thymocytes (which, by definition, must have in-frame rearrangements of Cγ1) cannot be their precursors. Instead, this biochemical analysis supports the notion that TCRγδ cells are a separate lineage from TCRαβ cells.

Nonetheless, the presence of γ gene rearrangements in mature cells with the αβ receptor, as well as partial β gene rearrangements (D-J) in cells that bear the γδ receptor, indicates that both lineages derive from a common precursor. One model accounting for all these findings (together with the early expression of TCRγδ relative to TCRαβ in ontogeny) postulates that once the product of a productively rearranged γ and δ gene is synthesized, further rearrangement of α and β genes

is prevented. If the rearrangement of γ or δ is nonproductive, no full-length protein could be produced, and the thymocyte would continue to rearrange its α and β genes to produce an $\alpha\beta$ heterodimer (Fig. 5). This model also explains the high frequency of nonproductive γ rearrangements in mature TCR$\alpha\beta$-bearing cells. Recently, de Villartay *et al.* (1988) have obtained evidence in humans that commitment to the TCR$\alpha\beta$ lineage may also involve deletion of unrearranged δ loci mediated by recombination between an element 5′ of Dδ1 and a Jα-like element 3′ of Cδ.

The presence of TCR$\gamma\delta$ cells in the periphery suggests an important function for this lineage outside of the thymus. The majority of splenic TCR$\gamma\delta^+$ cells appear to be thymus derived (Pardoll *et al.*, 1988). Thus, young athymic (nude) mice possess no detectable TCR$\gamma\delta^+$ cells in their periphery. After thymus engraftment, TCR$\gamma\delta^+$ cells of host origin first appear in the donor thymus and, subsequently, populate the periphery at levels comparable to an age-matched euthymic mouse. In addition, there is an extrathymic pathway of differentiation which can be seen operating in old nude mice. Whether the repertoire of extrathymically-derived TCR$\gamma\delta^+$ cells differs from those that are thymically derived remains to be determined.

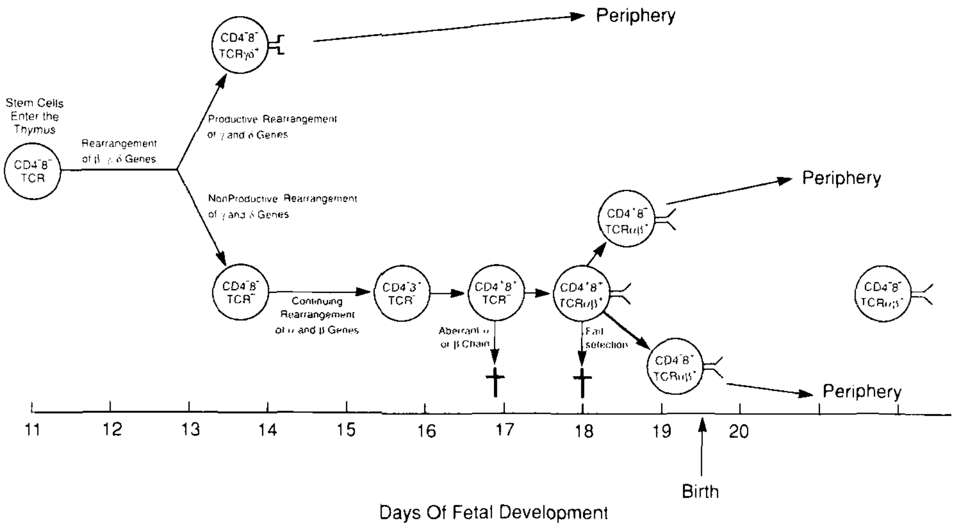

FIG. 5. Time line illustrating the ontogenic appearance of murine thymocytes as defined by CD4, CD8, and T cell receptor. Lineage relationships and selective events are depicted for which there exist reasonable experimental data.

C. $CD4^+8^+$ THYMOCYTES

Probably more than any other subset, the $CD4^+8^+$ thymocytes have been the most controversial and difficult to understand with regard to growth requirements, precursor product relationships, and selection mechanisms that determine their survival and differentiation. Historically speaking, the most provocative characteristic of these cells was their inordinate amount of intrathymic cell death (McPhee *et al.*, 1979; reviewed in Scollay and Shortman, 1985). This phenomenon most probably results from a combination of nonproductive rearrangements of the T cell receptor genes and selection mechanisms operating on TCR-bearing cells. Over the last 3 years, several important experimental approaches have been developed which are starting to unravel the mysteries of this very interesting stage of T cell development.

1. Phenotype and Function of $CD4^+8^+$ Thymocytes

Approximately 80–85% of adult thymocytes are defined by coexpression of the accessory molecules, CD4 and CD8, and have a cortical location. About one-half of these cells express low-level TCR (Roehm *et al.*, 1984; Lanier and Weiss, 1986; Bluestone *et al.*, 1987). The phenotype of the cells is predominantly $CD4^+$, $CD8^+$, PNA^{hi}, $H\text{-}2K^+$, $CD5^+$, $Thy\text{-}1^{hi}$, HSA^+, Pgp^-, $IL\text{-}2R^-$, ThB^+, and $CD1^+$ (T6) or TL^+ (Tables I and II). By forward light-scatter analysis, approximately 20% of these cells are larger and, by morphology and *in vivo* labeling studies, are dividing (reviewed in Shortman and Scollay, 1985). The larger cells are predominantly TCR^- (J. Allison, personal communication; Scollay *et al.*, 1988). It has been known for quite some time that double-positive thymocytes are steroid sensitive. Steroid sensitivity distinguishes the more sensitive $CD4^+8^+$ cells which reside in the cortex from the single-positive cells which reside in the medulla, which have phenotypes and functions more like peripheral T cells (Blomgren and Andersson, 1969).

Many of the earlier functional analyses of double-positive thymocytes *in vitro* were plagued by the technical difficulties of making pure populations for assay. It is widely acknowledged that the double-positive thymocytes die rapidly in culture, while single-positive thymocytes preferentially survive. This extreme selection pressure against the $CD4^+8^+$ thymocytes demands very highly purified populations of cells for analysis of growth, differentiation, and function. With the advent of flow cytometry, electronic cell sorting, and functional analysis by limiting dilution, it became possible to more reliably assess the growth and functional capacity of these thymocytes *in vitro*. Recent studies on the fetal and adult thymus demonstrate that cells of $CD4^+8^+$ phenotype are essentially devoid of

cytotoxic lymphocytes (CTL-P) or IL-2-producing cells capable of responding to allogenic stimuli (Ceredig *et al.*, 1982a; Kisielow *et al.*, 1982; Ceredig *et al.*, 1983a). Limiting dilution analysis of sorted populations for proliferation and CTL-P in response to mitogen similarly demonstrate that the cortical-type cells are nonfunctional (Chen *et al.*, 1982, 1983). Moreover, highly purified populations of double-positive thymocytes have not been induced to proliferate to a variety of thymic extracts, culture supernatants, or hormone preparations (Andrews *et al.*, 1985). In the rat, the mAb MRC OX-44 binds all peripheral T cells, but binds only 10–15% of thymocytes (Paterson *et al.*, 1987). Double-positive thymocytes are essentially OX-44$^-$. All the functional capacity in the rat thymus resides in the OX-44$^+$-sorted fraction.

The lack of functional activity in the CD4$^+$8$^+$ thymocytes might be somewhat unexpected, given that half of these cells express CD3-associated TCR$\alpha\beta$; however, the level of receptor expression is lower than on single-positive thymocytes or peripheral T cells (Roehm *et al.*, 1984; Bluestone *et al.*, 1987). CD4$^+$8$^+$ thymocytes fail to proliferate, produce IL-2, or express IL-2R, even in the presence of phorbol ester and calcium ionophore (Lugo *et al.*, 1986; Havran *et al.*, 1987; Finkel *et al.*, 1987; Weiss *et al.*, 1987). Anti-CD3 stimulation of these cells does illicit a rise in cytoplasmic free calcium (an early event in the activation of mature T cells). While double-positive cells appear to mobilize calcium, the response is lower than that of mature peripheral T cells.

When tritiated thymidine is injected intravenously, the bulk of the labeled cells in the thymus are found among the large CD4$^+$8$^+$ thymocytes (Scollay *et al.*, 1984). Similar results have been obtained using bromodeoxyuridine labeling in the rat (Paterson *et al.*, 1987) or in thymic fetal organ cultures in the mouse (Sékaly *et al.*, 1983). Cell cycle analyses of isolated large double-positive cells also indicate that a subset of CD4$^+$8$^+$ thymocytes is actively dividing *in vivo* (Sékaly *et al.*, 1983; Rothenberg, 1982); Nevertheless, double-positive thymocytes do not express IL-2R, and growth factors responsible for the *in vivo* division have not been determined.

As stated in many reviews, it is widely accepted that the bulk of double-positive cells die in the thymus (Scollay and Shortman, 1983, 1985; Mathieson and Fowlkes, 1984; Scollay *et al.*, 1984; Shortman and Scollay, 1985; Rothenberg and Lugo, 1985). The experimental evidence comes from *in vivo* studies that examine the reutilization of thymidine label, thymic input/output, and turnover rates (reviewed in Scollay and Shortman, 1985; Rothenberg and Lugo, 1985). It has been proposed that this intrathymic cell death is largely the result of nonproductive TCR gene rearrangements (Kronenberg *et al.*, 1986) or because they fail selection (reviewed in Sprent *et al.*, 1988).

2. *Analysis of $CD4^+8^+$ Thymocytes for Precursor Product and Lineage Relationships*

Because the apparent renewal and survival capacity of most double-positive thymocytes is so low both *in vitro* and *in vivo*, it has been extremely difficult to establish the precursor product and lineage relationship of double positives to other thymocytes. Much of the evidence used to support lineage models has come from experiments using unseparated or partially purified populations of cells. Such populations have been used to make interpretations and conclusions based on experiments analyzing the loss or gain of radioactive or fluorescent labels or of surface markers. Given the extreme heterogeneity in the thymus, and the added complexity of differential growth and survival, many of the data on this issue are uniformly unconvincing. Even in the case where double positives have been highly purified, controls have not been used to rule out selective survival of contaminants. Nevertheless, some of these studies are informative and provocative and influence current opinion on these issues.

Ontogeny studies reveal that the appearance of double-positive thymocytes is preceded by a population of cells that are $CD4^-8^+$ (Ceredig *et al.*, 1983a; Kisielow *et al.*, 1984; MacKay *et al.*, 1986). Ceredig and his colleagues (1983b) observed fetal mouse thymocytes with this phenotype prior to development of $CD4^+8^+$ cells or of immunocompetency, consistent with the interpretation that CD8 is expressed prior to CD4 on cells becoming double positive. It was additionally proposed that early $CD4^-8^+$ cells in fetal sheep were precursors to the double positives, since they could be distinguished by their coexpression of CDl from thymocytes of the same phenotype which develop later (MacKay *et al.*, 1986). Once antibodies to TCR became available in the mouse, a $CD4^-8^+$ TCR^- thymocyte subset was observed in the adult (Bluestone *et al.*, 1987; Crispe *et al.*, 1987b). This population was further characterized as $CD5^{lo}$, HSA^+, Thy-1^{hi}, Mel 14^-, Pgp-1^-, unresponsive to mitogens and anti-TCR antibody (anti-Vβ8), and lacking alloreactive CTL-p (Wilson *et al.*, 1987; Crispe and Bevan, 1987; MacDonald *et al.*, 1988a; Shortman *et al.*, 1988). These large, dividing $CD4^-8^+$ thymocytes are similar to $CD4^+8^+$ thymocytes in their cortisone sensitivity, cortical location, phenotype, and failure to be stimulated with a variety of stimuli including PMA plus ionomycin (Shortman *et al.*, 1988; MacDonald *et al.*, 1988a). This subset expresses no IL-2R, but is $RL73^+$, a marker for thymocytes rapidly dividing *in situ* (MacDonald *et al.*, 1988a). Studies with isolated $CD4^-8^+3^-$ thymocytes in the rat (distinguished from $CD4^-8^+3^+$ as OX-44^-) indicate that these cells differentiate to $CD4^+8^+$ cells after 18 hours of culture (Paterson and

Williams, 1987). This differentiation occurs in virtually all the cells in the absence of added growth factors, stimulants, or accessory cells, and with high cell recoveries. *In vivo* studies analyzing BUdR labeling and phenotype during thymic regeneration after irradiation supported the conclusion that these $CD4^-8^+$ cells are precursors to the $CD4^+8^+$ population. More recently, *in vitro* differentiation of $CD4^-8^+3^-$ thymocytes was demonstrated using mouse thymocytes (MacDonald *et al.*, 1988a; Scollay *et al.*, 1988). MacDonald *et al.* (1988a) observe that the $CD4^+8^+$ progeny of early $CD4^-8^+$ thymocytes which arise in culture are $CD3^-$; however, one group has demonstrated that these $CD4^-8^+$ thymocytes are able to generate both $CD4^+8^+$ and $CD4^+8^-$ cells after intrathymic injection of isolated $CD4^-8^+$ thymocytes (C. J. Guidos *et al.*, unpublished observations). This result suggests that at least some of these thymocytes have greater differentiation potential than had been demonstrated *in vitro*.

Differentiation within the double-positive population has not been as extensively addressed. Labeling studies either *in vivo* or in thymic organ culture suggest that the large $CD4^+8^+$ give rise to the small $CD4^+8^+$ thymocytes. Most of these smaller progeny are not cycling by *in situ* analysis and, presumably, are the bulk of the cells which die in the thymus (reviewed by Scollay and Shortman, 1985; Rothenberg and Lugo, 1985; Ceredig and MacDonald, 1985). The question remains as to whether most of the large $CD4^+8^+3^-$ thymocytes also die *in situ* and whether any or all serve as intermediate precursors to the $CD3^+$ progeny.

Probably the most controversial issue involving precursor product and lineage relationships among developing thymocytes has been whether $CD4^+8^+$ thymocytes are a completely dead-end lineage or contain any or all of the precursors to functional single-positive thymocytes. While numerous groups have claimed to induce *in vitro* differentiation under various culture conditions (Irlé *et al.*, 1978; Hardt *et al.*, 1980; Blue *et al.*, 1985; reviewed by Kruisbeek, 1979, and Shortman and Scollay, 1985), outgrowth of contaminating mature thymocytes, rather than true differentiation, was not ruled out in these studies.

Assessment of precursor activity of isolated $CD4^+8^+$ thymocytes *in vivo* requires intrathymic transfer, since these cells are unable to home to the thymus after intravenous injection (Fowlkes *et al.*, 1985). Some have observed the generation of single-positive thymocytes after intrathymic transfer of isolated $CD4^+8^+$ thymocytes (B. Mathieson, unpublished observations), while others observe no repopulation or differentiation to more mature phenotypes (Scollay *et al.*, 1988; B. Fowlkes and L. Hausmann, unpublished observations). Since it is believed that successful differentiation in this compartment is a fairly inefficient process, fractionation of the $CD4^+8^+$ thymocytes for transfer might be

the most reasonable approach. Although there have been several reports that a small number of $CD4^+8^+$, $CD3^{hi}$ thymocytes exist (Blue *et al.*, 1987; Crispe *et al.*, 1987b; Havran *et al.*, 1987), this specific subset has not been isolated and analyzed for precursor activity.

An alternative approach to analyzing precursor potential of double-positive thymocytes was made by Smith (1987), who showed that *in vivo* treatment with anti-CD8 antibody administered during development eliminated the $CD4^+8^-$ subset, suggesting a $CD4^+8^+$ precursor stage. This study utilized allogenic tetraparental radiation bone marrow chimeras in which the donor mice possessed allotypic differences at both the CD8 and Thy-1 loci. While very elegant in design, these experiments are flawed by the failure to use a fully syngeneic system and to analyze the thymocytes as well as the peripheral T cells. The elimination of the $CD4^+8^-$ progeny of the treated parental type could be the result of a graft versus graft reaction rather than a developmental block. Also, in other studies (Fowlkes *et al.*, 1988; MacDonald *et al.*, 1988c; B. Fowlkes and A. Kruisbeek, unpublished observations), *in vivo* treatment with either anti-CD8 or anti-CD4 with a variety of monoclonal antibodies (including one used in the Smith study) fails to eliminate the double-positive thymocytes. While it can be demonstrated that the $CD4^+8^+$ thymocytes are saturated with antibody *in vivo*, only single-positive and peripheral cells bearing the antigen against which the antibody is directed are diminished by the treatment. For example, in the case of anti-CD8 treatment, only $CD4^-8^+$, and not $CD4^+8^+$ cells are affected.

Given that $CD4^-8^+$ thymocytes do indeed develop in the presence of anti-CD4 treatment, Fowlkes *et al.* (1988) have demonstrated the precursor nature of $CD4^+8^+$ thymocytes by showing that *in vivo* anti-CD4 treatment alters the repertoire of $TCR(\alpha\beta)$ expression in the $CD4^-8^+$ subset. This experiment is described below in more detail because of the importance of its bearing on the developmental stage at which tolerance generation occurs (see Section V,A).

D. $CD4^+8^-$ and $CD4^-8^+$ Thymocytes

The next population to appear in fetal development after the double positives are $CD4^+8^-$, followed shortly by the $CD4^-8^+$ thymocytes. Single-positive thymocytes are similar to peripheral $CD4^+$ and $CD8^+$ T cells in both phenotype and function; however, evidence is mounting that these populations may be more heterogeneous than previously believed. Relevant to this issue, some recently described phenotypic markers are said to distinguish thymic and peripheral single-positive T cells.

1. Phenotype and Function of Single-Positive Thymocytes

Thymocytes which are $CD4^+8^-$ and $CD4^-8^+$ constitute about 10-15% of the adult thymus, are generally intermediate in size, reside mainly in the medulla, and are phenotypically PNA^{lo}, Thy-1^{lo}, $CD5^{hi}$, H-$2K^{lo}$, and $CD1^-$ (Shortman and Scollay, 1985). In the thymus, virtually all of the $CD4^+8^-$ cells express TCR($\alpha\beta$), while only about one-half of the $CD4^-8^+$ cells express TCR (Roehm *et al.*, 1984; Bluestone *et al.*, 1987; Crispe *et al.*, 1987b). In contrast to double-positive thymocytes, these cells express high levels of antigen receptor. While the ratio of $CD4^+8^-$ to $CD4^-8^+$ is generally 2:1 in the periphery, it is closer to 3:1 in the thymus, with some variation among mouse strains. Scollay *et al.* (1978) have designed an assay to reveal which cells in the thymus seed the peripheral lymphoid organs. They were able to detect labeled cells in the major lymphoid organs within a few hours after direct intrathymic injection of fluorescein isothiocyanate. The phenotype of the recent migrants was essentially medullary type.

It has been known for quite some time that many of the same functions attributed to mature peripheral T cells can be elicited from medullary single-positive thymocytes. Cortisone treatment *in vivo*, or *in vitro* separations based on physical characteristics or antigen expression, have been used to enrich for immunocompetent thymocytes. The question that arises is whether all thymocytes with a single-positive phenotype are functionally mature.

There is, in fact, mounting evidence for heterogeneity within the $TCR\alpha\beta^+$ single-positive thymocyte subsets. Single-positive thymocytes are generally thought to be cortisone resistent, but the fact that only 3-5% of thymocytes remain after *in vivo* treatment suggests that some single-positive thymocytes are also cortisone sensitive. Even excluding the cortisone-sensitive $CD4^-8^+3^-$ subset described above, other single-positive thymocytes would have to be cortisone sensitive, suggesting heterogeneity with respect to steroid sensitivity within $CD3^+$ single-positive thymocytes.

Despite initial reports to the contrary (Scollay *et al.*, 1984; Crispe and Bevan, 1987; Crispe *et al.*, 1987a), two distinct subsets of $CD4^+8^-$ thymocytes with respect to HSA expression and functional properties have been identified and characterized (B. Fowlkes, S. Sharrow, and M. Jenkins, unpublished observations). While all isolated $CD4^+8^-$ cells express CD3, the $CD4^+8^-$ thymocytes possess two subsets with reciprocal expression of HSA and Qa-2 (Tables I and II). In functional analysis of isolated subsets, only the Qa-2^+, HSA^- cells ($\sim$30% of the

$CD4^+8^-$) proliferate in response to immobilized anti-CD3 + PMA; however, both the Qa-2^+, HSA^- and the Qa-2^-, HSA^+ subsets will respond to these stimulators when IL-2 is added to the culture. The results suggest that the HSA^+, Qa-2^- subset of $CD4^+8^-$ thymocytes is unable to respond by making IL-2 when stimulated through the TCR, but is able to use exogenously added IL-2. Both subsets proliferate when stimulated with PMA plus calcium ionophore, even in the absence of exogenous IL-2. Interestingly, all peripheral $CD4^+8^-$ T cells are HSA^- and Qa-2^+ and will respond to immobilized anti-CD3 plus PMA in the absence of exogenously added IL-2. It is possible therefore that the Qa-2^-, HSA^+ single-positive subset in the thymus represents a stage of T cell development which has not yet achieved complete functional maturity, rather than a separate lineage. There is also heterogeneity among $CD4^-8^+3^+$ thymocytes. That is, there are two subsets, like the $CD4^+8^-$ thymocytes, which reciprocally express HSA and Qa-2 (B. Fowlkes, unpublished observations). Whether these subsets are heterogeneous with respect to functional capacity has not yet been determined.

The differences between peripheral T cells and thymocytes, in response to anti-CD3 stimulation plus PMA, are reminiscent of the results obtained by Ramarli *et al.* (1987). In the latter study, $CD3^+$ thymocytes, as opposed to peripheral T cells, responded poorly to anti-CD3 plus PMA in the absence of exogenously added IL-2; however, $CD3^+$ thymocytes, like peripheral T cells, could respond to mitogenic anti-CD2 plus PMA in the absence of exogenous IL-2.

2. *Phenotypic Differences between Single-Positive Thymocytes and Peripheral T Cells*

Differential marker expression between the thymus and the peripheral lymphoid organs has not been extensively investigated. Certainly, many acute activation antigens (i.e., antigens which appear transiently as a result of stimulation) have been described. These antigens probably have very important functional relevance but are beyond the scope of this review. More important to the present discussion is the issue of prethymic versus postthymic maturation and/or lineage diversification. Most notably, differential antigen expression between thymic and peripheral T cells has been delineated by antibodies to the varied protein forms of T200, either due to variation of T200 gene transcription or translational or posttranslational modification of T200. Anti-CD45R antibodies which reportedly bind peripheral T cells but not thymocytes include antibodies such as 2H4 (human), OX-22 (rat), and 14.8 and RA3-2C2 (mouse) (LeFrancois and Goodman, 1987) (Table I).

Presumably this differential expression could be the result of postthymic maturation. Markers for 4B4 (CDw29) and 2H4 (CD45R) are reported to delineate helper-inducer and suppressor-inducer subsets, respectively, in the human peripheral T cell pool (Morimoto *et al.*, 1985a,b). Whether these represent antigens that mark discrete functional subsets is a matter of controversy. Others contend that expression of such antigens is altered by a maturational event which occurs when a naive T cell is activated for the first time (Serra *et al.*, 1988; Sanders *et al.*, 1988a; Akbar *et al.*, 1988). The $CD4^+8^-$ or $CD4^-8^+$ mouse thymocytes lack the marker Pgp-1, while 10–40% of peripheral T cells express the antigen (Budd *et al.*, 1987a). After *in vivo* immunization, it was found that the antigen-specific responses are concentrated in the Pgp-1^+ subset. Expression of Pgp-1 persists long after the primary stimulation (perhaps permanently). These markers, therefore, appear to identify memory T cells. Sanders *et al.* (1988b) propose that these are not lineage markers that functionally subset the $CD4^+$ or $CD8^+$ lineages, but, instead, discriminate between naive and memory T cells. Since many other surface markers are also stably altered in expression as a result of activation (LFA-3, CD2, LFA-1, CD45, and CDw29), they further propose that the increase in expression of adhesion molecules results in enhanced responsiveness during subsequent activation.

Thus, it would appear that a postthymic, stable maturation event can occur, as a result of primary activation. Whether there are also stable and discrete functional subsets of $CD4^+$ or $CD8^+$ T cells and whether this differentiation occurs in the thymus is not as yet clear. Although no discriminating markers have yet been described, it is possible that T helper ($CD4^+$) types 1 and 2 clones (Mosmann *et al.*, 1986) exemplify such lineage diversification.

E. $CD4^-8^-$ $TCR\alpha\beta^+$ Thymocytes

A second group of T3-bearing cells among double-negative thymocytes use $\alpha\beta$ T cell receptors (Budd *et al.*, 1987b; Fowlkes *et al*, 1987; Crispe *et al.*, 1987a,b; Ceredig *et al.*, 1987; Toribio *et al.*, 1988). Early interest in the cells stemmed from the possibility that they might be early precursors which expressed $TCR\alpha\beta$ prior to expression of accessory molecules. While this issue is still a matter of controversy, it is now apparent that these cells appear late in ontogeny and have many of the characteristics of mature T cells, but are distinguished by a restricted TCR $V\beta$ repertoire.

1. *Phenotype and Function of* $CD4^-8^-3^+$ $TCR\alpha\beta$ *Thymocytes*

The $TCR\alpha\beta$-bearing subset constitutes 2–30% of $CD4^-8^-$ thymocytes in different mouse strains, and displays a $CD3^+$, HSA^-,

$CD5^{hi}$, $IL\text{-}2R^-$, Pgp^+, $B220^-$, and $Qa\text{-}2^+$ phenotype (see Tables I and II) (Fowlkes *et al.*, 1987; Howe and MacDonald, 1988; Wilson *et al.*, 1988a,b). Anti-CD3 immunoprecipitation of isolated $CD4^-8^-$ or $CD4^-8^-$ HSA^- thymocytes reveals CD3-associated material with a molecular mass of 38–43 kDa, suggestive of the $\alpha\beta$ heterodimer. More than one-half of $CD3^+$ cells within $CD4^-8^-$ thymocytes use $V\beta8$-bearing TCR as detected by the F23.1 mAb (Staerz *et al.*, 1985). Given that some of the $CD3^+$ cells use $TCR\gamma\delta$, the proportion of $CD4^-8^-TCR\alpha\beta^+$ thymocytes which use $V\beta8$ is even higher. Overexpression of $V\beta8$ has also been confirmed by Northern blot analysis of cultured HSA^- $CD4^-8^-$ thymocytes (Budd *et al.*, 1987b). These cells appear to be in a resting state *in situ*, as they do not express IL-2R or the activation antigen, RL73, and have a low percentage of cells in cycle by *in vivo* labeling and propidium iodide cell cycle analysis (Ewing *et al.*, 1988; MacDonald *et al.*, 1988c). Nevertheless, the $TCR\alpha\beta$ double-negative thymocytes appear to have a functional TCR, in that these cells respond in culture to Con A, phorbol ester plus calcium ionophore, or anti-TCR antibodies (anti-CD3 or anti-$V\beta8$). IL-1 also appears to be an important comitogenic factor for this population (Fowlkes *et al.*, 1987; MacDonald *et al.*, 1988c). Stimulated $TCR\alpha\beta^+$ double-negative cells make IL-2 and IL-3 and are capable of promoting cytotoxic lysis of appropriate FC-bearing targets (Ceredig *et al.*, 1987; MacDonald *et al.*, 1988c). Upon stimulation, these cells display a phenotype similar to abnormal $CD4^-8^-$ $TCR\alpha\beta$ cells which accumulate in the lymph nodes of autoimmune *lpr* mice (Morse *et al.*, 1982). It is interesting that the majority of the *lpr* cells also appear to express products of the $V\beta8$ gene family. The expanded *lpr* population also express some B cell markers (B220, PC-1), even though there is no rearrangement of immunoglobulin heavy chain genes. Thymic $TCR\alpha\beta$ double-negative cells from normal mice begin to express these same B cell markers upon activation (Fowlkes *et al.*, 1987). Thus, the $TCR\alpha\beta$-bearing $CD4^-8^-$ thymocytes may be the normal counterpart of the defective $CD4^-8^-$ T cells found in the *lpr* autoimmune mouse. Relevant to this point, it has been shown that thymectomy of *lpr* mice prevents lymphadenopathy (Steinberg *et al.*, 1980).

2. *Precursor–Product and Lineage Relationship of $CD4^-8^-3^+$ $TCR\alpha\beta^+$ to Other Thymocytes*

The place of the $TCR\alpha\beta^+$ $CD4^-8^-$ cell in T cell development is not clear. The question of whether $TCR\alpha\beta^+$ double-negative thymocytes are precursors to mature $TCR\alpha\beta$ single-positive cells or constitute a separate lineage is a crucial one, since they potentially include $TCR\alpha\beta$-bearing cells which have not yet passed through the thymic selection

process. Alternatively, if these cells are the mature progeny of a separate lineage, they may represent a distinct effector component in the immune response.

Data have been presented using human thymocytes which suggest that the TCR$\alpha\beta^+$ double-negative cells are precursors which differentiate to CD4$^+$8$^-$ and CD4$^-$8$^+$ T cells after *in vitro* culture with IL-2 or lectin (de la Hera *et al.*, 1986; Toribio *et al.*, 1988). Surprisingly, no double-positive cells were obtained after short-term culture, as is usually obtained with mouse double-negative thymocytes (Ceredig *et al.*, 1983c; Fowlkes *et al.*, 1984; Nakano *et al.*, 1987). The failure to generate double positives in culture may be related to the fact that CD1$^+$ (T6) cells (a subset of the double negatives) were removed prior to culture.

Some results, however, point away from TCR$\alpha\beta$ CD4$^-$8$^-$ thymocytes being precursors. Crispe *et al.* (1987a) reported that in contrast to CD4$^-$8$^-$3$^-$ thymocytes, TCR$\alpha\beta^+$ CD4$^-$8$^-$ thymocytes (isolated by cytotoxic treatment of CD4$^-$8$^-$ thymocytes using mAb J1ld) do not differentiate to single-positive cells after intrathymic injection. Intrathymic injection is required for this assay, since CD3$^+$ (TCR$^+$) thymocytes do not home to the thymus after intravenous injection (Nakano *et al.*, 1987; Scollay *et al.*, 1988). MacDonald *et al.* (1988c) find no further differentiation of these cells after transfer into nude mice. Moreover, the fact that these cells come up late in ontogeny (Fowlkes *et al.*, 1987; Ceredig *et al.*, 1987; Wilson *et al.*, 1988b), have the phenotype of mature T cells (HSA$^-$, CD5^{high}, Qa-2$^+$), and are immunocompetent (i.e., they proliferate in response to anti-TCR antibodies and mitogens, produce lymphokine, and are capable of cytotoxic lysis) supports the notion that these cells represent a late rather than early stage in T cell development. Others (C. J. Guidos *et al.*, unpublished observations) find TCR$\alpha\beta$ CD4$^-$8$^-$ cells of donor type in lymph nodes after intrathymic injection of CD4$^-$8$^-$ thymocytes into irradiated hosts. These cells persist in the periphery for >20 weeks, indicating that they are able to migrate to the periphery and are long lived.

The fact that these cells express TCR and are functionally mature, but lack the CD4 or CD8 accessory molecules (typical of TCR$\alpha\beta$ cells), suggested the possibility that they arise from the loss of accessory molecules. In this regard there is preliminary evidence from *in vivo* transfer experiments that these cells are the progeny of single-positive thymocytes (D. Pardoll and B. Fowlkes, unpublished observations). It is also worth noting that the bulk of these cells are Pgp-1$^+$, a marker usually found on a subset of single-positive thymocytes or peripheral T cells which have undergone previous activation (Budd *et al.*, 1987a). Taken together, it is interesting to speculate that the $\alpha\beta^+$ double-

negative cells are generated by an intrathymic activation event involving immature single-positive thymocytes which leads to down modulation of accessory molecules and acquisition of Pgp-1.

V. Mechanisms of Cellular Selection within the Thymus

Having reviewed the early molecular events of T cell development leading to expression of TCR ($\gamma\delta$ and $\alpha\beta$) and the studies on precursor-product relationship and lineage divergence, it is now appropriate to discuss the processes of thymocyte selection. We begin with the premise that cellular selection (stage 2 of development as outlined in Section I) operates upon a pool of thymocytes initially expressing the whole spectrum of surface TCRs that the germ line is capable of encoding. The set of TCRs which emerges from the selection stage constitutes what has been termed the T cell repertoire. Because the repertoire is defined on the basis of functional antigen-specific MHC-restricted reactivity, essentially all of the relevant studies pertain to T cells expressing TCR$\alpha\beta$. As the ligand specificity of TCR$\gamma\delta$ is, at this time, largely undefined, it is difficult to study selection within the TCR$\gamma\delta^+$ subset.

A. Negative Selection

The most clearly understood form of thymic selection is negative selection. Negative selection was initially proposed to explain how the developing immune system learns to distinguish self from non-self, i.e., to react to foreign antigens yet be tolerant of self-antigens. Probably the most compelling evidence that tolerance occurs during intrathymic development was the demonstration that T cells maturing in fetal thymic organ culture react against allogeneic stimulator cells to a significantly greater extent than against syngeneic stimulator cells (Robinson and Owen, 1977). Subsequently, the postulate that negative selection might involve recognition of thymic (self) MHC antigens (Schwartz, 1978) was borne out by experiments which showed that developing T cells are tolerant to self-nominal antigens only in the context of thymically expressed MHC antigens (Dos Reis and Shevach, 1983; Groves and Singer, 1983; Rammensee and Bevan, 1984; Matzinger *et al.*, 1984).

1. *Evidence for Deletion of Self-Reactive Clones*

Three general mechanisms have been proposed to account for self-tolerance: (1) suppression of self-reactive clones, (2) functional inactivation of self-reactive clones, and (3) physical deletion of self-reactive clones. Definitive evidence for the latter mechanism (clonal deletion) has been recently obtained. The initial breakthrough came from the identification of a mAb (KJ23) recognizing a murine Vβ segment (Vβ17a) that

confers a high probability of reactivity with the MHC class II, I-E molecule (of any haplotype) (Kappler *et al.*, 1987b). This allowed the direct detection of TCRs with a known ligand specificity. Most mouse strains that possess a functional Vβ17a segment in their germ line are I-E$^-$ (due to a defective I-Eα gene); however, the C57BR strain, which is both I-E$^+$ and possesses a functional Vβ17a gene, has virtually no peripheral T cells that express Vβ17a$^+$ TCRs. T cells expressing Vβ17a$^+$ TCRs were also found to be eliminated in F_1 progeny of I-E$^-$ $\times$ I-E$^+$ strains and in transgenic mice in which a functional I-Eα gene was introduced into an I-E$^-$ host (Kappler *et al.*, 1987a; Widera *et al.*, 1987; Marrack *et al.*, 1988b). In all these cases, Vβ17a$^+$ cells are absent from among the CD3^{hi} single-positive thymocytes, implying that their elimination occurred during intrathymic development rather than after dissemination to the periphery. Subsequent to the description of KJ23, there have been a number of additional examples of mAbs that recognize specific Vβ products that confer a high probability of reactivity to known antigens. These include Vβ11, which also confers anti-IE reactivity (O. Kanagawa, personal communication); Vβ8.1 and Vβ6, which confer anti-Mls^a reactivity (Kappler *et al.*, 1988; MacDonald *et al.*, 1988e); and Vβ3, which confers anti-Mls^c reactivity (J. Kappler and P. Marrack, personal communication). In all of these cases, mouse strains that express the particular stimulating antigen have very few peripheral T cells or single-positive thymocytes expressing the corresponding Vβ product. All of the above examples demonstrate deletion of TCRs that are either (self) class II reactive or class II restricted. Evidence for deletion of class I-restricted TCRs comes from the construction of transgenic mice expressing both α and β TCR chains from a CTL clone reactive with the male H-Y antigen + H-2D^b (Kisielow *et al.*, 1988a). Female H-2^b mice that expressed the transgenes have relatively normal numbers of single-positive thymocytes, whereas male littermates expressing the transgenes have greatly diminished numbers of single-positive thymocytes.

2. *Developmental Stage of Clonal Deletion*

A critical question is at which developmental stage clonal deletion occurs. The advent of mAbs that mark TCRs of known specificity has allowed this issue to be addressed. Though totally absent in the single-positive thymocyte populations, Vβ17a is expressed on a significant proportion of double-positive thymocytes in the I-E$^+$ C57BR mouse strain (Kappler *et al.*, 1987a). This finding in and of itself is equally compatible with the two opposing theories of T cell development: (1) that CD4$^+$8$^+$ thymocytes are a distinct dead-end lineage that is nonfunctional and, therefore, need not be subject to selection (Shortman and

Jackson, 1974), or (2) that the $CD4^+8^+$ population does contain precursors to mature single-positive thymocytes and selection occurs at or after this stage (von Boehmer, 1986; Marrack and Kappler, 1987). It is particularly noteworthy that in $V\beta17a^+$, I-E$^+$ mice, $V\beta17a$-bearing cells are deleted from the $CD4^-8^+$ as well as the $CD4^+8^-$ subset. One explanation for this finding is that, unlike the majority of anti-class II-reactive T cells, TCRs that use $V\beta17a$ do not require CD4 for anti-I-E reactivity and would, therefore, be autoreactive when expressed by either CD4 or CD8 single-positive cells. An alternative explanation is that many $V\beta17a^+$ TCRs do require CD4 for anti-I-E reactivity and $V\beta17a$ is deleted from the $CD4^-8^+$ subset because these cells express CD4 earlier in their developmental history (i.e., as $CD4^+8^+$), at a time when they are subject to negative selection. A specific prediction of the second hypothesis is that functionally blocking the CD4 molecule at the $CD4^+8^+$ developmental stage might interfere with I-E recognition and clonal deletion and, thus, result in the appearance of $V\beta17a^+$ TCR in the $CD4^-8^+$ subset. To test this prediction, Fowlkes *et al.* (1988) used anti-CD4 mAb to treat irradiated C57BR mice reconstituted with syngeneic bone marrow. This anti-CD4 treatment inhibited the deletion of $V\beta17$-bearing cells from the $CD4^-8^+$ thymocytes. These experiments indicate that clonal deletion requires the participation of the accessory molecules (CD4 in this case) and that deletion occurs at the double-positive stage. Equivalent results have also been obtained for $V\beta6$ expression in Mls^a mice treated *in vivo* with anti-CD4 (MacDonald *et al.*, 1988b; F. Ramsdell and B. Fowlkes, unpublished observations).

The anti-H-Y + H-$2D^b$, $TCR\alpha + \beta$ transgenic experiments described above are most compatible with the idea that thymocytes bearing anti-self class I-reactive (or restricted) TCR are likewise deleted at the $CD4^+8^+$ stage. In order to interpret the results of these studies, it must be remembered that this TCR requires CD8 coexpression for reactivity. In the male transgenics, the absolute number of $CD4^+8^+$ and $CD4^+8^-$ thymocytes, as well as $CD4^-8^+$ thymocytes, is significantly decreased (most of the remaining thymocytes are either $CD4^-8^-$ or $CD4^-8^{lo}$). The tremendous depletion of $CD4^+8^+$ transgenic thymocytes is perhaps surprising, given the relative lack of depletion of $V\beta17a^+$ cells in the $CD4^+8^+$ subset of I-E$^+$ strains such as C57BR. This may be because many of the $V\beta17a^+$ TCRs in the $CD4^+8^+$ subset lack appropriate MHC reactivity and are, therefore, incapable of either being positively selected or deleted.

Given that clonal deletion at an immature $CD4^+8^+$ precursor stage appears to be a major mechanism for intrathymic tolerance induction, three important questions remain to be answered: (1) what mechanisms

underlie this deletional event; (2) which thymic stromal components interact with $CD4^+8^+$ thymocytes to mediate deletion; and (3) are there additional nondeletional tolerance mechanisms that operate in the thymus?

A critical event that logically must occur is the engagement of self-MHC antigens (with or without associated self-nominal antigen) by TCR$\alpha\beta$ plus CD4 or CD8 expressed on the $CD4^+8^+$ thymocyte. This interaction must lead to a signaling event whose consequences are cell death (or paralysis) rather than the typical mitogenic response of mature T cells. In order to account for this paradox, Bretscher and Cohen (1970) proposed that the consequences of TCR engagement depended on whether or not a second costimulatory signal was simultaneously delivered to the T cell. TCR engagement in the absence of this second signal would lead to tolerance (via paralysis or death), whereas TCR engagement in the presence of the second signal would lead to mitogenic activation. Applied to thymocytes, their model would suggest that the tolerance process is mediated by interactions with stromal elements that do not efficiently deliver a second costimulatory signal, such as epithelial cells; however, this does not appear to be the case.

3. *Stromal Elements that Mediate Negative Selection*

In brief, the thymic stromal elements can be divided into bone-marrow-derived and non-bone-marrow-derived components. The major non-bone-marrow-derived stromal elements consist of cortical and medullary epithelial cells which express both MHC class I and MHC class II antigens. The major bone-marrow-derived elements are dendritic cells which express both class I and class II MHC antigens and thymic macrophages, which generally only express MHC class I antigens. Dendritic cells are found predominantly at the corticomedullary junction and also in the medulla, whereas thymic macrophages have a predominantly medullary location (Barclay and Mayrhofer, 1981; Jenkinson *et al.*, 1981; reviewed in Adkins *et al.*, 1987).

A number of cellular and molecular techniques have been used to vary MHC expression on specific stromal elements in an attempt to determine which ones mediate MHC-specific selection. As thymic epithelial cells tend to be far more radioresistant than bone-marrow-derived cells, radiation chimeras using allogeneic or semiallogeneic bone marrow reconstitution results in a chimeric stroma whose epithelial cells bear host MHC antigens and whose bone-marrow-derived elements bear donor MHC antigens (reviewed in Sprent, 1988). A second technique for achieving thymic stromal MHC chimerism is transplantation of deoxyguanosine (dGuo)-treated thymuses (Jenkinson *et al.*, 1982). Since dGuo is

selectively toxic to the bone-marrow-derived elements (particularly thymocytes), transplantation of dGuo-treated fetal thymuses into an allogeneic recipient produces a situation in which thymocytes of recipient origin develop in a thymus whose epithelial cells bear donor MHC antigens and whose bone-marrow-derived stromal cells bear recipient MHC antigens. There are two potential problems with both of these techniques. First, it is difficult to be sure that the lymphoid-depleting treatment (radiation or dGuo) does not partially damage epithelial MHC expression or functional competence. Second, it is difficult to verify that all bone-marrow-derived stromal elements are completely eliminated. Somewhat cleaner systems have been developed in other species.

Flajnik *et al.* (1985) produced frog chimeras by fusing the posterior half of a 24-hour-old *Xenopus* embryo, which contains all of the bone marrow anlagen, with the anterior half of an allogeneic 24-hour-old embryo, which contains the thymus anlagen. This procedure yields a chimeric thymic stroma in which all the bone-marrow-derived cells express MHC antigens of the posterior half genotype and the epithelial cells express MHC antigens of anterior half genotype.

In an analogous system, Ohki *et al.* (1987) surgically removed the thymic rudiment from embryonic chickens and replaced it with an embryonic xenogeneic quail thymic rudiment. Embryos which survived and hatched had quail thymus epithelium and bone-marrow-derived stromal cells of chicken origin.

Because the frog and chick/quail chimeras are produced prior to the time that bone-marrow-derived elements have entered the thymus, these systems have an advantage over the radiation or dGuo techniques, which attempt to selectively destroy preexisting syngeneic bone-marrow-derived cells before repopulation with allogeneic bone-marrow-derived cells.

Another system employed more recently to assess which stromal components mediate intrathymic tolerance has been the introduction of MHC genes into transgenic mice. By deleting various transcriptional control elements flanking the coding portion, it has been possible to create MHC transgenic mice that selectively express the MHC transgene product on different thymic stromal cells (Widera *et al.*, 1987; van Ewijk *et al.*, 1988). Needless to say, the power of these transgenic systems is dependent upon the level of cell type specificity achieved for the expression of the transgene.

When tolerance induction has been assessed in each of these systems, the invariable result has been that developing thymocytes are always tolerant to the MHC antigens expressed on the bone-marrow-derived stromal elements. This applies for both class I and class II MHC antigens (Ready *et al.*, 1984; von Boehmer and Schubiger, 1984; Jenkinson *et al.*,

1985; Flajnik *et al.*, 1985; Ohki *et al.*, 1987; Widera *et al.*, 1987; van Ewijk *et al.*, 1988). Furthermore, using the dGuo thymus engraftment system, it has been possible to demonstrate tolerance to epithelial cell-derived minor histocompatibility antigens in the context of major histocompatibility antigens expressed by bone-marrow-derived elements (von Boehmer and Hafen, 1986). Analysis of Vβ17a expression in the dGuo thymus engraftment system has verified that tolerance to I-E expressed only on bone-marrow-derived stromal cells occurs via a deletional mechanism (Marrack *et al.*, 1988b). These studies provide elegant confirmation for the classic experimental results of Billingham *et al.* (1956), who demonstrated that the critical elements for induction of neonatal tolerance were, in fact, the hematopoietic cells. Recently, MacDonald *et al.* (1988e) have directly demonstrated that neonatal tolerance induced by injection of Mls^a spleen cells is associated with clonal deletion of Vβ6-bearing T cells.

The fact that hematopoietic stromal cells (particularly dendritic cells), which are excellent APCs for mature T cells (Kyewsky *et al.*, 1986), are capable of inducing tolerance in developing thymocytes implies that the tolerance process is not necessarily due to the absence of a constimulatory (second) signal. Rather, tolerance by deletion appears to involve a property intrinsic to developing ($CD4^+8^+3^+$) thymocytes such that recognition of self antigens induces deletion rather than stimulation.

In contrast, it is not as clear what the role of the epithelium is in tolerance generation, either via deletion or other mechanisms. Different systems have yielded different results and, even within individual systems, results have varied depending on the tolerance assay used. The dGuo thymus engraftment system, as well as the frog chimera system, have indicated that the thymic epithelium induces a partial or "split" form of tolerance. Thus, even though T cells from these chimeras do react to splenocyte stimulators expressing the epithelial-type MHC antigens (either class II-specific mixed lymphocyte reaction or class I-specific cytolysis), the chimeras nonetheless do not reject either the thymus graft itself or skin or organ grafts expressing epithelial-type MHC antigens (Ready *et al.*, 1984; von Boehmer and Schubiger, 1984; Flajnik *et al.*, 1985). These results could be interpreted in different ways. One possibility is that T cells qualitatively "see" MHC antigens differently on epithelial cells versus hematopoietic cells (e.g., due to different sets of peptides displayed in Bjorkman's groove). An alternative interpretation is based on the quantitative argument that the thymic epithelium may only tolerize high-affinity self-reactive clones. The remaining low-affinity anti-self clones would mount a response to strong stimulator cells such as splenocytes, but not to the more weakly stimulatory epithelial cells of

the transplanted thymus or organ graft. The fact that the thymus and organ grafts are rejected after priming the chimeras with highly stimulatory splenic dendritic cells expressing thymic epithelial type MHC (J. J. T. Owen, personal communication) argues in favor of the latter quantitative argument.

Two additional studies should be noted which conclude that thymic epithelium can tolerize even by the mixed-lymphocyte reaction (MLR) assay. T cells from I-E^d transgenic mice that express detectable I-E^d only on thymic epithelium failed to produce an MLR against splenocytes expressing the transgenic I-E^d (Widera *et al.*, 1987). Also, T cells from athymic *nu/nu* mice transplanted with low-temperature cultured allogeneic thymuses (another method of selectively depleting hematopoietic cells) did not mount an MLR against splenic stimulators expressing the thymic epithelial type MHC (Jordan *et al.*, 1985).

Recently, other studies have sought to determine the capacity of epithelial cells to specifically delete self-reactive TCRs. In both the epithelial-expressing I-E transgenic mice described above, as well as in dGuo I-E^+ thymus transplants, less than 25% deletion of Vβ17a was observed (Marrack *et al.*, 1988b). The presence of near-normal Vβ17a levels in these transgenic mice is quite interesting, given the absence of an anti-I-E MLR, and suggests that a nondeletional tolerance mechanism may be operating in these mice. Conversely, J. Sprent (personal communication) has found that in radiation bone marrow chimeras expressing I-E only on the thymic epithelium, Vβ11 (also anti-I-E) is 75% deleted. The remaining V$\beta 11^+$ T cells mounted a weak anti-I-E MLR easily blocked by anti-I-E mAb, suggesting that epithelial cells may only delete high-affinity clones.

Regarding the ability of epithelial cells to mediate deletion of thymocytes expressing TCRs against non-MHC self-antigens, S. Webb (personal communication) has found that when Mls^a recipients were irradiated and reconstituted with Mls^b bone marrow, or when dGuo-treated Mls^a thymuses were transplanted into Mls^b recipients, there was virtually no deletion of (anti-Mls^a) V$\beta 6^+$ TCRs. Thymocytes from these chimeras mounted a significant anti-Mls^a MLR. The failure of thymic epithelium from Mls^a mice to delete T cells expressing Vβ6 may, however, be due to a lack of expression of the Mls^a gene product by epithelial cells.

Despite the variable results outlined above, the consensus appears to be that thymic epithelium has at least some tolerizing capability, though not as much as bone-marrow-derived stromal elements. Whether epithelium-induced tolerance primarily involves deletion or some other mechanism remains to be completely resolved. The third thymic element

with potential toleregenic capacity is the thymocyte. Interestingly, Shimonkevitz and Bevan (1988) have shown that $CD4^-8^-$ (Thy-1^+) thymocytes can tolerize to class I but not to class II MHC antigens. Finally, S. Webb (personal communication) has found that *in vivo* treatment of Mls^a mice with anti-IgM antibodies (in order to specifically eliminate B cells) inhibits the deletion of Vβ6, raising the possibility that B cells can enter the thymus and mediate deletional tolerance.

B. Positive Selection

In addition to negative selection, a second selective process appears to operate during thymocyte development which favors T cells capable of responding to foreign antigens in association with self-MHC antigens. The concept that T cell repertoire selection involves a complementarity between TCR and thymic MHC was first proposed by Niels Jerne (1971). The classic experiment to demonstrate this phenomenon showed that minor antigen-specific T cells from an A $\times$ B $\rightarrow$ A radiation bone marrow chimera preferentially lysed targets expressing these minor antigens in association with A-type MHC antigens relative to targets with B-type MHC antigens (Bevan, 1977). Equivalent chimera experiments demonstrated a skewing toward host MHC for MHC-restricted B cell helpers (Sprent, 1978). This phenomenon could not be due to priming, since peripheral APCs expressed both A- and B-type MHC antigens. Subsequent experiments, in which A thymuses were grafted into thymectomized A $\times$ B hosts, demonstrated that the skewing of the repertoire toward self-MHC restriction occurred during intrathymic development (Fink and Bevan, 1978; Zinkernagel *et al.*, 1978; Singer *et al.*, 1982). It should be noted that the concept of positive selection is not universally agreed upon. A handful of studies using fully allogeneic radiation bone marrow chimeras (Matzinger and Mirkwood, 1978) or depleting alloreactive cells with BUdR + light (Ishi *et al.*, 1982) have claimed to reveal significant numbers of T cells restricted to MHC antigens not expressed in the thymus. It could be argued, nevertheless, that all developing T cells are selected on self-MHC but that many cross-react on foreign MHC.

The major mechanistic enigma regarding positive selection is how the system chooses TCRs that will recognize the complex of self-MHC + a foreign antigen not encountered at the time of selection. If T cells recognized nominal and MHC antigens with separate receptors, the process would be simple to understand. The reality is, however, that nominal antigen + MHC are recognized as a complex by a single TCR. The commonly proposed mechanism to account for positive selection presumes that TCRs reactive with self-MHC + X will have at least a low affinity for self-MHC alone. Thus, TCRs with any affinity (low or

high) for MHC antigens expressed on the thymic stroma would be engaged, signaling the thymocyte to either expand or be salvaged from dying. The thymocyte would then pass into a negative selection stage in which a cell bearing TCR with high affinity for thymic MHC antigens would be deleted. The result is that only clones with low affinity for self-MHC (and high affinity for self-MHC + X) survive both positive and negative selection. Such a model is somewhat cumbersome, for it requires the developing thymocyte to pass through two selection stages associated with opposite consequences of TCR engagement.

An alternative version of the low-affinity (positive selection)/high-affinity (negative selection) model has been proposed by Singer and colleagues (1986) based on their observations that differences in MHC class I expression can affect the class II-specific repertoire. They have hypothesized that positive selection results from low-affinity interactions of thymocytes with self-MHC + self nominal antigens. Thus, the T cell repertoire might not only be shaped by thymically expressed MHC antigens but also by thymically expressed nominal antigens.

Finally, Marrack *et al.* (1988b) have proposed a modification of the Singer model which obviates the need for low-affinity versus high-affinity interactions. They have hypothesized that both positive and negative selection are mediated by high-affinity interactions between TCR and self-MHC + self X. Positive selection is proposed to be exclusively mediated by thymic epithelium (see below) which produces an epithelial-specific set of "educating peptides" displayed on epithelial MHC. These peptides must mimic the universe of potential foreign Xs. Negative selection would then be mediated by bone-marrow-derived elements displaying a nonoverlapping set of "deleting peptides" displayed on their MHC. This model requires that no other peripheral APCs display any of these epithelial-specific peptides, or they would become the targets of an autoimmune response. One testable prediction of the epithelial-specific, educating-peptide hypothesis is that antigen-specific clones from a given strain might be stimulated by syngeneic thymic epithelial cells.

The issue of whether the thymic epithelium does, indeed, mediate positive selection is somewhat controversial. The skewing of the repertoire toward thymically expressed MHC in radiation bone marrow chimeras was initially taken as evidence that the radioresistant epithelium mediates this process. Longo and colleagues have argued, however, that these results could be due to T cells selected on residual host hematopoietic elements present for some time after bone marrow reconstitution. They found that, after higher radiation doses and depletion of the first wave of T cells to develop after bone marrow reconstitution, there was a shift in restriction specificity toward donor MHC antigens

(Longo and Schwartz, 1980; Longo and Davis, 1983; Longo *et al.*, 1985). This data was taken as evidence that positive selection is instead mediated by bone-marrow-derived cells. Unfortunately, no other group has reproduced these results. On the contrary, most have found that replacement of host-derived with donor-derived hematopoietic cells is fairly rapid (2-3 weeks), even at standard (~900 R) radiation doses.

The most definitive evidence favoring a critical role for the thymic epithelium in positive selection comes from the dGuo system. When dGuo-treated fetal thymuses from A mice were grafted into thymectomized A × B F_1 recipients, the thymus was recolonized with bone-marrow-derived cells expressing both A- and B-type MHC antigens. Nonetheless, T cells developing in these chimeric thymuses showed preferential restriction of A-type MHC antigens (Lo and Sprent, 1986).

Another thymic selection process, which is probably related to positive selection as classically defined, results in the correlation between CD4 expression and class II restriction, and CD8 expression and class I recognition. *In vivo* anti-MHC treatments have provided powerful evidence that the development of mature CD4 and CD8 single-positive thymocytes requires interaction with thymic class II and class I MHC antigens, respectively. Thus, *in vivo* treatment of neonates with anti-class II mAbs selectively inhibits development of $CD4^+8^-$ thymocytes, whereas *in vivo* treatment with anti-class I mAbs selectively inhibits development of $CD4^-8^+$ thymocytes (Kruisbeek *et al.*, 1985; Marusic-Galesic *et al.*, 1988b). These findings were directly correlated with the classic phenomenon of positive selection by an experiment in which A × B F_1 neonates were treated with mAb specific for either A- or B-type class II MHC. Thymocytes from these treated animals were adoptively transferred into irradiated A × B recipients, primed with keyhole limpet hemocyanin (KLH), and assayed for MHC-restricted B cell helper activity. In F_1 neonates blocked with mAb against A-type class II molecules, the majority of developing KLH-specific helper cells were restricted by B-type class II molecules. In neonates blocked with mAb against B-type class II molecules, the majority of developing KLH-specific helper cells were restricted by A-type class II molecules (Marrack *et al.*, 1988a).

Taken together, these experiments indicate that positive selection results from a requirement of developing thymocytes to engage polymorphic portions of thymic MHC molecules in order to become functionally mature T cells. Coparticipation of the appropriate accessory molecules in this recognition (just as in negative selection) is probably important in "driving" class II-specific thymocytes toward a $CD4^+8^-$ phenotype and class I-specific thymocytes toward a $CD4^-8^+$ phenotype (von Boehmer, 1986). This latter hypothesis is supported by the fact that in

H-2^b female littermates of the H-Y + D^b (class I)-specific TCRα + β transgenic mice (described in Section V,A), the transgenic receptor is found almost exclusively in the CD4^-8^+ subset (Teh *et al.*, 1988). Furthermore, when bone marrow from H-2^b mice bearing the transgenic TCR is used to reconstitute irradiated H-2^k recipients, which do not express the D^b-restricting element on the thymic epithelium, then none of the mature donor-derived thymocytes (neither CD4^+8^- nor CD4^-8^+) express the transgenic receptor on their surface (Kisielow *et al.*, 1988b). Direct evidence for the role of the accessory molecules in this process comes from the fact that *in vivo* treatments with nondepleting anti-CD4 mAbs (i.e., mAbs that do not induce antibody-dependent cell-mediated cytotoxicity (ADCC)-type elimination) nonetheless block the development of CD4^+8^- thymocytes (F. Ramsdell, personal communication).

VI. Conclusions and Frontiers

As with most scientific work, the recent elucidation of lineage pathways and of selection mechanisms involved in T cell development has posed new questions and opened new frontiers. The lineage map in Fig. 5 represents a summary of events for which there is now reasonable consensus based on multiple experimental approaches. Although the time line is based on murine fetal ontogeny, the temporal and lineage relationships appear to be evolutionarily conserved. Some of the important questions that remain to be answered are as follows: (1) What is the molecular nature of the signals that prevent or promote TCR gene rearrangement? (2) How is the coordinate expression of TCR$\alpha\beta$ and CD4/CD8 regulated? (3) Do early TCR$\gamma\delta^+$ cells play a direct role in thymic development? (4) What intracellular signals induce clonal deletion upon interaction of thymocytes with self antigens? (5) Are there nondeletional mechanisms responsible for self-tolerance? (6) What is the mechanism responsible for positive selection, and at which developmental stage does it occur? (7) What is the role of other developmentally regulated surface antigens? Clearly, the thymus is no longer a black box.

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ADVANCES IN IMMUNOLOGY, VOL. 44

Molecular Biology and Function of CD4 and CD8

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I. Introduction

CD4 and CD8 are cell surface glycoproteins expressed primarily on subsets of thymocytes and mature T lymphocytes. These proteins have been referred to as "accessory molecules" because of their role in T cell activation, and as "differentiation antigens" because of their pattern of expression during T cell development. The most immature thymocytes ($\approx 5\%$) express neither CD4 nor CD8 and are referred to as "double negatives." The majority of thymocytes ($\approx 80\%$) express both molecules and are called "double positives." The most mature cells in the thymus express either CD4 ($\approx 10\%$) or CD8 ($\approx 5\%$) and are referred to as "single positives" (Reinherz and Schlossman, 1980; Reinherz *et al.*, 1980b; Ceredig *et al.*, 1983; Dialynas *et al.*, 1983; Mathieson and Fowlkes, 1984; Scollay *et al.*, 1984). Most cells that are double positive die within the thymus (McPhee *et al.*, 1979; Scollay *et al.*, 1984). Although it has been controversial whether mature, single-positive T cells derive directly from double-negative cells or pass through a stage of being double positive, recent data provide strong evidence that at least some of the double-positive thymocytes are the precursors of mature single-positive T cells (Smith, 1987; Kisielow *et al.*, 1988). These double-positive thymocytes also appear to play a role in the induction of tolerance to self-antigens (Kisielow *et al.*, 1988). In general, expression of CD4 or CD8 in the periphery divides mature T cells into two mutually exclusive subpopulations with distinct properties. Early studies suggested that peripheral T cells that express CD4 ($\approx 65\%$) are helper or inducer cells while those that express CD8 ($\approx 35\%$) are cytotoxic or suppressor cells (Cantor and Boyse, 1976; Reinherz *et al.*, 1979a,b, 1980a). However, this subdivision has proved not to be entirely correct, and the best correlation of expression of these surface proteins is with the class of major histocompatibility complex (MHC) protein recognized by the T cell. With few exceptions, CD4 cells recognize or are restricted by class II MHC proteins (HLA-DP, -DQ, or -DR in humans; IA or IE in mice), while CD8 cells recognize or are restricted by class I MHC proteins (HLA-A, -B, or -C in humans;

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H-2 K, D, or L in mice) (Swain, 1981, 1983; Swain *et al.*, 1981; Engleman *et al.*, 1981a,b; Meuer *et al.*, 1982a,b; Krensky *et al.*, 1982a,b; Dialynas *et al.*, 1983). Thus, while most cytotoxic T cells (CTLs) are specific for class I proteins and express CD8, there are some CTLs that are specific for class II MHC proteins and express CD4 (Moretta *et al.*, 1981; Spits *et al.*, 1982; Meuer *et al.*, 1982a,b; Krensky *et al.*, 1982a,b; Biddison *et al.*, 1982; Dialynas *et al.*, 1983). Similarly, there are examples of helper phenotype T cell clones that express CD8 and are specific for class I MHC proteins (Swain and Panfili, 1979; Swain, 1983; Swain *et al.*, 1984). The correlation between CD4 and CD8 expression and the class of MHC protein recognized led to the hypothesis that CD4 and CD8 might be receptors for nonpolymorphic (or relatively nonpolymorphic) regions on class I and class II MHC proteins, respectively (Krensky *et al.*, 1982a; Meuer *et al.*, 1982a,b; Ball and Stastny, 1982; Spits *et al.*, 1982; Biddison *et al.*, 1982; Engleman *et al.*, 1983; Swain, 1983; Marrack *et al.*, 1983; Greenstein *et al.*, 1984). Recent data have provided extremely strong evidence in favor of this notion. In the case of CD8, Rosenstein *et al.* (1988) have demonstrated a direct and specific interaction between human CD8 and class I MHC proteins when each was incorporated into distinct nylon matrix vesicles coated with a lipid membrane. Convincing evidence for binding between human CD4 and class II MHC proteins has been obtained in transfection experiments (Doyle and Strominger, 1987) described below (see Section III,E).

The initial evidence that CD4 and CD8 play a role in T cell function came from studies using antisera and subsequently monoclonal antibodies (Mabs) specific for these proteins. These antibodies were found to block all antigen-driven functions (e.g., cytotoxicity, proliferation, and lymphokine release) by T cells which express the corresponding molecule and are specific for the appropriate class of MHC protein (Shinohara and Sachs, 1979; Nakayama *et al.*, 1979; Hollander *et al.*, 1980; Evans *et al.*, 1981; Reinherz *et al.*, 1981, 1983; MacDonald *et al.*, 1982; Landegren *et al.*, 1982; Engleman *et al.*, 1981a, 1983; Dialynas *et al.*, 1983; Swain, 1983). However, there is clear heterogeneity in the ability of various T cell clones to be blocked (MacDonald *et al.*, 1982; Biddison *et al.*, 1982, 1984; Moretta *et al.*, 1984; Shaw *et al.*, 1985b). In the case of cytotoxicity, anti-CD8 Mabs were found to block the formation of conjugates between class I-specific CTLs and target cells (Hollander, 1982; Bonavida *et al.*, 1983; Tsoukas *et al.*, 1982; Landegren *et al.*, 1982; Platsoucas, 1984). Anti-CD4 Mab could induce dissociation of preformed conjugates between class II-specific, CD4$^+$ CTLs and target cells (Biddison *et al.*, 1984). These results suggested a role for CD8 and CD4 in the recognition step as opposed to the lytic machinery, and led to the hypothesis

that the function of CD4 and CD8 is to stabilize or increase the avidity of the interaction between T cells and antigen-presenting or target cells by binding to class II or class I MHC molecules, respectively (MacDonald *et al.*, 1982; Swain, 1983; Marrack *et al.*, 1983; Biddison *et al.*, 1984; Shaw *et al.*, 1985b). In accord with this idea was the finding that in some cases inhibition of cytotoxicity by anti-CD8 or anti-CD4 could be overcome by the approximation of the CTLs and target cells by lectins (Meuer *et al.*, 1982b; Spits *et al.*, 1982). This hypothesis could also explain the observed heterogeneity in the ability of different T cell clones to be blocked by anti-CD8 or anti-CD4 Mabs, as well as the finding that bulk cultures of CTLs from primary antigen responses could be blocked more easily by anti-CD8 than those from secondary antigen responses (MacDonald *et al.*, 1982; Moretta *et al.*, 1984; Shaw *et al.*, 1985b). As would be predicted by this model, T cells bearing T cell receptor (TCR) molecules with apparently low affinity for antigen/MHC appear to be more dependent on CD4 or CD8 interactions (i.e., their function is more easily blocked) than are T cells with apparently higher affinity TCRs (Marrack *et al.*, 1983; Biddison *et al.*, 1984; Shaw *et al.*, 1985b; van Seventer *et al.*, 1986; Portoles and Janeway, 1988).

More recent studies have suggested that CD4 and CD8 may not be simply cellular adhesion molecules and that they may additionally be involved in pathways of signal transduction. LFA-1 and CD-2 have been shown to play the major role in the initial, antigen-independent formation of conjugates between T cells and other cells (Spits *et al.*, 1986; Springer *et al.*, 1987). While CD8 also plays a role in this process, van Seventer *et al.* (1986) and Blanchard *et al.* (1987) both found that Mab specific for CD8 blocks cytotoxicity not only during the phase of conjugate formation, but also at a postbinding step. Similarly, Mab specific for CD4 was shown to inhibit cytotoxicity by class II-specific $CD4^+$ CTL clones at a postbinding step and had only a small effect upon the initial formation of conjugates (Blanchard *et al.*, 1988). As had been previously observed by Biddison *et al.* (1984), anti-CD4 was able to induce the dissociation of preformed conjugates, although this dissociation was much slower than that induced by anti-LFA-1 Mab and required 37°C incubation, while anti-LFA-1 could dissociate at either 20 or 37°C (Blanchard *et al.*, 1988).

A variety of studies have shown that Mabs specific for CD4 or CD8 can inhibit T cell activation induced by lectins or by Mabs specific for CD3 or the TCR, despite the absence of the appropriate ligand for CD4 or CD8 in these systems (Welte *et al.*, 1983; Hünig, 1984; Bank and Chess, 1985; Wassmer *et al.*, 1985; Beckoff *et al.*, 1985; Fleischer *et al.*, 1986b; Tite *et al.*, 1986; van Seventer *et al.*, 1986). These findings led to the

hypothesis that CD4 and CD8 might function to deliver a negative signal to the T cell, thereby inhibiting activation (Welte *et al.*, 1983; Bank and Chess, 1985; Wassmer *et al.*, 1985; Beckoff *et al.*, 1985; Fleischer *et al.*, 1986b; Tite *et al.*, 1986; van Seventer *et al.*, 1986). In support of this hypothesis, anti-CD4 Mabs have been shown to block the lectin- or antigen-induced rise in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) in a $CD4^+$ T cell clone or hybridoma (Tite *et al.*, 1986; Rosoff *et al.*, 1987) and the anti-CD3-mediated mobilization of $[Ca^{2+}]_i$ in human peripheral blood T cells (Ledbetter *et al.*, 1988). However, there is still no direct evidence for transmission of a negative signal via CD4 or CD8. The studies supporting this model involve Mab blocking as opposed to physiological conditions, and hence steric hindrance must be considered among the possible explanations for the observed results. Furthermore, the mere absence of a positive signal does not imply the presence of a negative signal.

The possibility that Mabs binding to CD4 or CD8 could interfere sterically with T cell activation has become increasingly credible based on the growing body of evidence that there may be a physical association between CD4 or CD8 and the TCR/CD3 complex. Janeway *et al.* (1987, 1988) found that anti-CD4 could inhibit activation of the mouse T cell hybridoma D10.G4.1 (D10) in response to Mabs specific for only one particular epitope of the TCR and not others, suggesting a steric mechanism. Anti-TCR Mabs with high potency (where potency refers to the amount of Mab required to bind to the TCR to achieve half-maximal activation) could co-cap CD4 and the TCR or comodulate both molecules off the surface of the D10 hybridoma, while those with lower (20- to 500-fold) potency could not, despite causing activation (Saizawa *et al.*, 1987; Janeway *et al.*, 1988). Saizawa *et al.* (1987) found that the decrease in cell surface CD4 was approximately 30% under conditions in which the cell surface TCR decreased by about 50%. Based upon knowledge of the number of binding sites for the respective Mabs, they were able to calculate that two molecules of CD4 modulated from the cell surface for each TCR molecule (Saizawa *et al.*, 1987). In a more physiological system Weyand *et al.* (1987) demonstrated that antigenic stimulation resulted in loss of 50% of cell surface CD4 from a human T cell clone. Surface CD4 could also be decreased by treatment with soluble anti-CD3 Mab or with phorbol myristate acetate (PMA), although PMA did not have this effect on D10 cells in the study by Saizawa *et al.* (1987). Similarly Anderson *et al.* (1988) found that modulation of all cell surface CD4 with anti-CD4 Mab followed by goat antimouse immunoglobulin (Ig) resulted in loss of 5% of surface CD3, while modulation of all the surface CD3 with anti-CD3 and goat antimouse

Ig resulted in loss of 10% of surface CD4. Antigen-induced comodulation has also been shown for CD8 and the TCR/CD3 complex. Takada and Engleman (1987) found that incubation of a human T cell clone with the relevant target cell resulted in modulation of 20–30% of the surface CD3 and CD8. Modulation of CD3 could be blocked by soluble anti-CD8 Mab and, similarly, modulation of CD8 could be blocked by soluble anti-CD3 Mab. Anti-CD3 followed by goat antimouse Ig could also modulate both CD3 and CD8. Although comodulation of CD4 or CD8 with the TCR/CD3 complex does not directly imply a physical association, it is consistent with one, and studies of O'Neill *et al.* (1987) suggest that such an association might be physiologically significant, at least in the case of CD4. They found that CD4 and the TCR did not significantly comodulate on the surface of a mouse thymoma cell line, but under capping conditions Mabs specific for either of these two molecules could modulate binding sites for the cell's cognate retrovirus from the cell surface. These results suggested that in this T cell line a small percentage of the surface CD4 and TCR forms complexes (either preexisting or induced) that are involved in retroviral binding. Further evidence for a physical link comes from studies of Kupfer *et al.* (1987) in which CD4 has been found to cocluster with the TCR to the area of intercellular contact between the mouse D10 T cell clone and an antigen-presenting cell bearing the class II/peptide antigen combination recognized by the D10 TCR. This coclustering did not occur when either the class II protein or the peptide antigen was inappropriate.

An association between CD4 and/or CD8 and the TCR/CD3 complex raises the possibility that CD4 and CD8 might be involved directly or indirectly in the transmission of positive signals to activate T cells. In support of this idea is the evidence cited above from Blanchard *et al.* (1987, 1988) that anti-CD8 and anti-CD4 Mabs both inhibit a postbinding step in CTL triggering. CD4 and CD8 have also both been shown to be rapidly phosphorylated on serine residues and then dephosphorylated upon activation of human T cell clones by specific antigen or PMA (Acres *et al.*, 1986, 1987). For both molecules, peak phosphorylation occurs within 10 minutes. CD8 was found to be constitutively phosphorylated in the cells examined, while CD4 was not. Phosphorylation was accompanied by modulation for CD4 but not for CD8. Mab specific for class I or class II MHC proteins could inhibit the antigen-induced phosphorylation of CD8 and CD4, respectively (Acres *et al.*, 1986, 1987). Additional evidence for involvement of CD4 and/or CD8 in signal transduction pathways derives from data that cross-linking of CD4 or CD8 to the TCR/CD3 complex with Mabs can activate resting T cells and induce proliferation under conditions in which anti-CD3 alone does

not (Emmrich *et al.*, 1986, 1988; Anderson *et al.*, 1987; Eichmann *et al.*, 1987; Owens *et al.*, 1987b; Ledbetter *et al.*, 1988). A heteroconjugate Mab specific for CD4 and CD3 has been shown to mobilize $[Ca^{2+}]_i$ in resting $CD4^+$ human peripheral blood T cells at concentrations two orders of magnitude lower than either the anti-CD3 Mab alone or anti-CD3 homoconjugates, and this mobilization could be blocked by pretreatment with free anti-CD4 Mab (Ledbetter *et al.*, 1988). This heteroconjugate could also stimulate synthesis of inositol phosphates (IP_1, IP_2, and IP_3) far better than anti-CD3 alone, and again this stimulation was blocked by soluble anti-CD4 Mab. In contrast, anti-CD4 Mab did not block the changes in phosphoinositol turnover induced by phytohemagglutinin (PHA) or antigen in a mouse T cell hybridoma (Rosoff *et al.*, 1987), although this difference could be cell related. While such results do not imply direct transmission of a signal via CD4 or CD8, they do suggest that these molecules may enhance signal transduction by their association with the TCR/CD3 complex.

As indicated above, the roles of CD4 and CD8 in T cell recognition and activation are not yet fully defined. However, the availability of the cloned genes encoding these molecules has already provided a great deal of knowledge about their structure and function, and future functional studies will be greatly aided by the use of these tools. The balance of this review consists of an attempt to summarize what we have already learned about CD8 and CD4 through molecular genetics, followed by a discussion of how these data fit together with those derived from other approaches and what questions still need to be addressed.

II. Molecular Biology of CD8

A. CD8 Polypeptide Structure and Subunit Composition

The polypeptide structure of the CD8 molecule is dependent both upon species and tissue. Mouse CD8 has been traditionally referred to as Ly-2,3. This molecular complex consists primarily of two forms of heterodimers on the surface of thymocytes: one Ly-2 chain, either α (38 kDa) or α' (34 kDa), is disulfide linked to a Ly-3 (also called β) chain (28-30 kDa) (Ledbetter *et al.*, 1981; Ledbetter and Seaman, 1982; Jay *et al.*, 1982; Walker *et al.*, 1984a,b). Some tetramers containing equimolar amounts of Ly-2 ($\alpha + \alpha'$) and Ly-3 (β) chains are also present on thymocytes (Ledbetter and Seaman, 1982). For the purpose of consistency, the terms Ly-2 and Ly-3 will be used from here on to refer to the antigenic determinants on these polypeptides, while the polypeptides themselves will be referred to using the CD8 nomenclature

(i.e., CD8α, CD8α', and CD8β). The amounts of CD8α and CD8α' chains immunoprecipitated from a total thymocyte population are close to equal. In contrast, if one examines peripheral T cells from spleen or lymph node, very little α' chain is detectable in immunoprecipitates of cell-surface-labeled CD8 complexes (Walker *et al.*, 1984b; Tagawa *et al.*, 1986; Zamoyska and Parnes, 1988). As discussed below, the two chains bearing the Ly-2 determinant (CD8α and CD8α') are the products of alternatively spliced mRNA species from a single CD8α gene, and these two polypeptide chains differ only in their cytoplasmic tail (Zamoyska *et al.*, 1985; Tagawa *et al.*, 1986). The polypeptide bearing the Ly-3 determinant is the product of a distinct but closely linked CD8β gene (Itakura *et al.*, 1972; Gorman *et al.*, 1988). All three of these polypeptides are glycosylated, with three N-linked glycan units on α and α' and one on β (Rothenberg and Triglia, 1983; Leuscher *et al.*, 1985). Two alleles have been defined serologically for both CD8α (Ly-2.1 and Ly-2.2) and CD8β (Ly-3.1 and Ly-3.2). The rat CD8 (OX8) complex similarly consists of heterodimers between two chains (32 and 37 kDa) that have been shown by molecular genetics to be homologous to the mouse CD8α and CD8β chains, respectively (Johnson *et al.*, 1985; Johnson and Williams, 1986). As a result of the glycosylation pattern, the rat β chain is the larger of the two. No equivalent of the mouse α' chain has been described in rat. The structure of human CD8 (originally designated T8 or Leu-2) has appeared to be distinct from that in rodents. Biochemical studies of CD8 on human peripheral blood T cells have indicated that the molecule consists of homodimers (and some higher multimers) of a single 34-kDa polypeptide chain (Snow and Terhorst, 1983; Snow *et al.*, 1983). As discussed below (see Section II,B), this chain could be shown to be the homolog of mouse CD8α (Zamoyska *et al.*, 1985), and will therefore be referred to as human CD8α. This is the polypeptide that is recognized by most anti-human CD8 Mabs (e.g. OKT8, anti-Leu-2a, anti-Leu-2b) (DiSanto *et al.*, 1987). Although the human CD8α polypeptide is glycosylated, the carbohydrate is all O linked rather than N linked (Snow and Terhorst, 1983). On human thymocytes the same apparent homodimer has been identified, but higher multimers have been found to contain an additional disulfide-linked polypeptide of 46 kDa that is not found in the periphery (Snow and Terhorst, 1983). This larger polypeptide has been identified as CD1, a protein of unknown function expressed on human cortical thymocytes and some tumor lines (Ledbetter *et al.*, 1985; Snow *et al.*, 1985). CD1 is related to class I MHC molecules, although it is encoded on a different chromosome (Calabi and Milstein, 1986), and like class I proteins, it was initially identified as a heterodimer noncovalently associated with β_2-microglobulin

(Ziegler and Milstein, 1979). However, the CDl that is disulfide linked to CD8 to form heteromultimers in human thymus is devoid of β_2-microglobulin (Ledbetter *et al.*, 1985; Snow *et al.*, 1985). An association with CDl has not been found for CD8 in mouse or rat thymus. Recently, a noncovalent association between CD8 and class I MHC proteins has been described on the surface of human T cell clones (Bushkin *et al.*, 1988). The physiological significance of such an association is not known. Although it might be tempting to consider that this association could be related to the postulated interaction between CD8 on T cells and class I proteins on target or antigen-presenting cells, one would have to account for the obvious difference in the spatial arrangements of these molecules when they are on the same, as opposed to interacting, cells. Biochemical studies have not been able to detect a human homolog of either mouse CD8α′ or CD8β, but recent molecular genetic and transfection studies have demonstrated the existence of a human CD8β polypeptide (Shiue *et al.*, 1988) (see below).

B. CD8α cDNA Structure and Predicted Protein Sequence

Complementary DNA (cDNA) clones encoding human CD8α were initially isolated by subtractive hybridization techniques using cDNA libraries constructed from mRNA of mouse fibroblasts (L cells) that had been transfected with total human genomic DNA and selected for cell surface expression of CD8 (Kavathas *et al.*, 1984; Littman *et al.*, 1985). The predicted amino acid sequence of human CD8α was then determined from the DNA sequence of these clones (Littman *et al.*, 1985; Sukhatme *et al.*, 1985a). The most striking conclusion to be drawn from the protein sequence was that CD8α is a member of the Ig gene superfamily by virtue of its possession of an amino-terminal domain that is homologous to Ig variable (V) regions, especially light chain V regions. This region contains many of the conserved residues of members of the Ig gene superfamily, including the centrally placed disulfide loop that is so characteristic of Ig homology units. Furthermore, computer analyses of the structural characteristics of this V-like domain suggest that this region can fold in a manner similar to folding of Ig domains (Sukhatme *et al.*, 1985a). CD8α was the first example of a T cell differentiation antigen that was found to be a member of the Ig gene superfamily, a finding that has now been extended to many other T cell surface markers. The predicted CD8α protein has a signal peptide of 21 amino acids followed by a mature protein sequence of 214 amino acids (Littman *et al.*, 1985; Sukhatme *et al.*, 1985a). The external portion of the protein consists of the 96-amino acid V-like domain and a membrane-proximal, 65-amino acid hingelike region or connecting peptide. This is followed by a

24-amino acid hydrophobic transmembrane segment and a highly basic 29-amino acid cytoplasmic tail. Although the sequence suggests the possibility of one N-linked glycosylation site, (Asn-X-Ser or Asn-X-Thr), the presence of proline as the variable residue (Asn-Pro-Thr) most likely accounts for the lack of usage of this site. The protein is predicted to contain nine cysteine residues: three in the V-like region (two of which form the Ig-like disulfide loop) and two each in the hinge, transmembrane region, and cytoplasmic tail. Biochemical studies of Snow and Terhorst (1983) demonstrated that the cysteines in the V-like region are not involved in interchain disulfide bridges. Fujimoto *et al.* (1983, 1984) found that CD8 is released from the surface of human T cell tumor lines as a 27-kDa monomer, indicating that the disulfide bridge(s) most likely involve cysteines within the cytoplasm or the plasma membrane. This conclusion is supported by a recent study in which a hybrid cDNA clone was constructed and transfected, resulting in the cell surface expression of human CD8 with its transmembrane region and cytoplasmic tail replaced by the carboxy-terminal domain of decay accelerating factor, including the portion specifying a glycolipid linkage to the membrane (Tykocinski *et al.*, 1988). Immunoprecipitation of this hybrid protein under nonreducing conditions was consistent with it being a monomer, again indicating that the dimers and higher multimers of CD8 are formed by disulfide linkages involving cysteines in the transmembrane region and/or cytoplasmic tail. Similarly, transfection of a cDNA construct encoding mouse CD8α without its transmembrane region and cytoplasmic tail results in a monomeric polypeptide chain (Zamoyska and Parnes, unpublished results). Furthermore, the α' chain of mouse CD8 contains no cysteine residues in its cytoplasmic tail, implying that it must use one or both of the cysteines in its transmembrane region for disulfide linkage to CD8β.

Mouse cDNA clones homologous to human CD8α were isolated by cross-hybridization to the human clones (Zamoyska *et al.*, 1985; Nakauchi *et al.*, 1985). Zamoyska *et al.* (1985) then isolated and transfected the corresponding mouse gene and could show that the mouse homolog of human CD8α encoded polypeptides bearing the Ly-2 and not the Ly-3 determinant. These studies further demonstrated that the two forms of polypeptide chain that were known to express the Ly-2 determinant, i.e., α and α', were products of that same (hence, CD8α) gene. In accordance with these findings, the isolated mouse cDNA clones could be shown to be of two types: one fully corresponded to human CD8α, while the other had a 31-bp deletion in sequence encoding the cytoplasmic tail (Zamoyska *et al.*, 1985). This deletion shifted the frame, resulting in early termination. The encoded protein would have only 4 rather than

29 amino acids in the cytoplasm, resulting in a size difference consistent with the shorter clone representing the α' chain. Zamoyska *et al.* (1985) found by Sl nuclease mapping that mRNA corresponding to both forms of cDNA was present in normal mouse lymphoid tissues and cell lines. Transfection of the two forms of cDNA clones in expression vectors confirmed that they indeed represented the α and α' polypeptides (Tagawa *et al.*, 1985; Zamoyska and Parnes, 1988). The mature polypeptide chain of mouse CD8α is slightly larger than its human counterpart (220 versus 214 amino acids), but the proteins are quite similar in overall structure and sequence (Zamoyska *et al.*, 1985; Nakauchi *et al.*, 1985). All of the cysteine residues are conserved between the two. The amino-terminal V-like domains of these molecules are the least conserved portions, with only 42% identical residues. However, both are predicted to fold into structures similar to Ig V regions. The connecting peptides contain 59%, the transmembrane regions 79%, and the cytoplasmic tails 55% identical residues. The sequence predicts three N-linked glycosylation sites, consistent with the results of biochemical analysis of the protein. The rat CD8α sequence has also been determined from cDNA clones and is very similar to that of the mouse (Johnson *et al.*, 1985).

C. CD8α Gene Organization and mRNA Splicing Patterns

The CD8α gene organization is consistent with that of other members of the Ig gene superfamily. The mouse CD8α gene consists of five exons which correlate roughly with the functional domains of the protein, although surprisingly there is no intron separating the leader (signal peptide) sequence from the sequence encoding the V-like domain (Liaw *et al.*, 1986; Nakauchi *et al.*, 1987b) (Fig. 1). Significantly, the entire V-like domain is encoded within a single exon (exon I). As in other members of the Ig gene superfamily, the splicing junctions all split codons between the first and second nucleotides (1/2 codon split) with the exception of intron 4, which separates two exons (IV and V) encoding the cytoplasmic tail. The structure of the gene confirmed the hypothesis that the α and α' forms of mouse CD8 mRNA arise from alternative splicing, because the 31 nucleotides missing in α' mRNA constitute exon IV of the gene (Liaw *et al.*, 1986). Therefore, inclusion of that exon results in α mRNA, while splicing from exon III to exon V results in α' mRNA (Fig. 1). Although the difference in size between the mRNA species encoding α and α' is too small to be distinguished by Northern blot analysis, two different sizes of mRNA ($\approx$1.7 and $\approx$3.5 kb) can be seen on Northern blots using a CD8α probe (Zamoyska *et al.*, 1985; Nakauchi *et al.*, 1985). These different species are believed to originate from usage of alternative polyadenylation sites (R. H. Seong and J. R. Parnes,

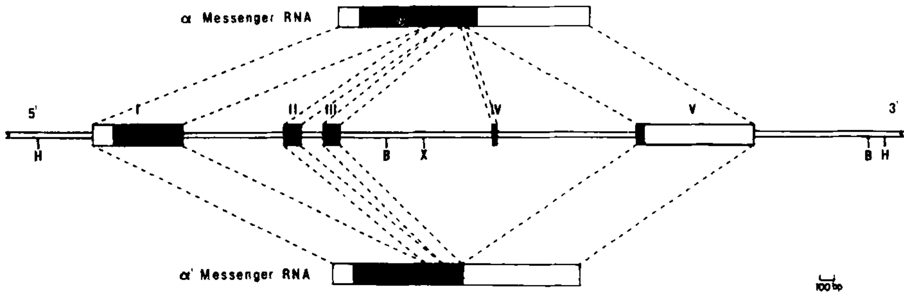

FIG. 1. Structure and alternative splicing patterns of the mouse CD8α gene. The structure of the mouse CD8α (Ly-2) gene is shown in the center of the figure. The exons are shown as boxes and are numbered in Roman numerals. The shaded boxes represent the protein-coding regions and the open boxes represent the 5′ and 3′ untranslated regions. The *Hin*dIII (H), *Bam*HI (B), and *Xba*I (X) recognition site are also indicated. The two forms of mRNA encoded by this gene are shown above (α) and below (α′) the gene. Dotted lines indicate where genomic exons are represented in the two forms of mRNA. Reprinted from Liaw *et al.* (1986) with permission of the publisher.

unpublished results). Nakauchi *et al.* (1987b) have mapped the start site of transcription of the mouse CD8α gene 334–335 bp upstream of the initiation codon. The sequence TATTAA is located 29 bp upstream of the transcription initiation site and may constitute a promoter region (Nakauchi *et al.*, 1987b). The genomic sequence necessary for expression of the 1.7-kb mRNA (and both α and α′ polypeptides) upon transfection is all contained within a 5.4-kb fragment of DNA (Zamoyska *et al.*, 1985), but this fragment is not sufficient for expression of the 3.5-kb mRNA species (R. H. Seong and J. R. Parnes, unpublished results).

The sequences of the two serologically described allelic forms (Ly-2.1 and Ly-2.2) of mouse CD8α have been determined (Liaw *et al.*, 1986). These sequences show very few differences, and a single base pair substitution and consequent amino acid change at residue 78 (Val, Ly-2.1; Met, Ly-2.2) accounts for the difference in serological properties. The human CD8α gene is similar in structure to that of the mouse, with the exception that the human gene, like most other members of the Ig gene superfamily, does contain an intron separating the sequence encoding the signal peptide from that encoding the V-like domain (Littman *et al.*, 1985; Liaw *et al.*, 1986). Although one predominant mRNA species (2.5 kb) is seen on Northern blots of CD8$^+$ human cells (Kavathas *et al.*, 1984; Littman *et al.*, 1985), an alternative form of mRNA splicing has also been identified for human CD8α (Littman, 1987; K. C. Sizer and J. R. Parnes, unpublished results). However, it is not the cytoplasmic

tail that is altered in this instance, but rather the transmembrane region. The inclusion or exclusion of exon IV sequence of the human CD8α gene (corresponding to mouse exon III) does not alter the reading frame of the resultant mRNA, but encodes a protein that either contains or is missing the transmembrane region. It is not known whether this transmembrane-minus form of human CD8α is physiologically secreted from cells *in vivo*, although it can be secreted by transfected cells (Littman, 1987). This form of protein should be distinguished from the 27-kDa single chain fragment of CD8α that has been found to be released from the surface of human T cell tumor lines (Fujimoto *et al.*, 1983, 1984).

The mouse CD8α gene had been known for many years to map to chromosome 6, closely linked to the Ig K light chain locus (Itakura *et al.*, 1972; Gottlieb, 1974; Claflin *et al.*, 1978; Gibson *et al.*, 1978). The isolation of human CD8α probes allowed the chromosomal mapping of that gene to the short arm of chromosome 2 (2p12) at a location closely linked to the human Ig K light chain locus (Sukhatme *et al.*, 1985b). This close linkage to K in both mice and humans supports the hypothesis that CD8α and K derived from a common ancestral precursor. Despite their similarities to Ig genes, the mouse and human CD8α genes are both single copy and do not require rearrangement for expression (Zamoyska *et al.*, 1985; Littman *et al.*, 1985; Sukhatme *et al.*, 1985a).

D. CD8β cDNA Structure, Predicted Protein Sequence, and Gene Linkage

cDNA clones encoding CD8β were first isolated in the rat (OX8, 37 kDa) (Johnson and Williams, 1986). The nucleotide sequence predicted a protein of 187 amino acids, with 141 amino acids external to the cell, a 27-amino acid transmembrane region, and a 19-amino acid highly basic cytoplasmic tail. As in the case of the α chain, CD8β was shown to possess an amino-terminal external domain (101 amino acids) that is homologous to Ig and TCR V regions. Strikingly, this region of the protein is followed by a 12-amino acid segment that is extremely similar to Ig joining (J) segments, with as many as 10 out of 12 residues identical to a human λ J sequence. The Ig-like portions are joined to the membrane by a connecting peptide of 28 amino acids. The protein contains six cysteine residues: two in the V-like region (forming the Ig-like disulfide loop), one in the connecting peptide, two in the transmembrane region, and one in the cytoplasmic tail. Although CD8α and CD8β are both members of the Ig gene superfamily and show similarities throughout, they are not very closely related to one another, with only 21% identical residues in the rat (Johnson and Williams, 1986). The

mouse CD8β cDNA has also been cloned and sequenced by several groups, and is very similar to the rat CD8β (78% identical residues) both in sequence and organization (Nakauchi *et al.*, 1987a; Panaccio *et al.*, 1987; Gorman *et al.*, 1988; Blanc *et al.*, 1988). All of the cysteine residues are conserved. The mouse sequence contains only one N-linked glycosylation site, in contrast to three in the rat. Two predominant mRNA species of mouse CD8β are evident on Northern blots of all mouse strains examined (Nakauchi *et al.*, 1987a; Panaccio *et al.*, 1987; Gorman *et al.*, 1988; Blanc *et al.*, 1988). As in the case of mouse CD8α, these result from usage of alternative polyadenylation sites and do not alter the encoded protein (Gorman *et al.*, 1988). Gorman *et al.* (1988) examined a large panel of mice and noted that the sizes of these mRNA species differ among inbred strains in a striking way depending upon the particular combination of serologically defined alleles expressed at both the CD8α and CD8β loci. Thus, the CD8β mRNA sizes for the three described serological phenotypes are 1.6 and 2.4 kb for mice that are Ly-2.1, Ly-3.1; 1.6 and 2.6 kb for mice that are Ly-2.1, Ly-3.2; and 1.2 and 2.6 kb for mice that are Ly-2.2, Ly-3.2. In transfection studies the mRNA size polymorphisms could be shown to be encoded within the structural gene for CD8β and not to be a function of the mouse strain in which the gene is expressed (Gorman *et al.*, 1988). Consequently, these polymorphisms define three different allelic forms of the mouse CD8β gene. As in the case of human CD8α mRNA, there is also an alternative form of splicing of mouse CD8β mRNA that deletes the sequence encoding the transmembrane region (Gorman *et al.*, 1988). It is not yet known whether this mRNA results in a secreted form of CD8β or what the function of such a protein might be.

Classical genetic studies led to the early conclusion that the genes encoding mouse CD8α and CD8β are closely linked both to each other and, as discussed above, to the Ig K light chain locus on chromosome 6. Gorman *et al.* (1988) have recently linked the mouse CD8α and CD8β genes to each other at the molecular level by chromosomal walking. These two genes are in the same transcriptional orientation and are separated by 36 kb of DNA, with the CD8β gene located to the 5′ side of the CD8α gene (Gorman *et al.*, 1988). The CD8β gene spans approximately 16 kb of DNA, and hence is much larger than the CD8α gene (Gorman *et al.*, 1988; Blanc *et al.*, 1988). Like the CD8α gene, the CD8β gene is single copy and does not require rearrangement for expression (Nakauchi *et al.*, 1987a; Gorman *et al.*, 1988). The distance between these genes and the K locus has not yet been determined.

As discussed above, human CD8 has been thought to consist of homodimers and homomultimers of a single polypeptide chain on

peripheral T cells, and biochemical studies did not reveal the presence of a polypeptide equivalent to mouse and rat CD8β. Johnson (1987) used a rat cDNA probe to demonstrate that there is a human gene homologous to rat CD8β and that mRNA corresponding to this gene is present in human thymus. This gene was further shown to be located on human chromosome 2, suggesting the possibility that the CD8α and CD8β genes might be linked in the human genome as they are in the mouse genome (Spurr *et al.*, 1988). However, the sequence data obtained for the gene were incomplete and did not allow an assessment of whether mRNA transcribed from the gene could be translated into protein. Shiue *et al.* (1988) have recently isolated and sequenced human thymocyte cDNA clones corresponding to CD8β. These clones are fully in frame (with respect to the mouse and rat cDNA sequences) and encode a mature protein of 189 amino acids with 143 residues external to the cell, and transmembrane and cytoplasmic domains of 27 and 19 residues, respectively. The predicted protein is very similar to mouse and rat CD8β, with approximately 56% identical amino acids. The mRNA corresponding to this protein was shown to be present not only in thymus, but also in peripheral blood mononuclear cells (Shiue *et al.*, 1988). A second form of human CD8β cDNA (CD8β′) with a longer cytoplasmic tail (55 amino acids) could be shown to derive from use of an alternative splicing acceptor site for the last exon of the gene, resulting in a frame shift (Shiue *et al.*, 1988). The CD8β cDNA was then inserted into an expression vector and transfected into mouse L cells either alone or together with a human CD8α construct. To determine whether a human CD8β protein was expressed by these transfectants, the cells were stained with an anti-CD8 Mab (2ST8-5H7) which had been shown not to bind to L cells transfected with the CD8α gene (DiSanto *et al.*, 1987). It was reasoned that this Mab might be specific for or require the presence of a CD8β chain. Indeed, 2ST8-5H7 only bound to cells that had been transfected with both CD8α and CD8β constructs, and not cells transfected with either alone (Shiue *et al.*, 1988). This might be because the Mab is specific for a combinatorial determinant expressed only on heterodimers between the two chains, or because CD8β may need to be complexed with CD8α to be expressed on the cell surface (see below). In either case, these findings demonstrate that human CD8β protein can be expressed on the cell structure. Shiue *et al.* (1988) further demonstrated that 2ST8-5H7 positively stains the same population of peripheral blood mononuclear cells as does the anti-CD8α Mab OKT8, indicating that virtually all human peripheral blood cells that have been identified as $CD8^+$ express both CD8α and CD8β. It is not yet known what proportion of CD8 molecules on the surface of human cells are α chain homodimers or homomultimers versus α/β heterodimers or heteromultimers.

E. Regulation of Expression of CD8

Studies to date have shown that mRNA encoding CD8α is found only in cells that express the corresponding protein on the cell surface (Zamoyska *et al.*, 1985; Nakauchi *et al.*, 1985; R. H. Seong, C. W. Liaw, and J. R. Parnes, unpublished results). As will be discussed below, this is not the case for mouse CD8α' or for CD8β. It is likely that the major level of regulation of CD8α expression is transcriptional, but this will need to be confirmed by nuclear run-on assays. Carbone *et al.* (1988, and unpublished results) have shown that sequences at the 5′ end of the mouse CD8α gene are more heavily methylated in cells that do not express mRNA and protein as compared to cells that do.

In contrast to the α chain, the α' chain of mouse CD8 undergoes a developmental alteration in the manner in which its expression is regulated. Although close to equal levels of α and α' have been demonstrated on the cell surface of mouse thymocytes, very little α' is found on the surface of mature peripheral T cells (Walker *et al.*, 1984a; Gallagher *et al.*, 1986; Tagawa *et al.*, 1986; Zamoyska and Parnes, 1988). This is not a result of differences in the way in which the mRNA transcript is spliced in the periphery as compared to the thymus, since the ratio of α to α' mRNA is essentially identical in thymus and peripheral T cells (Zamoyska *et al.*, 1985). Similarly, the ratio of metabolically labeled α to α' polypeptide does not vary between the thymus and the periphery, and the turnover of α' chain is not greater in peripheral T cells as compared to bulk thymocytes (Zamoyska and Parnes, 1988). The α' polypeptide found within peripheral T cells appears to be properly glycosylated and associated with the β chain (Zamoyska and Parnes, 1988). However, a novel sorting mechanism results in only (or predominantly) α/β and not α'/β heterodimers traversing from the Golgi to the cell surface in peripheral T cells (Zamoyska and Parnes, 1988). In contrast, double-positive ($CD8^+$, $CD4^+$) cells in the thymus, and those few single-positive CD8 thymocytes which have an immature phenotype (i.e., $CD8^+$, $CD4^-$, $J11D^+$) (Crispe and Bevan, 1987), do not distinguish between the two forms of CD8 heterodimers, both of which have equal access to the cell surface. The population of $CD8^+$ thymocytes which has a mature phenotype (i.e., $CD8^+$, $CD4^-$, $J11D^-$) is similar to mature peripheral $CD8^+$ cells in restricting cell surface expression to heterodimers containing α as opposed to α' chains (Zamoyska and Parnes, 1988). It is tempting to speculate that α'/β heterodimers might have a function distinct from that of α/β heterodimers, but it should be noted that α' chains have not been described in either humans or rats.

Intracellular expression of CD8β polypeptide chains is regulated at

the mRNA level, as it is for CD8α, but cell surface expression of the polypeptide is regulated at a posttranslational level. Studies in the mouse system have demonstrated that cell surface expression of CD8β requires concomitant expression of CD8α, presumably to form heterodimers (Gorman *et al.*, 1988; Blanc *et al.*, 1988). Gorman *et al.* (1988) found that human CD8α can substitute for the mouse chain to allow cell surface expression of mouse CD8β. It appears that cell surface expression of human CD8β probably also requires CD8α expression, although as discussed above, one cannot yet be certain whether the Mab used for detection of the human protein might require the heterodimer for binding and hence detection of surface expression (Shiue *et al.*, 1988). In contrast, both mouse and human CD8α (and CD8α' in the mouse) can be expressed on the cell surface as homodimers in the absence of CD8β mRNA or protein (Kavathas *et al.*, 1984; Littman *et al.*, 1985; Zamoyska *et al.*, 1985; Tagawa *et al.*, 1986).

F. CD8 Function

The function of mouse CD8 has been explored in a series of gene transfer studies into functional T cell hybridomas. Dembić *et al.* (1986) transferred the genes encoding the α and β chains of the TCR from a mouse CD8$^+$ CTL clone, BDFL1.1.3, specific for D^d plus the hapten fluorescein (FL), into the CD8$^-$ mouse cytotoxic T cell hybridoma SPHI.3, specific for K^k plus the hapten *p*-sulfophenyldiazo-4-hydroxyphenyl acetic acid (SP). While killing by the donor cell (BDFL1.1.3) could be blocked by anti-CD8α Mab, killing by the nontransfected recipient (SPHI.3) could not. After the TCR gene transfer they found that the transfectant, BD7-S17, could not kill targets expressing normal amounts of D^d + FL [i.e., fluoresceinated D^d-expressing lipopolysaccharide (LPS)-induced lymphoblasts], but could kill fluoresceinated L cell targets which had been selected for overexpression of a transfected D^d gene. These results suggested that the affinity of the transferred TCR for D^d + FL was low, and that the absence of CD8 in the recipient cells might be the explanation for the failure to kill target cells expressing normal levels of D^d + FL. The mouse CD8α gene was then transfected into the recipient hybridoma cells, resulting in cell surface expression of the encoded protein(s) (Dembić *et al.*, 1987). The ability to kill LPS-induced lymphoblast targets expressing D^d + FL was indeed restored in BD7-S17-Ly2(2), a cloned CD8α^+ transfectant (Fig. 2). Furthermore, this killing could be blocked by Mab specific for mouse CD8α to the same extent as killing by the original donor cell. These findings demonstrate that the product(s) of the transferred CD8α gene can either increase the avidity of the interaction between a cytotoxic T cell hybridoma and its target, or in some other way enhance the reactivity

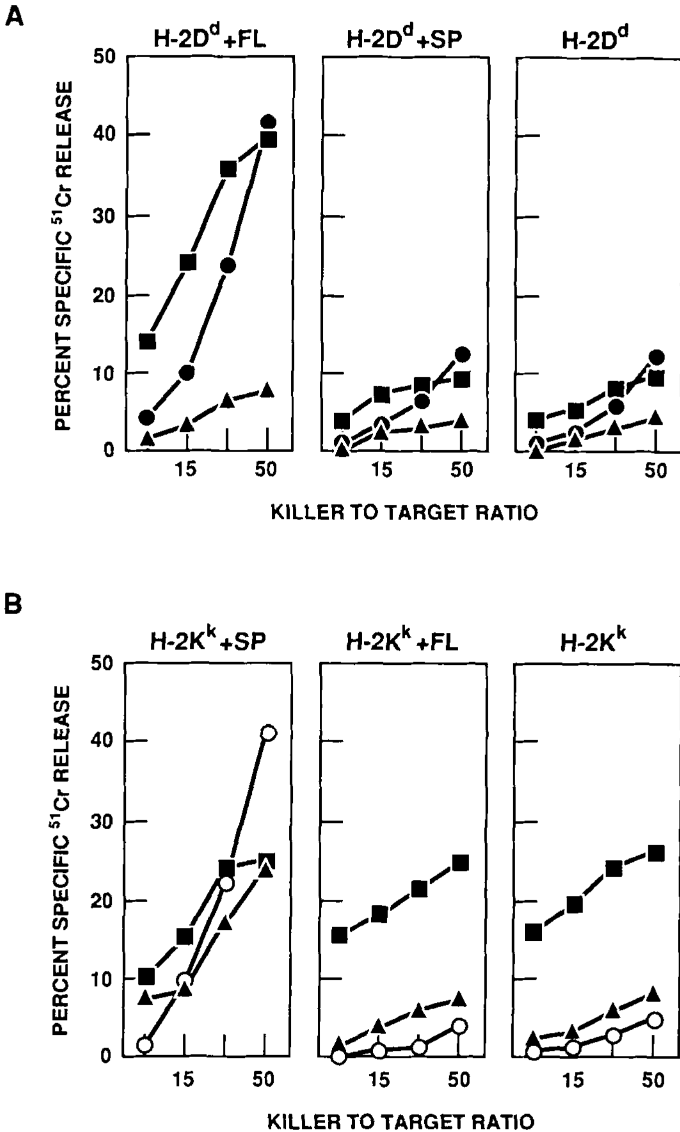

FIG. 2. Cytolytic activity of cytolytic T cell clones on (A) DBA/2 (H-2[d]) and (B) CBA/J (H-2[k]) ^{51}Cr-labeled LPS-induced lymphoblasts. Effectors were (A) ●, BDFL1.13 (donor T cell hybridoma); ▲, BD7-S17 (recipient hybridoma expressing transfected TCR from the donor); ■, BD7-S17-Ly2(2) (recipient hybridoma expressing TCR genes and CD8α gene); (B) ○, SPH/1.3 (nontransfected recipient hybridoma); ▲, BD7-S17; ■, BD7-S17-Ly2(2). Reprinted by permission from Dembić *et al.* (1987), *Nature (London)* 326, 510–511. Copyright © 1987 Macmillan Journals Limited.

of the T cell hybrid so that killing can be accomplished with a lower concentration of cell surface antigen. Dembić *et al.* (1987) also observed a new response to K^k alone in the transfectants that had received the CD8α gene. It was not determined whether this specificity involves usage of the TCR from SPH1.3, which typically recognizes K^k plus the hapten SP, or whether it involves a hybrid TCR using some combination of the various TCR α and β chains within the transfectant. In either case, though, the response to isolated K^k was dependent upon the expression of CD8α. These data illustrate the ability of CD8α not only to decrease the required density of antigen for T cell activation, but also to facilitate responses to antigens that cannot trigger the specific T cell in the absence of CD8α.

These conclusions have been extended to a mouse helper hybridoma system by Gabert *et al.* (1987) (Table I). In these studies the α and β chain genes of the TCR from a CTL clone (KB5-C20) specific for the allogeneic class I MHC molecule K^b were transfected into a $CD4^+$ T cell hybridoma (DO-11.10) specific for Ia^d plus ovalbumin. As in the experiments described above, the donor CTL clone, KB5-C20, was $CD8^+$ and its cytotoxic function was blockable by Mab specific for mouse CD8α, while the recipient hybridoma, DO11.10, was $CD8^-$, and its normal function [as measured by interleukin-2 (IL-2) release] was not blocked by anti-mouse CD8 Mab. As shown in Table I, transfer of the KB5-C20 TCR genes alone (transfectant DC27.15) did not result in transfer of the ability to respond to the antigen K^b as presented by either spleen cells or L cells expressing a transfected K^b gene (Gabert *et al.*, 1987). However, the transfected TCR genes were expressed in a functionally relevant manner in that an antiidiotypic Mab, Désiré-1, specific for the transfected TCR could stimulate release of IL-2 by the transfectants when bound to Sepharose beads. In contrast, when the CD8α gene was transfected into the recipient cells in addition to the TCR genes (transfectants DC41.8 and DC43.12), the resulting cloned transfectants could respond to K^b presented either on spleen cells or on transfected L cells by releasing IL-2 (Table I). Furthermore, this response could be blocked by Mab specific for CD8α, but not by Mabs specific for either Thy-1 or mouse CD4 (Gabert *et al.*, 1987). These results extend the previous study by showing that the functional requirement for CD8α is independent of the particular type of T cell, but rather is dependent on the specific TCR (and the antigen being recognized by this TCR). In this instance the KB5-C20 TCR could not functionally respond to antigen (K^b) in the absence of CD8α or when CD8α was blocked by Mab, independent of whether it was in a CTL or a helper hybridoma. The most likely explanation is that the affinity of this TCR is too low for it to be stimulated by its specific antigen in the absence of an interaction between CD8α and its ligand (class I MHC).

TABLE I
IL-2 SECRETION BY TRANSFECTED CELLS IN RESPONSE TO STIMULATION[a]

Transfected responder cells			IL-2 Secreted (units/ml) in response to stimulants						
Name	Parental cell	Gene(s) transfected	Ltk$^-$	CA60.6.4 (K^b-positive L cell transformant)	CBA/J splenocytes (H-2^k)[b]	B10.MBR splenocytes (K^b, I^k, D^q)[b]	F23.1-Sepharose[c]	Désiré-1-Sepharose[c]	Leucoagglutinin
DO-11.10	–	None	<7.5	<7.5	<7.5	<7.5	>135	<7.5	>135
DC25.11	DO-11.10	CD8α	<7.5	<7.5	<7.5	<7.5	>135	<7.5	>135
DC27.10	DO-11.10	KB5-C20 TCR	9	<7.5	9.5	<7.5	18	>135	>135
DC41.8	DC25.11	KB5-C20 TCR (CD8α)[d]	<7.5	48	9.5	122	21	>135	>135
DC43.12	DC27.10	CD8α (KB5-C20 TCR)[d]	14	>135	<7.5	>135	41	>135	>135

[a]Cells were transfected with the CD8α and/or T cell receptor genes. Stimulation was via H-2K^b-transfected L cells, irradiated spleen cells, or anticlonotypic antibodies. The amounts of IL-2 produced by the T cell hybridomas were assessed after 24 hours. Results were expressed as units of IL-2 per milliliter of undiluted supernatant. The minimum detectable amount in this assay was 7.5 units/ml. Reprinted from Gabert *et al.* (1987) with permission of the publisher.

[b]Irradiated, T-depleted spleen cells.

[c]Mab coupled to Sepharose beads at 3 mg/ml of beads.

[d]Parentheses indicate that these genes were already transfected into the cells.

Similar findings have been obtained for enhancement of T cell responses by gene transfer of human CD8α. Ratnofsky *et al.* (1987) studied a mouse T cell hybridoma, BY155.16, which produces IL-2 in response to human class II HLA-DR antigens. The human CD8α cDNA was transferred into this cell line by use of a retroviral expression vector, resulting in cell surface expression of human CD8α. All three resulting CD8α^+ hybridoma lines responded to human JY (HLA-DR$^+$) stimulator cells with enhanced IL-2 production (about 10-fold), and IL-2 secretion could be detected with 10-fold fewer stimulator cells. This enhanced response could be blocked by Mabs specific for human CD8α or for the class I MHC molecules expressed by the JY stimulator cells. Furthermore, the response to Daudi cells, which express human HLA-DR but lack cell surface class I MHC proteins, was increased no more than 2-fold in hybridomas expressing CD8α, as compared to 10-fold when stimulators expressing class I proteins (i.e., JY) were used. Therefore, expression of class I MHC molecules by the stimulators is important for the enhancement effect mediated by CD8.

Notably the findings in all three sets of experiments indicate a positive function for CD8α; expression of the gene either increases the magnitude of measured T cell responses, or results in new responses that do not occur at measurable levels in its absence. Although one cannot conclude that a positive signal is being transmitted through the CD8α molecule, one can conclude that in these systems the function is clearly not to transmit a negative signal and turn off a response. Finally, the functional activity being measured in all three systems is in the absence of the CD8β chain. It remains to be determined whether the physiologically more relevant heterodimers between CD8α and CD8β polypeptide chains might either function better or differently than CD8α homodimers.

These studies do not address the issue of whether a direct interaction is necessary between CD8 and the TCR, or whether CD8 must bind to the same or different class I molecules as the TCR. However, the system studied by Ratnofsky *et al.* (1987) involves human CD8 and class I MHC molecules and a mouse TCR. Therefore, if a direct interaction between the TCR and CD8 is necessary, this can occur across species in the combination mouse TCR/human CD8.

III. Molecular Biology of CD4

A. CD4 Polypeptide and cDNA Structure

The CD4 molecule was first identified on rat cells by use of Mab W3/25 (Williams *et al.*, 1977; White *et al.*, 1978; Bernstein *et al.*, 1980) and subsequently on human cells (where it was originally designated T4 or

Leu-3) by use of Mab OKT4 (Reinherz *et al.*, 1979a,b; Terhorst *et al.*, 1980). Studies of the homologous protein in the mouse (originally designated L3T4) were delayed until the generation and characterization of the rat antimouse Mab GK1.5, which is specific for this T cell surface protein (Dialynas *et al.*, 1983). Homologs have also been described in sheep (Classon *et al.*, 1986b), pig (Pescovitz *et al.*, 1985), and chicken (Chan *et al.*, 1988). The CD4 molecule is a glycoprotein of approximately 55 kDa, which, in contrast to CD8, immunoprecipitates as a single polypeptide chain (Terhorst *et al.*, 1980; Dialynas *et al.*, 1983; Classon *et al.*, 1986a,b; Chan *et al.*, 1988). However, in sheep a small percentage of CD4 immunoprecipitates as a disulfide-linked homodimer (Classon *et al.*, 1986a,b). It is not known whether this is a physiological form of the protein or an artifact created *in vitro*.

cDNA clones encoding human CD4 were isolated in much the same way as human CD8 clones (Maddon *et al.*, 1985). Mouse (Tourvieille *et al.*, 1986; Littman and Gettner, 1987) and rat (Clark *et al.*, 1987) cDNA clones were subsequently isolated by cross-hybridization to human probes. From the nucleotide sequences of these clones, the mature CD4 polypeptide chain is predicted to be 435 amino acids in humans and 431 amino acids in mice, and is preceded by a 23- or 26-amino acid signal peptide in each of these two species, respectively. The extracellular portion of CD4 consists of 374 amino acids in humans and 368 amino acids in mice. This is followed by a hydrophobic transmembrane segment (26 amino acids in humans, 25 in mice) and a highly basic cytoplasmic tail (38 amino acids in both species). The human molecule has two potential N-linked glycosylation sites, while the mouse has four. Each has six cysteine residues external to the cell, and biochemical studies on both mouse and sheep CD4 have shown that these form three intrachain disulfide loops connecting adjacent cysteines (Classon *et al.*, 1986a). There are six additional cysteines in human CD4 (two in the transmembrane region and four in the cytoplasmic tail) and seven additional cysteines in mouse CD4 (four in the transmembrane region and three in the cytoplasmic tail) (Maddon *et al.*, 1985; Tourvieille *et al.*, 1986). The cytoplasmic tail is the most highly conserved domain of CD4 between mouse and man, with 79% identical residues, while the external domains share only 55% identical residues between these two species (Tourvieille *et al.*, 1986).

Comparisons of the predicted protein sequence of CD4 indicated that, like CD8α and CD8β, it too is a member of the Ig gene superfamily, although it is not as similar to true V regions as the homology units of the CD8 chains (Maddon *et al.*, 1985; Tourvieille *et al.*, 1986; Littman and Gettner, 1987). The amino terminal domain of CD4 (approximately 100 amino acids) is the part of the protein most closely related to Ig V regions (up

to 35% identical residues to certain K light chain V regions), and it contains the two cysteine residues characteristic of Ig homology units. Computer predictions of secondary structure potential suggest that this region may fold in a fashion very similar to folding of Ig and TCR V regions. This V-like region is followed by a short sequence that is similar to Ig J segments, although it is not nearly as closely related as that in CD8β. In contrast to the structure of the CD8 polypeptides, CD4 contains a second V-like domain (V′) just downstream of the J-like segment (Tourvieille *et al.*, 1986; Clark *et al.*, 1987). However, this domain is severely truncated, maintaining only a sequence related to the carboxy-terminal half of Ig V regions. Although this region contains two cysteine residues, the first of the two is not within the region that is clearly related to Ig V regions and the disulfide loop between the two spans only 30 amino acids. Comparisons to other Ig gene superfamily members indicate that this foreshortened domain is most similar to the analogous region of the amino-terminal or primary V-like domain of CD4. These results suggest that the V′ sequence may have been derived from a duplication of at least part of the amino-terminal V-like domain, or a duplication followed by deletion of sequence encoding the amino-terminal region of the homology unit. The V′ domain of CD4 is also followed by a J-like sequence (J′) (Tourvieille *et al.*, 1986). Sequence comparisons indicate that this second J-like sequence is the more convincing of the two, at least in the mouse, and it is followed by an intron in the appropriate location for Ig and TCR J segments. In contrast, the sequence of the J-like segment following the amino-terminal V-like domain is less closely related to Ig J segments, less conserved between species, and is interrupted at the DNA level by an intron (Parnes and Hunkapiller, 1987). The carboxy-terminal half of the external domain of mouse CD4 has been referred to as a connecting peptide (Tourvieille *et al.*, 1986). It has been suggested that this portion of the molecule may be constructed of two additional, albeit degenerate, V-like domains (Clark *et al.*, 1987). The more membrane distal of these two has no cysteine residues, while the membrane-proximal domain has a foreshortened disulfide loop (spanning only 43 amino acids in the mouse protein). If this connecting peptide region is derived from ancestral precursors of the Ig gene superfamily, it is clear that it has diverged far more than the amino-terminal portion of CD4 (Parnes and Hunkapiller, 1987). Williams *et al.* (1987) have noted that the membrane-proximal (fourth external) domain and transmembrane region of CD4 are also distantly related to their respective counterparts in another T cell surface molecule, CD2. CD2 may be another member of the Ig gene superfamily, as its amino-terminal domain also shows a low level of sequence similarity

to the amino-terminal domains of CD4 and other Ig-like domains, although it lacks the conserved cysteine residues (Williams *et al.*, 1987).

B. CD4 Gene Structure

CD4 is encoded by a single gene on human chromosome 12 (Kozbor *et al.*, 1986; Isobe *et al.*, 1986) and mouse chromosome 6 (Field *et al.*, 1987). Despite the fact that it is located on the same chromosome as several other members of the Ig gene superfamily [i.e., the TCR β chain locus (Lee *et al.*, 1984; Caccia *et al.*, 1984) and the linkage group including CD8α, CD8β, and K], the CD4 gene is not closely linked to any of these markers (Seldin *et al.*, 1988). As is the case for CD8α and CD8β, rearrangement of the CD4 gene is not required for expression (Maddon *et al.*, 1985; Tourvieille *et al.*, 1986). The mouse CD4 gene spans 26 kb of DNA and is composed of 10 exons (Gorman *et al.*, 1987) (Fig. 3). One unusual, although not unique, feature of this gene is the presence of an intron (8.6 kb) in the 5′ untranslated region. However, the most striking and unexpected finding was that a large intron (6.4 kb) divides the sequence encoding the amino-terminal V-like domain into

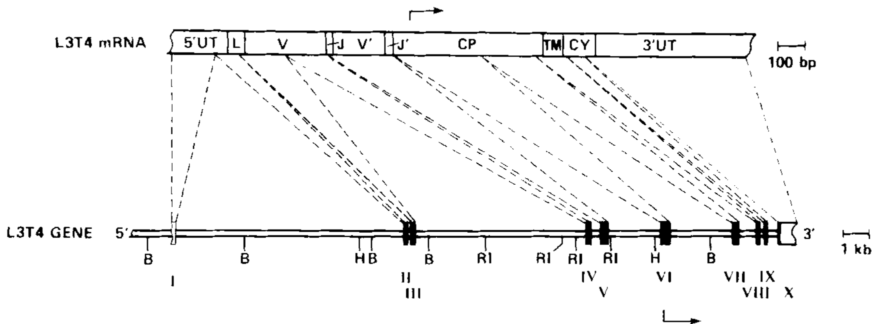

FIG. 3. Structure of the mouse CD4 gene. Bottom: the structure and partial restriction map of the mouse CD4 (L3T4) gene. The exons are indicated by boxes and are numbered with Roman numerals. Shaded boxes indicate protein-coding regions and open boxes represent 5′ and 3′ untranslated regions. Top: dashed lines indicate where the exons of the gene are represented along the structure of the mouse CD4 (L3T4) mRNA. The portions of the mRNA encoding the previously identified protein domains are indicated along the mRNA structure: UT, untranslated region; L, leader, V and V′, sequences homologous to immunoglobulin V regions; J and J′, sequences homologous to immunoglobulin J segments; CP, connecting peptide; TM, transmembrane region; CY, cytoplasmic tail. Arrows indicate the start site of the smaller mouse CD4 mRNA species found in brain. Restriction endonuclease recognition sites: B, *Bgl*II; H, *Hin*dIII; RI, *Eco*RI. Reprinted from Gorman *et al.* (1987).

two exons (exons III and IV) approximately half-way through the predicted protein domain (Gorman *et al.*, 1987; Littman and Gettner, 1987). Only one other member of the Ig gene superfamily, neural cell adhesion molecule (N-CAM) is known to have an intron within sequence encoding a homology unit, and this is the case for all five homology units of N-CAM (Owens *et al.*, 1987a). Like CD4, N-CAM also has an intron in its 5′ untranslated region (Owens *et al.*, 1987a). Despite these similarities, the nature of the evolutionary relationship between CD4 and N-CAM is uncertain (Parnes and Hunkapiller, 1987). The remainder of the intron/exon structure of CD4 correlates well with the predicted protein domains (Gorman *et al.*, 1987; Maddon *et al.*, 1987), as is characteristic for most members of the Ig gene superfamily. Interestingly, the connecting peptide region, which (as described above) may contain two additional domains ancestrally related to Ig V regions, is appropriately split between two exons (Fig. 3). As is true for other members of the Ig gene superfamily, introns interrupt codons between the first and second nucleotides (1/2 codon split) in all cases except between the exons encoding the evolutionarily unrelated cytoplasmic tail (Gorman *et al.*, 1987). The human CD4 gene structure has also been determined and is quite similar to the mouse, with the exception that an intron has not been described in the 5′ untranslated region (Maddon *et al.*, 1987). However, since the start of transcription of the human gene has not yet been characterized, it is quite possible that the full extent of the 5′ untranslated region is not known and that an analogous intron is present. The gene spans at least 33 kb of DNA (Maddon *et al.*, 1987).

C. CD4 Expression

In the mouse, CD4 protein has only been identified on thymocytes and mature T lymphocytes. Rat (Jefferies *et al.*, 1985) and human (Wood *et al.*, 1983; Moscicki *et al.*, 1983) CD4 have additionally been identified on macrophages and the related Langerhans cells, but this does not appear to be true in the mouse (Crocker *et al.*, 1987). The cellular distribution of CD4 mRNA has been examined in both mouse and human cells. Tourvieille *et al.* (1986) identified a 3.7-kb mouse CD4 mRNA species in thymus, spleen, and lymph node and in T cell lines that express CD4 protein. Expression of this mRNA was not detected in mouse liver, B cell lines, a macrophage cell line, F9 teratocarcinoma cells, or a rat glial cell line. A very small amount of this mRNA species was seen in mouse kidney, but it could not be excluded that this might be the result of expression by contaminating T cells.

Although CD4 protein has not been identified in mouse brain, Tourvieille *et al.* (1986) found that this tissue expresses low levels of a smaller

(2.7 kb) polyadenylated CD4 mRNA species. Maddon *et al.* (1986, 1987) detected low levels of this smaller mRNA in mouse forebrain from embryonic day 15 and on, with a sharp rise and sustained higher levels beginning on postnatal day 11. In the adult mouse forebrain the smaller CD4 mRNA was identified in cortex and, at higher levels, in striatum (Maddon *et al.*, 1987). No CD4 mRNA was detectable in midbrain, hippocampus, cerebellum, medulla, pituitary, or hindbrain. The particular cell type(s) that expresses this smaller mRNA species has not yet been identified. Gorman *et al.* (1987) used Northern blotting and S1 nuclease analysis to demonstrate that this mRNA contains sequences only from the 3′ half of the mouse CD4-coding sequence and the 3′ untranslated region. The start site could be mapped approximately to the sequence encoding amino acid 200 (±10 nucleotides). This site does not correlate with an exon/intron junction, but rather lies within the protein-coding sequence of exon VI (Fig. 3). The brain-specific transcript then continues colinearly with the T cell form of mouse CD4 mRNA. Although it could not be excluded that there might be a small amount of noncontiguous sequence at the 5′ end, these findings indicated that the smaller transcript could not be the product of a simple alternative pattern of mRNA splicing, and rather that it is most likely the product of an alternative transcriptional start site. In accord with this conclusion, the sequence TATAA is located also within exon VI, 32 bp upstream of the predicted start site, and may well serve as a promoter that is specifically regulated in brain (Gorman *et al.*, 1987). The first methionine codon (ATG) downstream from the predicted start site is located 43 bp more 3′ and would be the predicted initiation site for translation. This ATG is in the same frame as that of the full-length, T cell form of mouse CD4 mRNA. The predicted protein from the brain transcript would therefore be a truncated form of mouse CD4 beginning at amino acid 214 (within the connecting peptide) and continuing through the normal cytoplasmic tail. It would be missing the normal signal peptide and all of the sequences that are most similar to Ig V regions and J segments, i.e., the portion of the mature protein that is most likely involved in receptor function. Without a typical signal sequence, it is not known what the fate of such a protein would be. It is possible that another internal sequence could act in this capacity, or that the protein is not expressed on the cell surface. In the latter instance it might either be maintained within the cell or degraded. In any event, the predicted protein would certainly not function in the same manner as the full-length CD4 molecule on T cells.

In contrast to findings in the mouse, human brain tissue has been found to express not only a smaller form of CD4 transcript, but also

readily detectable quantities of full-length CD4 mRNA that comigrates with human T cell CD4 transcripts (Maddon *et al.*, 1986). Although the equivalent TATAA sequence that may promote transcription of the smaller transcript in mouse brain is conserved in the human CD4 gene, it is not yet known whether the shorter CD4 transcript in human brain is analogous to that seen in the mouse. If it is, it could not be translated into a protein similar to that predicted from the mouse sequence, because the equivalent methionine codon is absent (Maddon *et al.*, 1985). The expression of CD4 mRNA in human brain is of great interest because of the role human CD4 as the receptor for human immunodeficiency virus-1 (HIV-1), the retrovirus that causes acquired immune deficiency syndrome (AIDS) (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1986; Maddon *et al.*, 1986). Central nervous system involvement, especially a subacute encephalopathy, occurs with high frequency in patients with AIDS (Snider *et al.*, 1983; Johnson and McArthur, 1986), and HIV-1 can be isolated from the brain of affected patients (Ho *et al.*, 1985; Shaw *et al.*, 1985a; Levy *et al.*, 1985; Epstein *et al.*, 1985; Koenig *et al.*, 1986; Wiley *et al.*, 1986). It is most likely that receptors for HIV-1 in human brain result from translation of the full-length CD4 transcript. In accord with this, Mabs specific for human CD4 have been shown to stain human brain sections (Pert *et al.*, 1986; Funke *et al.*, 1987). Since human macrophages are known to express CD4 protein, they are most likely the predominant class of CD4$^+$ cells in brain. This is supported by data demonstrating the presence of HIV-1 mainly within mononuclear and multinucleated macrophage-like cells in the brains of patients with AIDS involving the central nervous system (Epstein *et al.*, 1985; Koenig *et al.*, 1986; Wiley *et al.*, 1986). However, CD4 may also be present on at least some neuronal and glial cells in human brain (Funke *et al.*, 1987), and on rare occasions HIV-1 has been observed in astrocytes in the brains of AIDS encephalopathy patients (Epstein *et al.*, 1985; Wiley *et al.*, 1986). *In vitro,* HIV-1 has been shown to infect some glioma cell lines and normal brain-derived cell cultures, and infection usually but not always correlated with detectable cell surface expression of CD4 (Cheng-Mayer *et al.*, 1987; Chiodi *et al.*, 1987; Dewhurst *et al.*, 1987a,b). In the case of the exceptions the level of surface expression may have been below that detectable by staining, and CD4 mRNA expression was not examined in all cases. HIV-1 also appears to infect capillary endothelial cells in the brain of AIDS patients, although expression of CD4 has not been described on these cells (Wiley *et al.*, 1986).

As expected from protein studies, human macrophages have been shown to express full-length CD4 mRNA (Maddon *et al.*, 1987). Similarly, some Epstein–Barr virus-transformed human B cell lines express CD4

mRNA (Maddon *et al.*, 1987) and detectable protein (Dalgleish *et al.*, 1984). However, full-length CD4 transcripts have also recently been demonstrated in human granulocytes, which are not known to express cell surface CD4 (Maddon *et al.*, 1987). The physiological role (if any) of CD4 on non-T cells is unknown.

D. CD4 Function

Gene transfer studies using CD4 cDNA constructs in expression vectors have provided experimental data that verify much of what had been inferred about CD4 function from Mab blocking studies. Doyle and Strominger (1987) have used the human CD4 cDNA to provide the strongest evidence to date for physical binding between CD4 and class II MHC molecules. CV1 fibroblasts expressing large amounts of human CD4 (10–15 times the number of molecules on the human T cell line HPB-ALL) were derived by infection with a recombinant SV40 virus vector containing the human CD4 cDNA driven by the SV40 late promoter. Four B cell lines expressing class II MHC molecules could be shown to bind directly to monolayers of these CD4$^+$ CV1 cells, but not to monolayers of cells infected with wild-type SV40 virus. Independently isolated class II$^-$ mutants of two of these B cell lines (Raji and T5-1) and other class II$^-$ B cell lines did not bind to the CD4$^+$ CV1 monolayers. The specificity of this binding for the CD4 and class II MHC molecules was confirmed by its complete inhibition by either anti-CD4 Mab or a mixture of anti-class-II Mabs. This study further indicates that the interaction between CD4 and class II that is responsible for this adhesion can take place in the absence of the TCR.

Sleckman *et al.* (1987) used the same system described above for their studies of CD8α function to investigate the role of CD4 in T cell activation. In this case the mouse T cell hybridoma By155.16, which produces IL-2 in response to HLA-DR (class II MHC) molecules on stimulator cells, was infected with a defective retrovirus vector containing the human CD4 cDNA. Cell lines expressing human CD4 were selected and found to respond to HLA-DR$^+$ stimulator cells with a 6- to 20-fold increased level of IL-2 release as compared to the CD4$^-$ parental line. This enhanced responsiveness could be blocked to near basal levels by addition of the anti-CD4 Mabs OKT4F or anti-Leu-3a, while Mab OKT4 did not inhibit as well. The human CD4$^+$ lines could also respond with detectable IL-2 release to 30-fold fewer stimulator cells than the parental line. These data provide strong support for the hypothesis that CD4 increases the avidity of the interaction between the T cell and class II MHC$^+$ stimulator cells, or in some other way enhances the signal transduction in such interactions. Interestingly, the anti-mouse CD4 Mab

GK1.5 did not block the response of either the human CD4$^-$ parental line or a human CD4$^+$ infected line, despite the fact that these lines express mouse CD4. These results suggest that the mouse CD4 molecule does not interact with the human stimulator cells in the same manner as does human CD4, and may imply an inability of mouse CD4 to (productively) bind to human class II molecules. More direct experiments will be necessary to test the latter interpretation.

Using the same system, Sleckman *et al.* (1988) have recently demonstrated that elimination of 31 of the 38 amino acids of the cytoplasmic domain of human CD4 (including all potential phosphorylation sites) does not affect the ability of the molecule to enhance responsiveness of Byl55.16 infectants to JY or Daudi cell stimulators. This implies that the cytoplasmic tail of human CD4 is not critical for function. Infectants expressing the deleted CD4 construct or full-length CD4 also showed enhanced responsiveness to HLA-DR presented on liposomes, but in this case a much higher level of expression of the deleted form (10-fold was examined) was required to see the enhancement. While it is unclear what role the cytoplasmic tail plays in this system, it must be considered that it may be involved in interactions that affect mobility of the molecule (and hence its ability to interact with the TCR) rather than in transmission of a signal. At the very least it can be concluded that not all functions of CD4 in enhancing T cell responses require the presence of the cytoplasmic tail.

Gay *et al.* (1987) have drawn similar conclusions concerning the ability of human CD4 to enhance responsiveness in the presence of class II MHC molecules using another xenogeneic system. They studied the IL-2 response of an unusual mouse CD4$^+$ T cell hybridoma, 3DT-52.5, which is specific for the mouse class I MHC molecule H-2D^d (Endres *et al.*, 1983). A CD4$^-$ variant of this hybridoma, 3DT-52.5.8, was derived by single-cell cloning, and a subclone of the latter, 3DT-52.5.8/S4, bearing much lower levels of the TCR was isolated (Gay *et al.*, 1987). The human CD4 cDNA was introduced into 3DT-52.5.8 by use of a retroviral expression vector. Infected cells expressing human CD4 were found to have decreased levels of TCR expression, in two cases equivalent to that of 3DT-52.5.8/S4. The responses (IL-2 release) of both these human CD4$^+$ infectants and 3DT-52.5.8/S4 to P815 (H-2D^d) stimulator cells were very low as compared to that of the "parental" mouse CD4$^-$, high-TCR-expressing 3DT-52.5.8. However, while the latter line and the low-TCR variant 3DT-52.5.8/S4 did not vary in their responses to P815 and to transfectant P815 cells that express cell surface human HLA-DR, the human CD4$^+$ infectants produced much greater levels of IL-2 in response to the HLA-DR$^+$ transfectant P815 cells. A third human

CD4$^+$ infectant which expressed almost undetectable levels of TCR and had no IL-2 response to P815 was able to respond well to the HLA-DR$^+$ P815 cells. As expected, Mab specific for H-2D^d could block the responses of all of these cells to either P815 or HLA-DR$^+$ P815. However, Mab specific for human CD4 or HLA-DR could only block when the appropriate molecule was expressed on the cell surface and an enhanced response was present. These results support the hypothesis that CD4 interacts with class II MHC molecules and that this interaction enhances T cell activation. Again the mechanism of this enhancement has not been elucidated. Importantly, in this study the TCR is specific for a class I and not a class II MHC molecule, and therefore the enhancement mediated by human CD4 cannot be the result of binding to the same MHC molecule as the TCR. This does not exclude the possibility that responses might be enhanced to an even greater extent when the TCR and CD4 can bind to the same MHC molecule. Finally, if an interaction is required between the TCR/CD3 complex and CD4, this interaction must be able to occur between mouse TCR/CD3 and human CD4.

Similar conclusions regarding the function of mouse CD4 have been obtained in a totally syngeneic system (Ballhausen *et al.*, 1988). Reske-Kunz and Ruede (1985) had generated a series of mouse T cell hybridomas specific for beef insulin on H-2^{bxk} antigen-presenting cells, and found upon subcloning that they fell into two classes. Some of the clones had a high reactivity (as measured by IL-2 release) to beef insulin and a lower level cross-reactivity to pork insulin. Clones with this reactivity pattern all expressed CD4. In contrast, other clones had a lower level of response to beef insulin and did not respond to pork insulin. Such clones lacked cell surface expression of CD4. Ballhausen *et al.* (1988) investigated the question of whether the difference in reactivity patterns was a result of the presence or absence of surface CD4. One of the CD4$^-$ clones (BI-R), which expressed no CD4 mRNA, was transfected with the mouse CD4 cDNA in an expression vector, and transfectants expressing cell surface CD4 were selected. A cloned transfectant (BI-T) showed a markedly increased response (IL-2 release) to beef insulin on the appropriate antigen-presenting cells, requiring 50-fold lower concentrations of beef insulin than the recipient (BI-R) clone (Fig. 4). Furthermore, the transfectant clone (BI-T) gained a new reactivity to pork insulin. Whereas the response of this clone to pork insulin could be completely blocked by a Mab specific for mouse CD4, the response to beef insulin could only be blocked down to the level observed in the CD4$^-$ parental line (Fig. 4). Although it is clearly the TCR which determines the specificity for antigen, these findings underscore the importance of CD4 in enhancing responses that would otherwise be undetectable. The result

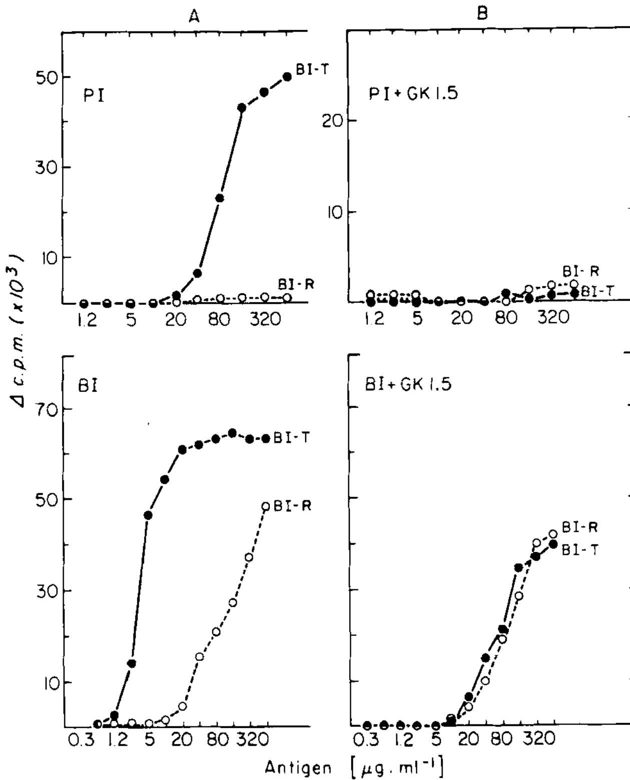

FIG. 4. Characterization of the antigen recognition pattern of BI-T and BI-R T cell hybrids. (A) Response to beef insulin (BI) and pork insulin (PI). (B) Response to BI and PI in the presence of anti-CD4 Mab GK1.5. Solid lines, BI-T (transfectant expressing mouse CD4); dashed line, BI-R (untransfected $CD4^-$ recipient T cell hybridoma). The medium controls were 1330 cpm for BI-T and 720 cpm for BI-R. The nonspecific inhibitory effect on BI and PI responses by the presence of rat IgG2b control Mab YR1/100 directed against rat class I MHC proteins was determined to be $<4.2\%$ in comparison to the unblocked response. Reproduced from Ballhausen *et al.* (1988), *The Journal of Experimental Medicine*, Vol. 167, pp. 1493–1498, by copyright permission of The Rockefeller University Press.

of the CD4 interaction in this case is an effective increase in the T cell repertoire, i.e., a T cell which could not otherwise respond to a physiological level of a given antigen (pork insulin) because of the low affinity of its TCR for that antigen can measurably respond to that antigen because of CD4 function.

E. CD4 AS THE RECEPTOR FOR HIV-1

As mentioned above, studies from a variety of laboratories have indicated that CD4 is the cell surface receptor used by HIV-1 for entry into human cells. *In vitro* infection of $CD4^+$ cells by HIV-1 can be inhibited by Mabs directed against CD4, but not by Mabs specific for other cell surface molecules (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985). Expression of CD4 introduced by gene transfer has been found to confer susceptibility to HIV-1 infection upon human cells that lacked CD4 expression and hence were resistant to HIV-1 (Maddon *et al.*, 1986). Cell surface expression of CD4 is required not only for viral infection, but also for cell fusion (syncytium formation) mediated by HIV-1 (Sodroski *et al.*, 1986; Lifson *et al.*, 1986; Maddon *et al.*, 1986). CD4 binds to gp120, the exterior envelope glycoprotein of HIV-1 (McDougal *et al.*, 1986). Kowalski *et al.* (1987) used mutagenesis to localize the binding site for CD4 to the carboxy-terminal half of gp120. They found that the important sequences are in three distinct regions of conserved sequence, suggesting an important role for tertiary structure in binding. Lasky *et al.* (1987) have further defined a region on gp120 that is important for binding CD4. They found that the epitope recognized by an anti-gp120 Mab that blocks CD4/gp120 binding is contained within amino acids 397–439 of gp120. Deletion of 12 amino acids (410–421) from this region completely abrogated binding to CD4, and a single amino acid substitution (Ala to Asp) at residue 417 significantly reduced binding (Lasky *et al.*, 1987). Notably, mouse CD4 does not bind HIV-1 despite its homology to human CD4 (Maddon *et al.*, 1986; Traunecker *et al.*, 1988), and mouse cells expressing human CD4 bind HIV-1 but are not susceptible to infection (Maddon *et al.*, 1986). Several recent studies have shown that genetically engineered soluble forms of human CD4 can bind gp120 and block infection by HIV-1 (Smith *et al.*, 1987; Fisher *et al.*, 1988; Hussey *et al.*, 1988; Deen *et al.*, 1988; Traunecker *et al.*, 1988; Berger *et al.*, 1988). In one case amino acids 1 through 152 of CD4 were sufficient to bind gp120 and block HIV infection (Traunecker *et al.*, 1988). The binding site has been further defined in a study of epitope mapping of CD4-specific Mabs using synthetic peptides. Jameson *et al.* (1988) found that Mab OKT4A, which had been

previously shown to block HIV-1 binding, recognizes a site between amino acids 32 and 47 on human CD4. This region was predicted to exist as a protruding loop assuming an Ig-like structure for this domain of CD4. A synthetic peptide containing amino acids 25–58 of CD4 inhibited HIV-1-induced cell fusion, providing strong evidence that the binding site for gp120 is indeed contained within this region of CD4. This conclusion is supported by results of Landau *et al.* (1988), who used mouse/human CD4 hybrid cDNA constructs to demonstrate that gp120 binding to transfected CHO cells requires human CD4 sequence from amino acids 37–83. Finally a mutational analysis by Peterson and Seed (1988) indicated that certain specific substitutions at amino acids 45 (Thr→Pro), 47 (Gly→Arg), or both 46 (Lys→Asn) and 47 (Gly→Val), or deletion of residues 42 through 49 of human CD4 abolish gp120 binding, while numerous other mutations at other sites and some substitutions at these sites do not. Although many of the Mabs which block binding of CD4 to gp120 also inhibit CD4 function, the degree of overlap between the binding site on CD4 for class II MHC molecules and that for gp120 is not yet known.

Binding of HIV-1 to cell surface CD4 has been found to induce rapid (within 5 minutes) phosphorylation of CD4, and this phosphorylation is maintained for up to 2 hours (Fields *et al.*, 1988). Binding of purified gp120 is not sufficient for induction of CD4 phosphorylation (Hoxie *et al.*, 1988; Fields *et al.*, 1988). A protein kinase C inhibitor, H7, can block HIV-1-induced phosphorylation without affecting cell surface expression of CD4. H7 was found to inhibit infectivity by HIV-1 and to cause an accumulation of HIV-1 bound to the cell surface (Fields *et al.*, 1988). These results suggested that CD4 phosphorylation might be important for viral entry. However, Bedinger *et al.* (1988) have shown that CD4 can still serve as a receptor for HIV when the cytoplasmic tail is deleted, when sequence C-terminal to amino acid 177 is replaced by the CD8 hinge, transmembrane, and cytoplasmic domains, or when each of the potential protein kinase C phosphorylation sites (Ser 408 and Ser 415) is specifically mutated. In the former two instances CD4 is no longer internalized in response to phorbol esters. These findings imply that phosphorylation of CD4 is not required for HIV infection, and suggest that receptor-mediated endocytosis may not be necessary for viral entry.

HIV-1 infection has also been shown to result in a loss or decrease of cell surface expression of CD4 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Folks *et al.*, 1986; Hoxie *et al.*, 1986; Stevenson *et al.*, 1987). Hoxie *et al.* (1986) concluded that the loss of surface CD4 is the result of decreased steady-state levels of CD4 mRNA secondary to HIV-1 infection. However, Stevenson *et al.* (1987) found that the reduction of CD4

expression is rapid and can occur without a concomitant decrease in CD4 mRNA. They demonstrated that loss of surface CD4 correlates with the presence of intracellular complexes between the envelope glycoproteins (gp120 and gp160) and CD4 (Stevenson *et al.*, 1988). These results suggest that the reduction of surface CD4 is a consequence of altered processing and localization of this protein in cells infected with HIV-1.

HIV-1 infection may disrupt the function of CD4$^+$ cells in a variety of ways. The numbers of CD4$^+$ cells decrease during HIV-1 infection (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Siegal *et al.*, 1981). This may be a result of several mechanisms: cell death following syncytium formation mediated by CD4–gp120 interaction (Sodroski *et al.*, 1986; Lifson *et al.*, 1986), direct cell death from infection (Somasundaran and Robinson, 1987; Stevenson *et al.*, 1988), antibody-dependent cellular cytotoxicity (ADCC) of cells bearing surface gp120 (Lyerly *et al.*, 1987), and disappearance of CD4 from the cell surface of infected cells as described above. Immune function would be compromised in all cases because of the central role of CD4$^+$ cells in immune responses and the functional importance of CD4 on those cells. However, additional mechanisms are most likely involved, since only a small proportion of CD4$^+$ cells are infected by the virus and immune responsiveness of CD4$^+$ cells decreases before cell numbers decrease. Soluble gp120 has been shown to inhibit PHA-induced proliferation of CD4$^+$ T cells (Mann *et al.*, 1987) and to block the CD4-dependent enhanced responsiveness of a mouse antihuman class II T cell hybridoma that was observed upon gene transfer of human CD4 (Diamond *et al.*, 1988). Thus gp120 mimics the inhibitory activity of many anti-CD4 Mabs. As discussed by Sattentau and Weiss (1988), these findings suggest that if any gp120 is released in infected patients, it could bind to cell surface CD4 and thereby decrease immune responsiveness directly, as well as making noninfected CD4$^+$ cells targets for destruction by ADCC.

IV. Summary and Discussion

The cloning of the cDNA and genes encoding CD4 and CD8 has provided the first detailed knowledge of their protein sequences and predicted structures. Both chains of CD8 and the single chain of CD4 were found to be members of the Ig gene superfamily, and the amino terminal domain of each most likely folds like an Ig V region. There is now strong evidence that these molecules do indeed bind to class II and class I MHC molecules, respectively. It is likely that it is the V-like portion of these molecules that is responsible for the binding. There is currently no direct evidence for the sites on class I and class II molecules which are bound

by CD4 and CD8, although this is a subject of current investigation. Human CD4 has also been demonstrated to have another ligand, namely the gp120 protein of HIV-1, and the availability of the cloned CD4 cDNA has led to a proliferation of studies that have increased our understanding of the interaction between HIV-1 and $CD4^+$ cells.

Molecular genetic studies of CD8 have led to two findings which have resolved the apparent discrepancies in the structure of this molecule in mouse versus man. First, the α' chain of mouse CD8, which has no counterpart in man, is derived from an alternative splicing pattern of CD8α mRNA. The resulting polypeptide chain differs from α only by the presence of a distinct and far shorter cytoplasmic tail. A posttranslational regulatory mechanism limits cell surface expression of this alternative polypeptide chain almost exclusively to immature thymocytes, with very little expression on mature T cells. The absence of an α' chain in humans would therefore be of no consequence in the function of mature peripheral T cells. Whether α' plays a unique role in mouse thymocyte development is not known. Second, the isolation and expression of cDNA clones encoding a human CD8β chain have demonstrated that the absence of such a chain in humans, as compared to mice and rats, was only apparent. CD8β protein is indeed present on virtually all human $CD8^+$ peripheral blood cells. While it has been shown that homodimers of the mouse or human CD8α chain can provide some of the functions that have been attributed to CD8, it is not yet known what the role of CD8β is and whether the normal heterodimeric form of CD8 functions better than or differently from CD8α homodimers.

For both CD4 and CD8 the isolation of molecular clones has added a new dimension to studies of the normal function of these proteins, providing an important complement to the Mab blocking studies. It is evident from the results of both types of analyses that these molecules play more than a single role in the complicated process of T cell recognition/activation. At a minimum they are involved both in adhesion and in T cell triggering. It is likely that the latter function involves an association between CD4 or CD8 and the TCR/CD3 complex. CD4 has also been suggested to play a role later in the activation process, although the mechanism(s) remains controversial. Carrera *et al.* (1987) found that anti-CD4 Mab could inhibit proliferation of peripheral blood mononuclear cells induced by PHA, anti-CD3 or ionomycin + PMA, even when added as late as 24 hours after the activation stimulus. IL-2 production was only slightly decreased in cells activated by PHA or anti-CD3 in the presence of anti-CD4, but IL-2 receptor expression was almost completely blocked when examined 3 days after stimulation. A late role for CD4 has also been described by Blue *et al.* (1988). They found that

proliferation in response to exogenous IL-2 of an IL-2-dependent, $CD4^+$, $CD8^+$ T cell clone could be partially blocked by anti-CD4 but not by anti-CD8 Mab, despite the fact that only anti-CD8 inhibited the function of this clone (see below). However, in this case the number of IL-2 receptors did not change with anti-CD4 treatment. Similarly, CD8 may also be involved in a late stage of the activation process. Welte *et al.* (1983) found that anti-CD8 Mab could inhibit IL-2 production (but not proliferation) by a $CD8^+$ human T cell clone in response to anti-CD3 Mab when added as late as 14–20 hours after anti-CD3.

It is also clear that CD4 and CD8 can enhance T cell responses whether they bind to the same or different MHC molecules as the TCR. Evidence for this comes from several sources. As discussed above, Gay *et al.* (1987) found that IL-2 release by a mouse class-I-specific hybridoma expressing human CD4 was increased if the antigen-presenting cells expressed human class II. Similarly, Ratnofsky *et al.* (1987) found increased IL-2 release by a mouse antihuman class II T cell hybridoma expressing human CD8 if human class I molecules were expressed on the antigen-presenting cells. In these two studies Mab specific for CD4 or CD8, respectively, blocked the enhanced response. Finally, Goldstein and Mescher (1988) found that at suboptimal antigen densities activation of a mouse $CD8^+$ CTL specific for allogeneic class I was enhanced as the level of an irrelevant class I molecule was increased on antigen-bearing membrane-coated beads. One possible interpretation is that the role played by these molecules differs depending upon whether they bind to the same or different MHC molecules as the TCR. Thus, CD4 and CD8 may be involved only in adhesion when they bind to MHC molecules distinct from those bound by the TCR, while they may be more directly involved in activation, i.e., signal transduction (as well as adhesion), when they bind to the same MHC molecule as the TCR. Possibly only in the latter case is a physiological complex formed between CD4 or CD8 and the TCR/CD3. In support of such a model are data obtained from rare double-positive ($CD4^+$, $CD8^+$) T cell clones. Fazekas de St. Groth *et al.* (1986) found that only anti-CD4 and not anti-CD8 inhibited antigen-induced lymphokine production by two class-II-restricted double-positive mouse T cell clones, suggesting that only CD4 is physiologically associated with the TCR/CD3 complex in these clones. Jones *et al.* (1987) similarly examined the roles of CD4 and CD8 in mouse class-II-restricted cytotoxic T cell clones. They found that both anti-CD4 and anti-CD8 could block cytotoxicity, but anti-CD4 was 25- to 100-fold more potent in blocking. Furthermore, the effect of anti-CD8 was only evident when suboptimal doses of antigen were used. These results suggest that, as in the clones described by Fazekas de St. Groth *et al.* (1986), only CD4 is functionally

associated with the TCR/CD3 complex. It is likely that the inhibition by anti-CD8 is acting at a different step, i.e., adhesion, which would presumably only become critical at low antigen density. Finally, Blue *et al.* (1988) studied a human double-positive CTL and found that only anti-CD8 Mab and not anti-CD4 blocked T cell triggering induced by anti-CD3 (as measured by the increase in $[Ca^{2+}]_i$) and lectin-facilitated cytotoxicity. In this instance one can conclude that only CD8 is associated functionally with the TCR/CD3 complex.

Regardless of whether there is a clear division of function based upon binding to the same or different MHC molecules, the bulk of evidence indicates that, depending upon the specific TCR involved (particularly its affinity for a given antigen/MHC) and the antigen/MHC density, some responses require binding of CD4 or CD8 to the same MHC molecule as the TCR, some only need binding at sites apart from the TCR/CD3 complex, and some are totally independent of CD4 or CD8 interactions. What remains unclear is the mechanism(s) by which these molecules enhance function beyond their role in adhesion, by which they presumably increase the avidity of the interaction between the T cell and target or antigen-presenting cells. It is possible that an association between CD4 or CD8 and the TCR/CD3 complex changes the conformation of the latter either to increase its affinity for antigen/MHC or to facilitate signal transduction. In this regard, Janeway *et al.* (1988) calculated, based on the 20- to 500-fold differences in potency between those anti-TCR Mabs that induce association between CD4 and the TCR (i.e., comodulation) and those that do not, that a physiological association between these molecules occurring during activation would theoretically reduce the number of ligands that the TCR must bind by an average of 100-fold. It is also possible that CD4 and/or CD8 might directly transduce positive signals when bound to their ligands. While the observed increase in CD4 and CD8 phosphorylation in response to activating stimuli might be relevant to signal transduction, it is too early to conclude what role (if any) this plays in the enhancement of T cell responses. A fourth possibility, which has been suggested by Fleischer and Schrezenmeier (1988), is that the observed modulation of surface CD4 or CD8 in response to activating stimuli might lower the threshold for triggering of the T cell. With regard to this hypothesis it should be noted that CD4 and CD8 comodulate with the TCR/CD3 complex in response to antibody cross-linking of the TCR/CD3 complex in the absence of any ligand binding to CD4 or CD8 (and vice versa) (see Section I). While this might lead one to question the role of comodulation, it is unlikely that such a situation would be a normal physiological occurrence in mature T cells, since the appropriate ligand for the expressed accessory molecule (CD4 or CD8) would typically be present

on the specific antigen-presenting or target cell. It would clearly be of interest to see whether both CD4 and CD8 modulate with the TCR/CD3 upon antigen-induced activation of the double-positive clones described above. Finally, modulation of neither CD4 nor the TCR can be an absolute requirement for T cell activation, even in cells in which CD4 appears to be physiologically linked to the TCR, since Saizawa *et al.* (1987) found that a low-potency anti-TCR Mab maximally stimulated D10 cells when provided at high concentrations but did not modulate either the TCR or CD4.

It has been suggested that CD4 might deliver a negative signal if it is cross-linked in the absence of cross-linking of the TCR/CD3 complex, and that this results in cell separation if the TCR is not bound to the appropriate antigen (Janeway *et al.*, 1988). There is currently no experimental evidence showing this to be the case, but several points are worthy of consideration. First, this hypothesis contains two separable parts: transmission of a negative signal (to turn off the T cell) and induction of cell separation. These two processes could be induced by entirely different mechanisms. As discussed in Section I, there is no direct evidence showing that CD4 (or CD8) can transmit a negative signal, and much of the evidence is against such a role physiologically. It has been suggested that inhibition by anti-CD4 or anti-CD8 of activation induced through "alternative pathways" supports transmission of a negative signal. For example, anti-CD4 Mab blocks activation of human T cells by anti-CD2 Mab plus PMA in the absence of accessory cells (Carrera *et al.*, 1987). Similarly, activation through Tp103 (Fleischer *et al.*, 1986a) and CD28 (Schrezenmeier and Fleischer, 1988) can be blocked by anti-CD4 or anti-CD8, and, in the mouse, activation by anti-Thy-1 Mab can be blocked by anti-CD4 (Pont *et al.*, 1987). However, it is not clear that these accessory pathways are truly independent of the TCR/CD3 complex. Heteroconjugate Mabs that cross-link CD2 with CD3 are as efficient at activating T cells as heteroconjugates between anti-CD3 and anti-CD4 or anti-CD8 (Emmrich *et al.*, 1988), and certain anti-CD4 or anti-CD2 Mabs have been found to comodulate surface CD4 and CD2 on human peripheral blood T cells (Bueso-Ramos *et al.*, 1988). Activation by anti-Thy-1 Mab is also dependent upon a functional TCR/CD3 complex (Schmitt-Verhulst *et al.*, 1987; Gunter *et al.*, 1987; Sussman *et al.*, 1988). It is therefore possible that the effects of anti-CD4 or anti-CD8 on such accessory pathways of activation are a result of steric interference with the formation of the appropriate molecular complexes as opposed to transmission of a signal to turn off the T cell.

Certainly the gene transfer experiments show that the major role played by CD4 and CD8 is to enhance T cell responses. However, the Janeway model limits negative signal transmission to one particular circumstance,

i.e., when CD4 is cross-linked in the absence of TCR/CD3 cross-linking. The requirement for cross-linking of CD4 in this model rests on the finding that only intact anti-CD4 and not monovalent Fab fragments could inhibit lectin-induced activation of the D10 T cell hybridoma (Haque *et al.*, 1987). The Fab fragments could inhibit activation by anti-TCR Mabs or by antigen, suggesting a steric mechanism in these instances (Haque *et al.*, 1987; Janeway *et al.*, 1988). It is not clear, however, that CD4 binding to class II results in cross-linking of either molecule in the absence of TCR/CD3 binding to the same class II molecule. It seems unlikely that there are two binding sites for CD4 on a single class II molecule and vice versa. It is possible, however, that two CD4 molecules must come together to form a single binding site for class II. This idea is attractive given the propensity of Ig homology units to pair and thereby create ligand-binding sites (e.g., Ig or TCR). The stoichiometry of comodulation of CD4 and the TCR from D10 cells (two CD4 molecules for one TCR molecule) (Saizawa *et al.*, 1987) is suggestive in this regard, but to date there has been no demonstration of association of two CD4 molecules on mouse or human cells. Against the hypothesis that CD4 is normally cross-linked upon binding to class II alone are data from Ledbetter *et al.* (1988), who found that independent but simultaneous cross-linking of CD4 and CD3 with Mabs diminishes signal transduction. Yet, as discussed above, CD4/class II interactions have been shown to enhance and not to diminish T cell responses even when the TCR cannot bind to the same MHC molecule as CD4 (e.g., when it is class I specific), suggesting that independent cross-linking of CD4 is not occurring under these circumstances. If CD4 is normally cross-linked merely by binding to class II, one might expect (based on the antibody cross-linking studies previously described) that this interaction would lead to comodulation of both CD4 and the TCR/CD3 complex even in the absence of a ligand for the TCR. This is certainly a testable hypothesis. In any event, there does not seem to be a need to postulate a negative signal when the TCR/CD3 complex is not cross-linked; the absence of a positive signal is sufficient to explain the lack of response when the TCR/CD3 complex is not engaged. This is not meant to imply that CD4 and CD8 are not capable of transmitting negative signals, but rather to stress that there is not yet any physiological evidence showing that they do.

The Janeway model further proposes that cells will separate if CD4 binds to class II in the absence of TCR/CD3 cross-linking. However, this is clearly not the case in non-T cells. The most direct demonstration of a role for CD4 in binding to class II is the study of Doyle and Strominger (1987) discussed above, in which a fibroblast line expressing large amounts of CD4 was able to bind only those B cell lines which

express class II. Since there is no TCR or CD3 in this system, one would have to postulate that the signal for separation is restricted to T cells (or at least absent in fibroblasts). As discussed in Section I, Blanchard *et al.* (1988) demonstrated that anti-CD4 Mab can induce a slow dissociation of preformed conjugates. If the Mab is mimicking a physiological response, it is as likely that the role of CD4 in cell separation is one that occurs after T cell activation and not as a means of dissociating nonspecific conjugates.

Progress on the understanding of CD4 and CD8 function has proceeded at an accelerating rate in recent years. Yet, as discussed above, there are still many open questions regarding how these molecules work. We also know very little about the mechanisms of regulation of expression of CD4 and CD8 during T cell development and the means by which T cells which are restricted by class I or class II MHC proteins become CD8 or CD4 single-positives, respectively. The latter most likely involves a selection process related to the ability of CD4 or CD8 on double-positive thymocytes to bind to the same MHC molecule as the expressed TCR, but how this happens mechanistically and developmentally remains obscure. One can only expect that the combination of molecular genetics, biochemistry and cell biology will eventually fill in the gaps in our current knowledge.

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ADVANCES IN IMMUNOLOGY, VOL. 44

Lymphocyte Homing

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"... Lymphocytes circulate and recirculate, so that the cells present in the blood at any one time are like the chorus of soldiers in a provincial production of the Opera *Faust*—they make a brief public appearance and then disappear behind the scenes only to re-enter by the same route." (Medawar and Medawar, 1977)

"...Though they circulate freely in the bloodstream and have opportunity for interaction elsewhere they are not promiscuous but faithfully seek out those blood vessels which provide entry into these tissues." (Woodruff and Kuttner, 1980)

I. Introduction

The secondary lymphoid organs are strategically positioned in the body so as to drain all potential portals of entry for foreign substances. Thus, associated with the skin, gastrointestinal tract, respiratory tract, and urogenital tract are lymphoid organs, which are specialized to collect, concentrate, and process antigens that enter the body in their vicinity, and then to present these antigens to potentially responsive lymphocytes. An essential component of this defense system is the continuous recirculation of lymphocytes from the blood into the widely distributed lymphoid organs and back to the blood again (Gowans, 1957, 1959). lymphocyte recirculation serves to maximize the probability of a productive interaction between an antigen sequestered in a particular lymphoid organ and the typically rare lymphocytes that can respond to it. The efficiency of this process depends upon a massive and continuous procession of lymphocytes. In the rat, for example, 4×10^7 lymphocytes per hour are returned to the blood via the thoracic duct, a major lymphatic vessel draining many of the secondary lymphoid organs. This rate is sufficient to replace the blood content of lymphocytes 10–20 times each day (Gowans, 1959; Ford, 1969). The vast majority of these cells are lymphocytes that had emigrated from the blood several hours earlier and now are being returned to the blood to initiate another cycle of recirculation. Joining the recirculating pool on each cycle, for either brief or extended stays, are lymphoblasts, memory cells, and terminally

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differentiated effector cells, which have been generated during immune responses within regional lymphoid organs.

The major portal of entry of blood-borne lymphocytes into secondary lymphoid organs is through a specialized postcapillary venule, which is characterized by a cuboidal, rather than the typical flat, endothelial lining (Gowans and Knight, 1964). An adhesive interaction of lymphocytes with these so-called high endothelial venules (HEV[1]) initiates lymphocyte extravasation. There is now considerable evidence (to be reviewed herein) that this cell-cell adhesive event is one of extraordinary specificity, depending upon various properties of the lymphocyte (subset, stage of development, state of activation, history of antigenic stimulation) as well as the anatomic site of the HEV (gut-associated lymphoid organs, peripheral lymph nodes, lung, etc). The lymphocyte cell-surface receptors underlying HEV attachment are termed homing receptors, a nomenclature chosen to reflect the tendency of different lymphocyte populations to migrate to and extravasate within particular lymphoid organs. Thus far, homing receptors of three distinct HEV-binding specificities have been identified and more are likely to exist. The adhesive determinants on HEV, complementary to the lymphocyte homing receptors, are referred to in this review as HEV ligands. This discussion will focus on four aspects of the lymphocyte-HEV interaction: (1) a brief description of the general characteristics of this cell-cell interaction, emphasizing its histological features; (2) a review of the specificity thus far demonstrated, or inferred, for this interaction, and the possible physiological significance and regulation of this specificity; (3) the identity and nature of the homing receptors and their corresponding HEV-associated ligands; and (4) the possible role for specific carbohydrates as cell recognition determinants in lymphocyte-HEV binding.

Other recent reviews are available on the subject of homing receptors and their associated HEV ligands (Butcher, 1986; Gallatin *et al.*, 1986; Jalkanen *et al.*, 1986a, 1987b; Kraal *et al.*, 1987; Weissman, 1986; Woodruff *et al.*, 1987). Also, more general surveys can be consulted on the broader topic of lymphocyte recirculation (Ford, 1975; Anderson *et al.*, 1982; Husband, 1988).

[1] Abbreviations: HEV, high endothelial venules; HEC, high endothelial cells; LuN, lung lymph node; MN, mesenteric lymph node; M1P, mannose-1-phosphate; M6P, mannose-6-phosphate; NeuAc, *N*-acetylneuraminic acid; PN, peripheral lymph node; PP, Peyer's patch; PPME, poly-phospho monoester core of the yeast wall mannan from *Hansenula holstii*; TC, trypsin-calcium.

II. General Description of Lymphocyte-HEV Interaction

A. Structure of HEV

HEV are found in most mammalian species examined, from a primitive monotreme to the human (Shulze, 1925; Miller, 1969). Their most diagnostic characteristics are a plump endothelial lining layer associated with adherent and infiltrating lymphocytes. Although the occurrence of lymphocytes within the wall of HEV was first noted in 1899 (Schumacher), the direction and physiological significance of lymphocyte transendothelial migration was not appreciated until the classical autoradiographic experiments of Gowans and Knight (1964). With the exception of spleen,[2] HEV are found in all secondary lymphoids organs including lymph nodes, Peyer's patches, tonsils, adenoids, appendix, and small aggregates associated with the mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts (Miller, 1969; Butcher and Weissman, 1984). HEV are absent in the primary lymphoid organs (bone marrow and thymus) and are normally not present in nonlymphoid organs. However, HEV-like vessels have been observed within many examples of chronically inflamed organs and are believed to support the infiltration of large numbers of lymphocytes into these tissues (Smith *et al.*, 1970a; Freemont, 1983; Freemont and Jones, 1983). One of the best documented examples of this phenomenon is in the inflamed synovium associated with rheumatoid arthritis (Graham and Shannon, 1972; Freemont *et al.*, 1983; Iguchi and Ziff, 1986).

Within lymph nodes, HEV are found between B-cell follicles, within the T-cell domains of the outer and inner cortex (Anderson *et al.*, 1982). Our description of the histology will emphasize HEV within lymph nodes of mice and rats, since most of the detailed studies have been carried out in these species; however, HEV in other lymphoid organs or in other species share many characteristics with these vessels (Freemont and Jones, 1983). In rat lymph node, HEV begin at the juncture of flat-walled venules in the cortex and end with large-diameter vessels draining into the medulla (Anderson *et al.*, 1982). Because of their position and diameter (7–30 μm), HEV are classified as postcapillary venules. In terms of luminal diameter, rate of blood flow, and concentration of blood cells, this general class of blood vessel optimizes the rate of collision between the blood cells that it carries and the vessel wall (Ottaway, 1988). High

[2] The extent of lymphocyte recirculation through the spleen is enormous, exceeding that of all the lymph nodes combined (Pabst, 1988). Yet the mechanisms by which lymphocytes are exchanged between the blood and spleen are understood relatively poorly.

endothelial cells (HEC) have domed surfaces that protrude into the HEV lumen, presumably augmenting the rate of lymphocyte collision. When collision does occur, the specialized functions of HEC are to secure a firm attachment of the lymphocyte and then to facilitate the migration of the lymphocyte across the endothelium.

Histologically, HEV exhibit a number of distinguishing characteristics (Anderson and Anderson, 1976; Anderson *et al.*, 1982). The HEC are cuboidal to columnar in height (10–12 μm) and are linked by discontinuous macular junctions at their apical and basal aspects. Zonula-type junctions are not observed. The luminal aspect of HEV presents a "cobblestone-like" appearance composed of the dome-shaped endothelial cells separated by deep intercellular clefts. Associated with the luminal surface is a prominent glycocalyx with a thickness approximately three times that of capillaries (Anderson and Anderson, 1975). This feature is noteworthy in view of the evidence (see Section V) that carbohydrates may serve as essential recognition determinants of the HEV ligands. The single layer of endothelial cells rests on a basement membrane, which is intimately apposed to a sheath of overlapping cell processes contributed by parenchymal reticular cells (Anderson and Anderson, 1975). Reticular fibers within the basement membrane merge with those in the parenchyma of the lymphoid organ. Attempts to characterize the basement membrane of HEV at the biochemical and ultrastructural level have been initiated (Freemont *et al.*, 1986). The basement membrane and its associated sheath of reticular cell processes appear to constitute the rate-limiting barrier to transmigrating lymphocytes (Wenk *et al.*, 1974; Anderson and Anderson, 1975). The mechanical and degradative mechanisms by which lymphocytes breach this barrier are not understood.[3] Another important issue, just beginning to receive considerable attention, concerns the receptors on lymphocytes mediating interactions with extracellular matrix components (e.g., laminin, fibronectin, collagens) (Cardarelli and Pierschbacher, 1987; Hemler, 1988). These receptors are undoubtedly of critical importance in controlling the migration and arrest of lymphocytes within the extravascular compartment. It is already evident that these matrix receptors are regulated as a function of antigen-dependent and -independent differentiation.

Ultrastructurally, HEC have the characteristics of metabolically active,

[3]It is somewhat puzzling that the penetration of leukocytes (lymphocytes and neutrophils) through the basement membranes of blood vessels, a normal and extensive process in the body, is not utilized more widely as a potential model for the breaching of basement membranes by metastasizing tumor cells.

secretory-type cells, exhibiting a prominent Golgi apparatus, abundant mitochondria, many ribosomes (frequently in polyribosome clusters), multivesicular bodies, and a large, rounded euchromatic nucleus with one to two nucleoli (Wenk *et al.*, 1974; Anderson and Anderson, 1975). Endothelial cells of other vessels typically have a flattened heterochromatic nucleus and a poorly developed Golgi apparatus. HEV further demonstrate several distinctive enzymatic activities, including elevated levels of a nonspecific esterase (Smith and Henon, 1959), acid phosphatase, and β-glucuronidase (Anderson *et al.*, 1976). The functional significance of these activities is not understood.

A further unusual property of HEC is their ability to synthesize and secrete a sulfated glycolipid, apparently in association with a nonsulfated glycoprotein (Andrews *et al.*, 1982, 1983). Very rapid metabolic labeling of this component can be demonstrated within HEV after addition of [^{35}S]sulfate to an organ culture of lymph node or following intravascular administration of the isoptope to rats. Rapid sulfate incorporation has also been demonstrated for HEV-like vessels at sites of chronic inflammation (Freemont, 1987). In lymph node HEC, a high concentration of incorporated label is found at early time points within the Golgi and its associated smooth vesicles (Andrews *et al.*, 1982). These labeled organelles are localized frequently in the apical region of the HEC, suggesting that the sulfated macromolecule may be secreted into the HEV lumen. Despite these intriguing findings, attempts to directly relate the sulfated compound to the process of lymphocyte extravasation have thus far been equivocal (Drayson and Ford, 1984; Ford *et al.*, 1984). Although the physiological significance of the unusual sulfate incorporation is unclear at present, this metabolic activity has been a useful diagnostic feature in the identification of HEC in primary cultures (Ager, 1987).

The identification of HEV by morphological criteria and enzyme histochemistry has now been complemented by the availability of monoclonal antibodies that selectively stain HEV and HEV-like vessels (see Section IV,B for a detailed account). MECA-325, the first of these described, reacts with the cell surface and cytoplasm of high endothelial cells in mouse lymph nodes and Peyer's patches, but is negative on other vascular endothelium (Duijvestijn *et al.*, 1987). This antibody also reacts with HEV-like vessels that are induced at sites of chronic lymphocyte infiltration and with thin-walled blood venules that are probable portals of lymphoblast extravasation within the lamina propria of the gut (Jeurissen *et al.*, 1987). An analogous antibody, reactive with HEV and HEV-like vessels in human tissues, has also been described (Duijvestijn *et al.*, 1988). These two antibodies do not appear to discriminate among

functional subsets of HEV, nor do they interfere with the lymphocyte adherence function of these vessels. However, a subsequent study (Streeter *et al.*, 1988) has reported a monoclonal antibody, called MECA-367, that stains differentially the HEV of mouse Peyer's patches as compared to those in peripheral lymph nodes. Moreover, as described in Section IV, the antibody blocks selectively lymphocyte attachment to Peyer's patch HEV without affecting lymphocyte attachment to peripheral lymph node HEV.

B. Lymphocyte–HEV Interaction

The interaction of lymphocytes with HEV has been studied in real time by direct observation. Individual, fluorescently labeled lymphocytes were observed by video fluoromicroscopy as they flowed through the postcapillary venules of Peyer's patches in an exteriorized loop of mouse small intestine (Bjerknes *et al.*, 1986). Secure attachments to HEV occurred within seconds after intravenous injection, whereas lymphocytes were not arrested in vessels of comparable size in nonlymphoid tissues. Ottaway (1988) has emphasized the stochastic or probabalistic nature of the process. A cell has a certain probability of colliding with the HEV wall and another probability of forming a secure contact after collision. A given cell may interact with the vessel wall several times until it is either secured within the HEV, or it exits the lymphoid organ with the blood. If a cell maintains its association with the wall of the HEV for over 12 sec, then the interaction will not be reversed and the cell will undergo extravasation. The overall efficiency of lymphocyte collection (i.e., the probability of a productive interaction for an incoming lymphocyte) for Peyer's patch HEV is about 25%. Comparable extraction efficiencies are reported for lymph nodes (Ottaway and Parrott, 1981). Immunological stimulation of a lymphoid organ dramatically affects the extraction of lymphocytes. For example, in sheep, the rate of cellular output measured in efferent lymph from an individual lymph node increases about 10-fold following antigenic stimulation (Hay and Hobbs, 1977). Since the vast majority of lymphocytes leaving a node through efferent lymph originate via recirculation from the blood (Hall and Morris, 1965), this dramatic increase is probably due to an enhanced influx of lymphocytes, which may occur as a result of increased blood flow in the organ (e.g., vasodilation), by increased shunting of blood flow to HEV through arteriovenous communications (Herman *et al.*, 1979), or angiogenic expansion of the HEV (Hay and Hobbs, 1977; Anderson *et al.*, 1975). The first two responses are seen within hours after stimulation, while angiogenesis requires several days.

Once securely bound within the HEV, the subsequent diapedesis of an arrested lymphocyte takes about 5–10 min (Smith and Ford, 1983).

Morphological studies reveal that lymphocytes form their initial contacts through numerous microvilli, which interdigitate with randomly positioned depressions in the HEC (Van Ewijk *et al.*, 1975; Anderson *et al.*, 1982). Stabilization of the surface coat material with alcian blue, which binds acidic glycoconjugates, reveals electron-dense, fibrillar material at sites of close contact (12-20 nm) between adherent lymphocytes and endothelial cells (Anderson and Anderson, 1976). These specializations presumably represent the sites of initial adhesive contacts, but localization of homing receptors or HEV ligands to these regions has not, as yet, been demonstrated. After initial contact, a lymphocyte assumes a smooth surface contour as it penetrates the endothelium (Van Ewijk *et al.*, 1975; Anderson and Anderson, 1976). The prevailing view based upon transmission electron microscopy of serial sections scanning electron microscopy and extracellular tracer studies is that lymphocytes pass between adjacent endothelial cells rather than migrating through the cells by transcytosis (Schoefl, 1972; Van Ewijk *et al.*, 1975; Wenk *et al.*, 1974; Anderson and Anderson, 1976). An alternative view is that lymphocytes begin their migration by invaginating directly into endothelial cells and then subsequently enter intercellular spaces (Farr and DeBruyn, 1975; Cho and DeBruyn, 1986). The finding that most (>80%) of the luminally bound lymphocytes are situated over intercellular clefts between adjacent HEC (Anderson and Anderson, 1976) argues that the transcellular mechanism does not generally apply during the initial stage of migration.

Based upon the dramatic changes in morphology of both transmigrating lymphocytes and HEC, it is clear that neither cell type is a passive partner during the process of diapedesis. As a lymphocyte penetrates the HEV, its plasma membrane becomes intimately apposed to that of the endothelial cells, an appearance that suggests a highly deformable and enveloping nature of the HEC (Schoefl, 1972; Anderson and Anderson, 1976). This intimate relationship, together with the augmented height of the endothelium, probably contributes to minimizing leakage of vascular contents as the lymphocytes squeeze between the endothelial cells (Schoefl, 1972; Anderson and Anderson, 1976). For much of its course through the vessel wall, a lymphocyte assumes an active migratory configuration, with its nucleus anterior followed by a thin uropod (Anderson and Anderson, 1976; Campbell, 1983). Interestingly, the macular adhesive junctions holding endothelial cells together are not broken, but are instead circumnavigated by transmigrating cells (Anderson and Anderson, 1976). Adhesive connections having the morphology of gap junctions may form between transmigrating lymphocytes and HEC (Campbell, 1983). The fact that lymphocytes exhibit directed migration

from the lumen to the parenchyma of the lymphoid organ strongly suggests the possibility that a haptotactic or chemotactic gradient directs the migrating cells (Carter, 1967; Czinn and Lamm, 1986). The nature of this putative guidance mechanism is a fascinating and important subject for future study.

Another important, but rarely studied, aspect of the lymphocyte/HEV interaction is how lymphocytes detach from the HEC once they have migrated across the vessel wall. Investigators examining this and other cell adhesive systems have addressed the possibility that adherent cells modify attachment-ligands in order to facilitate deadhesion (Stamper and Woodruff, 1977; Brandley *et al.*, 1987; Shur and Hall, 1982; and see Section IV,C,2). An additional possibility is that HEC or lymphocytes elaborate degradative enzymes that inactivate the adhesion molecules. Relevant to this issue is the finding that peripheral lymphocytes manifest a plasminogen activator activity that is stimulated by exposure of the cells to fucoidin, a polysaccharide that may be a structural mimic of an HEV ligand (Yednock *et al.*, 1988b). Degradative enzymes, produced by either the lymphocyte or the HEC, may also be required for lymphocyte penetration of the HEV basement membrane.

A further noteworthy feature of the lymphocyte-HEC relationship concerns the orientation of the Golgi apparatus within the endothelial cells. The majority of HEC in contact with lymphocytes have their Golgi complexes oriented toward the lymphocyte, whereas HEC not in contact have their Golgi's oriented toward the luminal surface (Van Deurs *et al.*, 1986). This arrangement may indicate an active secretory role of the HEC involved in the transmigration of lymphocytes. For example, cytotoxic T-cells orient their Golgi stacks toward adherent target cells, presumably to facilitate the efficient delivery of cytotoxic secretory products that have been packaged within this organelle (Kupfer *et al.*, 1983; Kupfer and Dennert, 1984). Possible secretory products targeted toward the transmigrating lymphocyte may include the sulfated glycolipid described above, or proteolytic enzymes to bring about lymphocyte detachment.

In general, flat vascular endothelia do not support high levels of lymphocyte extravasation (Gowans and Knight, 1964; Ford, 1975). However, the height of the HEC is clearly not the essential feature for either lymphocyte adherence or diapedesis; postcapillary venules in sheep lymph nodes have a relatively flat endothelium (Trevella and Morris, 1980), and yet appear to function as their high-walled counterparts in other species. The height of HEV is also diminished in nude rats with no apparent effect on the efficiency of lymphocyte influx (Fossum *et al.*, 1980). Additionally, as noted above, thin-walled blood venules in the intestinal lamina propria apparently are sites of lymphocyte (in particular,

lymphoblast) extravasation, as are similar vessels in the connective tissue of lactating mammary gland (Roux *et al.*, 1977). The height of the HEV probably represents a morphological adaptation principally for preventing vascular leakage (Anderson and Anderson, 1976).

A key issue concerns the nature of the factors involved in endowing these particular postcapillary venules with their specialized functions (see Section III,E for more detail). For lymph nodes, there is evidence that substances or cells (e.g., macrophages) provided by the afferent lymph supply are responsible for the maintenance of the HEV phenotype. Ligation of afferent lymphatics to a lymph node results in a dramatic reduction of lymphocyte influx across HEV, which is measurable within the first day following surgery; eventually, the HEV become flattened (Hendriks and Eestermans, 1983; Drayson and Ford, 1984). The application of an *in vitro* lymphocyte adherence assay (see Section III,B,1) clearly established that the decreased *in vivo* migration produced by the deafferentation is due to a loss in the ability of the HEV to support lymphocyte binding (Hendriks *et al.*, 1987). This reduction in adhesion appears to be reversible, since the introduction of antigen into a deafferented lymph node restores lymphocyte traffic (Hendriks and Eestermans, 1983). It is strongly suspected that mediators released by antigen-stimulated cells (e.g., macrophages), in the same or in upstream lymphoid organs, may act to induce adhesive ligands on HEV.[4] This possibility gains credence from the findings that exposure of cultured umbilical vein endothelium to various immunologic mediators (e.g., IL-1, IFN-γ, TNF) augments its ability to support lymphocyte attachment (Cavender *et al.*, 1987). Moreover, IFN-γ has been shown to induce an HEV-specific epitope (recognized by the monoclonal antibody MECA-325) on cultured microvasculature, although HEV ligand activity has not been demonstrated (Duijvestijn *et al.*, 1986).

HEV have distinctive morphological, ultrastructural, and biochemical characteristics that underlie their specialized function in lymphocyte adhesion and extravasation. Development of a cell culture system that preserves the unique properties of these endothelial cells is crucial to gaining an understanding of the factors that regulate HEV form and function (Ager, 1987). One of the most intriguing aspects of these vessels

[4] In this regard, it is interesting that in homing studies, lymphocytes localize exceptionally well within liver-associated lymph nodes (Smith *et al.*, 1980). This finding may reflect the fact that these nodes receive approximately 10-fold higher numbers of macrophages than other nodes via the afferent lymph (Smith *et al.*, 1970b). The inflammatory products released by these macrophages may induce "super" HEV that support increased levels of lymphocyte binding. Such a possibility is readily testable with the *in vitro* lymphocyte adherence assay.

is their remarkable organ specificity. Specific HEV ligands appear to direct selectively the homing of particular lymphocytes to lymphoid tissues at distinct anatomical sites, recently earning these molecules the designation of "vascular addressins" (Streeter *et al.*, 1988). The next section reviews the established specificity of this system, and explores its potential range and possible function.

III. Regional Specificity of Lymphocyte Homing

A. Early Evidence: The Selective Migration of Blast Cells

Organ-selective lymphocyte migration was documented 25 years ago in the classic study of Gowans and Knight (1964), where lymphocyte "homing" was first described. These investigators radiolabeled large lymphoblasts that were isolated from the thoracic duct lymph of rats, infused them intravenously into the same animals, and found these cells to localize selectively within the mesenteric lymph nodes and wall of the small intestine. Since that time, numerous investigators have employed similar techniques to reveal a system with remarkable specificity, the full range of which is yet to be appreciated.

Among the *in vivo* homing studies, those that examine the migration of lymphoblasts, isolated from various organ sources, are perhaps the most easily interpretated. Lymphoblasts have the unusual ability to extravasate through flat-walled vessels within tissue beds outside of organized lymphoid tissues (although they also interact well with HEV); for unknown reasons, the majority of small lymphocytes extravasate through such vessels at much lower levels (see Section III,F). However, as will be discussed below, the interaction of lymphocytes with flat-walled vessels in a particular organ appears to involve a binding specificity similar to that of the HEV within the associated lymphoid organs. Thus, vessels within the lamina propria of the small intestine share a binding specificity with gut-associated Peyer's patches and mesenteric lymph nodes. Although lymphoblasts comprise only 3-6% of most mature lymphocyte populations, they can be labeled selectively with [^{3}H]thymidine or [^{125}I]deoxyuridine, and can thereby be traced readily during *in vivo* homing studies. The exquisite selectivity with which these cells interact with different endothelial beds has proved to be diagnostic for revealing unique lymphocyte homing specificities. For example, complementing the results of Gowans and Knight (1964), investigators found that when lymphoblasts are isolated from peripheral lymph nodes and injected intravenously they will largely bypass the intestine to localize within cutaneous (peripheral) lymph nodes and the skin (Griscelli *et al.*, 1969; Smith *et al.*, 1980; Chin and Hay, 1980; Rose *et al.*, 1976).

A general concept to emerge from these and other studies has been the idea of separate "peripheral and mucosal immune systems" for lymphocyte homing and recirculation (McDermott and Bienenstock, 1979). According to this model, lymphocytes express one set of homing receptors for HEV in mucosal-associated lymphoid organs, such as the intestine and its associated lymphoid tissues, and another set of homing receptors for HEV within peripheral lymph nodes. We will review studies indicating that lymphocyte homing involves a complex network of regional specificities that is a great deal more refined than merely separate peripheral and mucosal homing systems. We discuss how the regulation of such a homing network may serve to orchestrate a unified immune surveillance among related organ systems, as well as to produce an efficient response within a single, localized site. Dissecting the complexity of organ-specific lymphocyte homing presents a formidable challenge, but due to its great accessibility for study, this system provides a potentially rich source of information concerning cellular interactions in general, including the developmental, hormonal, and environmental regulation of cell adhesion molecules.

B. Distinct Lymphocyte-Binding Specificities for HEV of Peripheral Lymph Nodes and Peyer's Patches

1. *A Model of Selective Lymphocyte Homing*

The confirmation of distinct homing specificities at the level of lymphocyte-HEV binding and an appreciation for the underlying cellular mechanisms have advanced dramatically due to an *in vitro* assay developed by Stamper and Woodruff (1976). In this assay, viable lymphocytes are layered over cryostat-cut sections of lymphoid tissues and are found to attach selectively to exposed profiles of HEV. The degree of binding can be quantified by counting the number of lymphocytes bound per HEV (Stamper and Woodruff, 1976), or per unit area HEV (Rosen *et al.*, 1985), or by comparing the number of cells bound relative to a fluorescently labeled internal standard population of lymphocytes (Butcher *et al.*, 1979a). The *in vitro* assay has been shown to reflect with remarkable fidelity the *in vivo* localization of lymphocytes within lymphoid tissues (Butcher *et al.*, 1979b; Gallatin *et al.*, 1983; Woodruff *et al.*, 1987). Results obtained with this assay have supported the hypothesis that high endothelial cells express organ-specific HEV ligands for lymphocyte attachment and that lymphocytes express complementary organ-specific homing receptors.

Butcher (1982) reported that lymphoblasts isolated from mesenteric lymph nodes (MN) bind to HEV in sections of gut-associated Peyer's patches (PP) 38-fold better than to HEV in peripheral lymph nodes (PN).

Reciprocal studies that examine the attachment of PN blasts to HEV *in vitro* have not been reported; however, numerous investigations have shown that PN and PP HEV express distinguishable HEV-ligands for lymphocyte binding. Organ-specific HEV attachment has most convincingly been demonstrated with cultured lymphoma cell lines; some lymphoma lines bind exclusively to HEV in Peyer's patches, others to HEV in peripheral lymph nodes, and still others bind well to HEV in both organs (Butcher *et al.*, 1980). These results led Butcher *et al.* (1980) to propose a model of lymphocyte/HEV binding, a modified version of which is depicted in Fig. 1. Among normal lymphocyte populations, highly differentiated lymphoblasts appear to possess the greatest ability to

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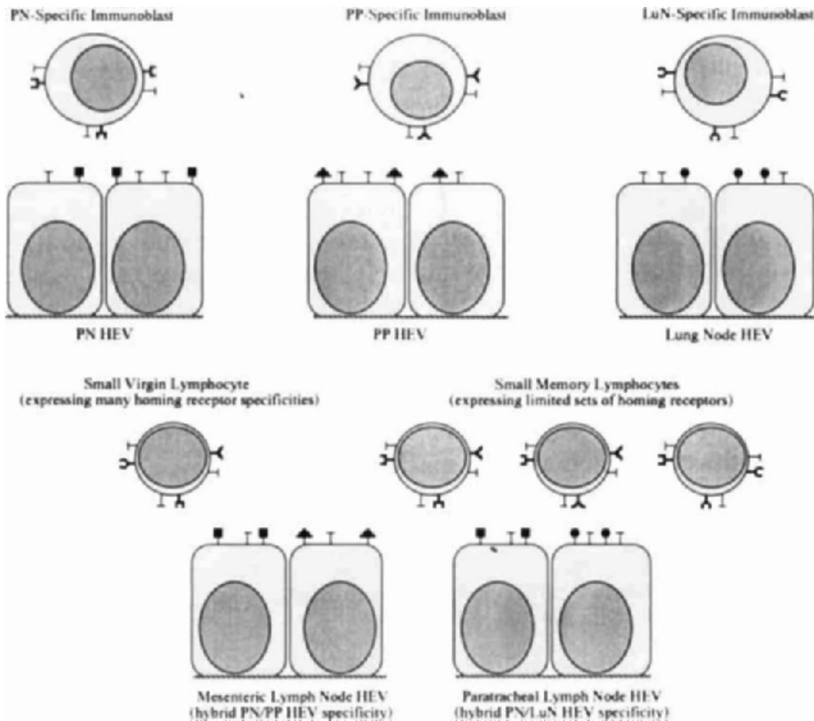
FIG. 1. Model for organ-specific lymphocyte attachment to HEV [based on a model originally presented by Butcher *et al.* (1980)]. Lymphocytes express specific cell adhesion molecules, called homing receptors, that mediate their attachment to organ-specific ligands associated with HEV of different lymphoid tissues. Candidates for PN-specific homing receptors include the MEL-14 antigen, its associated carbohydrate-binding activity, and $HEBF_{LN}$. A member of the 90-kDa MEL-14/Hermes family of glycoproteins and $HEBF_{PP}$ are possible PP-specific homing receptors (see Sections IV,A and V,B,2). Lymphocyte attachment to HEV also appears to involve general, adhesion-strengthening accessory molecules. These molecules are depicted as simple "platforms," not to suggest homophilic interaction necessarily, but rather to reflect their lack of tissue specificity and the fact that several different receptors may be involved. For example, LFA-1, CD4, CD8, and the CT4 antigen are molecules expressed by lymphocytes that could possibly function as accessory adhesion receptors (see Section IV,A,7). In addition, such molecules, acting in concert with the organ-specific homing receptors, may mediate lymphocyte adhesion to flat-walled vessels at sites of inflammation, or underlie the migration of blast cells through flat-walled vessels in normal tissues, such as the intestine, breast, and skin (Section III,F). Substantial evidence supports the proposal that PN and PP HEV exhibit distinct recognition ligands (see Section III,A), and data presented in Section III,C,2 suggest that a third set of unique ligands is expressed by lung-associated HEV. Mesenteric lymph nodes are thought to express both PN- and PP-like HEV ligands, perhaps reflecting the fact that these nodes receive lymphatic input from both the intestine and peritoneal cavity. Likewise, lymphocyte attachment to HEV within paratracheal lymph nodes, which receive lymphatic input from the peritoneal cavity and the lung, appears to share characteristics of lymphocyte binding to both PN and LuN HEV; hence, the possibility that paratracheal lymph node HEV expresses both PN- and LuN-like HEV ligands is depicted. As proposed in the text, the expression of different homing receptors by lymphocytes may, in some cases, become increasingly restricted with antigen-dependent differentiation (Section III,D,4). According to this proposal, virgin lymphocytes express several homing receptor classes, allowing promiscuous recirculation through many lymphoid organs. Upon exposure to cognate antigen in the context of a particular lymphoid organ, memory cells would be generated that exhibit preferential recirculation to the same and related lymphoid organs. Finally, terminally differentiated immunoblasts, such as plasma cell precursors, may express only one class of homing receptor, directing them to localize selectively within the organ where antigen was initially encountered.

distinguish among HEV attachment sites. To accommodate this observation within the model, blast cells are shown with only one type of homing receptor.

Other lymphocyte subpopulations have also been shown to exhibit preferential, but not absolute, HEV-binding tendencies. For example when B- and T-cells are compared, B-cells bind preferentially (twofold to threefold) to PP HEV, while T-cells bind better (1.5-fold) to PN HEV (Stevens *et al.*, 1982). Also, the CD4 and CD8 T-cell subsets show slight binding differences to HEV of PN (Kraal *et al.*, 1983). In the context of the model, each of these subpopulations would express both PN- and PP-specific homing receptors, but one receptor type would be represented on the cell surface to a higher degree than the other (as is proposed for memory cells in Fig. 1).

In vivo homing studies have demonstrated that the localization of the lymphocyte subsets within peripheral lymph nodes and Peyer's patches parallels closely their *in vitro* HEV-binding preferences (Stevens *et al.*, 1982; Kraal *et al.*, 1983). Furthermore, this localization pattern reflects



the normal *in vivo* distribution of lymphocyte subsets resident within lymphoid organs; thus Peyer's patches contain more B-cells than peripheral lymph nodes, while peripheral lymph nodes contain a higher percentage of T-cells than Peyer's patches.[5] Based on these results, Stevens *et al.* (1982) suggest that specificity of lymphocyte/HEV binding may regulate the localization, and hence, representation of the lymphocyte subsets within particular lymphoid tissues; thereby regulating the type of immune response generated by different lymphoid organs. For example, the fact that Peyer's patches are enriched for B-cells and T-helper cells is consistent with their primary function in mounting a humoral immune response (Kraal *et al.*, 1983). Furthermore, since the antibody response in Peyer's patches is largely of the IgA isotype, IgA-specific T-helper cells may exhibit a high degree of preferential homing to Peyer's patches as well as to other mucosal lymphoid organs (Butcher *et al.*, 1982a). The notion that lymphocyte/HEV interactions influence the type of immune response generated within a lymphoid organ raises a number of interesting issues concerning the regulation of expression of lymphocyte homing receptors and HEV-specific ligands. As discussed below, there are certain instances where a reverse type of regulation may apply; that is, where the lymphoid organ, and the type of immune response that it fosters, directs the expression of homing receptors by responsive resident lymphocytes.

2. *Lymphocyte Homing to the Breast: Hormonal Regulation of an "Intestinal" Specificity*

During lactation the number of IgA plasma cells within the breast increases 900-fold (Weisz-Carrington *et al.*, 1979). Roux *et al.* (1977) proposed that at the time of lactation, gut-derived blast cells in the circulation are diverted to the connective tissue of the mammary gland for IgA antibody secretion into the milk. It has been shown that plasma cells within breast tissue do, in fact, produce antibodies specific for gut-associated antigens (Stoliar *et al.*, 1976; Roux *et al.*, 1977); thus these antibodies presumably confer passive immunity to the intestinal tract of the suckling newborn.

Roux *et al.* (1977) confirmed that blast cells isolated from mesenteric lymph nodes exhibit remarkable specificity for localizing within the lactating breast. The migration of blast cells isolated from peripheral and

[5] In addition to preferential HEV interactions, factors such as the selective retention or expansion of lymphocyte subsets may also contribute to their unequal representation within particular lymphoid compartments.

mesenteric lymph nodes was compared following intravenous injection. As expected, MN blasts localized within the small intestine better (threefold to fourfold) than did PN blasts. While both cell types localized poorly within the nonlactating breast, just before the onset of lactation, MN blasts localized within this tissue 3- to 10-fold better than PN blasts. Localization of blasts within the intestine was not affected by the lactation cycle. McDermott and Bienenstock (1979) extended these observations by demonstrating that MN blast cells localize within the proestral cervix and vagina eight-fold better than do PN blasts.

The inference that lymphocyte homing to the lactating breast involves an endothelial-binding specificity similar to that of the small intestine has been supported by studies with the monoclonal antibody MECA-367 (Streeter *et al.*, 1988; see Section IV,B,2); this antibody identifies an antigen associated selectively with HEV in Peyer's patches and mesenteric lymph nodes, as well as small vessels within the lamina propria of the intestine and mammary gland. Since MECA-367 inhibits the binding of lymphocytes to PP HEV, but not to PN HEV, it is thought to identify a ligand involved in organ-specific lymphocyte binding. Based on the results of McDermott and Bienenstock (1979), expression of the MECA-367 antigen would also be predicted in the cervix and vagina.

C. A Common Mucosal Immune System?

1. *The Basis for the Hypothesis*

From the studies described above, the intestine, breast, vagina, and cervix appear to share a common specificity for lymphocyte homing. Based on such evidence, it has been proposed that these tissues are actually part of a "common mucosal immune system" for lymphocyte homing and recirculation (Bienenstock *et al.*, 1973; McDermott and Bienenstock, 1979). Such a system would functionally unite widely distributed mucosal surfaces, all of which produce protective secretory antibodies at sites of potential antigen entry. In further support of this proposition are the following findings: (1) Peyer's patches are anatomically similar to other mucosal-associated lymphoid tissues, such as those in the trachea and oral surfaces (Bienenstock *et al.*, 1973; Racz *et al.*, 1977; Nair and Schroeder, 1986); (2) all mucosal organs produce secretions containing IgA antibodies; furthermore, oral immunization leads to the appearance of antigen-specific IgA plasma cells not only in the intestine, but also in the respiratory tract, salivary gland, and the breast (Fuhrman and Cebra, 1981; Mestecky *et al.*, 1978; Weisz-Carrington *et al.*, 1979); (3) cells isolated from one mucosal-associated organ can home selectively to several types of mucosal tissues (Pierce and Cray, 1981;

McDermott and Bienenstock, 1979; McDermott *et al.*, 1980; Rudzik *et al.*, 1975); and (4) cells from Peyer's patches or from lung-associated lymph nodes can repopulate lymphoid tissue in the lung of irradiated mice, but repopulation of this tissue is not achieved with cells from peripheral lymph nodes (Rudzik *et al.*, 1975). Despite this evidence, careful evaluation of the literature reveals that the mucosal immune system is likely to be composed of several coordinated yet distinct specificities for lymphocyte homing. Such evidence is presented below for two mucosal organs, the lung and the colon.

2. *A Distinct Lymphocyte-Binding Specificity for Lung-Associated Lymphoid Tissues*

In as much as *in vivo* homing studies with lymphoblasts predicted the distinct lymphocyte recognition specificities for PN and PP HEV, similar studies have suggested the possibility of unique recognition specificities in other organ systems. Several reports have demonstrated that lymphoblasts isolated from lung-associated lymph nodes migrate preferentially to the lung following intravenous injection. McDermott and Bienenstock (1979) compared the localization pattern of lymphoblasts isolated from peripheral lymph nodes, mesenteric lymph nodes, and lung-associated lymph nodes (LuN) of the rat. As seen in previous studies, MN blasts localized within the intestine 10-fold better than did PN blasts. Significantly, LuN blasts also localized poorly within the intestine; instead, these cells entered lung-associated nodes 10 times better than did PN blasts and three times better than did MN blasts. In a study in sheep, Spencer *et al.* (1983) found that while MN and LuN blasts were retained equally well within the spleen or liver, MN blasts went to the intestine five times better than did LuN blasts, and LuN blasts went to lung twice as well as MN blasts. These results are supported further by van der Brugge-Gamelkoorn *et al.* (1986), who found that the distribution of specific antibody-containing cells after intratracheal antigen administration into rats was limited largely to the lung and its associated lymphoid tissues. A few antigen-specific cells were observed in the spleen, indicating that the cells had entered the circulation, but these cells were never observed in MN, PP, or PN.

Additional evidence for distinct homing specificities for the intestine and lung came from studies by Spencer and Hall (1984), who examined the recirculation of small lymphocytes obtained from lymph draining either the intestine or lung-associated lymph nodes in sheep. The cells were labeled with ^{51}Cr, injected intravenously, and their reappearance in the intestinal lymph and lung node lymph was compared. It was found that lung lymph cells returned to the lung lymph fivefold better than

did intestinal lymph cells, while the intestinal cells returned to intestinal lymph sixfold better than did the lung cells.

Crawford and Miller (1984) compared the prevalance of lymphocyte subsets resident within Peyer's patches and lung-associated lymphoid tissue in the rat. These investigators found that while Peyer's patches contain 57% B-cells and 21% T-cells (almost all of which are of the CD4 subset), the lung was composed of 15% B-cells and 62% T-cells (only 40% of which expressed the CD4 marker). The reciprocal representation of lymphocyte subsets within these lymphoid tissues may reflect their distinct binding preferences for lung-associated HEV versus PP HEV (as discussed above for PN and PP). van der Brugge-Gamelkoorn and Kraal (1985) used the Stamper-Woodruff *in vitro* assay to compare the attachment of B-cells and T-cells to HEV in bronchus-associated lymphoid tissue of the rat and guinea pig. Whereas B-cells bind preferentially to PP HEV and T-cells prefer to bind to PN HEV, both cell types bind equally well to HEV in the bronchus tissue. It was suggested that bronchus-associated HEV may express a hybrid mixture of PN- and PP-like HEV attachment sites, although an explanation based upon a distinct binding specificity is equally plausible.

These studies argue strongly for the existence of a unique lung homing specificity; that is, lymphocyte attachment to HEV within lung-associated lymphoid tissues would be distinguishable from lymphocyte attachment to HEV in either peripheral lymph nodes or in Peyer's patches. Using treatments that block selectively the activity of either the PN or PP homing receptor, in conjunction with the *in vitro* HEV-binding assay, Geoffroy *et al.* (1988) has confirmed the prediction of a unique lung HEV-binding specificity. For example, lymphocytes treated with trypsin in the presence of calcium bind normally to HEV in Peyer's patches (Yednock *et al.*, 1988a; and see Section IV,A,6); however, trypsin/calcium-treated lymphocytes are completely unable to bind to HEV in lymphoid aggregates within the lung or to HEV within the lymph nodes that drain the lung. Thus, the interaction of lymphocytes with lung-associated HEV is distinct from lymphocyte attachment to PP HEV. On the other hand, while treatment of lymphocytes with the monoclonal antibody MEL-14 inhibits their binding to PN HEV (see Section IV,A,1), it does not affect lymphocyte attachment to HEV in either lung lymphoid aggregates or lung lymph nodes; thus the lung interaction is also distinct from lymphocyte attachment to PN HEV. Several other reagents, affecting either the lymphocyte homing receptors or the HEV, have supported these conclusions.

Thus, the selective homing of lymphoblasts and the preferential recirculation of isolated lymphocyte populations *in vivo* predicted a unique

lymphocyte-binding specificity for lung-associated HEV that could be verified *in vitro*. This specificity is depicted in the model of Fig. 1. These studies with lung indicate that the idea of common mucosal immunity must be modified. Before further discussion of this issue, it will be useful to examine the role that organ-specific homing plays during an immune response.

D. Regional Lymphocyte Homing during an Immune Response

Two papers by Pierce and Cray (1981, 1982) provide considerable information concerning the homing tendencies of antigen-specific memory and effector cells during an immune response. Importantly, these studies also clearly demonstrate selective lymphoblast migration to the colon, exclusive of other mucosal sites such as the jejunum, ileum, and trachea. These studies will be reviewed in some detail because they illustrate several points that pertain to the general theme of organ-specific lymphocyte homing and recirculation, and because they predict a novel colon specificity for lymphocyte localization, perhaps within the larger sphere of "mucosal immunity."

1. Recirculation of Memory Cells

Pierce and Cray (1981) demonstrated that rats primed in the colon with cholera toxin produced antigen-specific memory cells that recirculated to populate the entire gut, as well as the trachea; thus, following colonic immunization, challenge with cholera toxin in the colon, jejunum, duodenum, or trachea produced an equally vigorous secondary immune response (scored histologically in tissue biopsies by quantifying the number of antigen-specific plasma cells). Thoracic duct lymphocytes were collected from a colon-primed animal and injected intravenously into a naive recipient; jejunal challenge of such an animal, either immediately after the cell transfer or 21 days later, produced a good secondary immune response. Thus, colonic priming produces specific memory cells that enter the circulation from the lymph to impart antigen-specific protection to distant mucosal sites. The memory cells apparently recirculate for extended periods since lymphocytes collected from the thoracic duct lymph 14 or even 56 days after priming could transfer specific immunity to naive recipients.

It should be appreciated that the recirculating population of memory cells is not large plasmablasts producing antibody, but are expanded populations of small lymphocytes that will produce blast T- and B-cells upon stimulation with specific antigen. Although Pierce and Cray showed that gut-derived memory cells can populate several mucosal tissues, they did not examine the ability of these cells to populate "peripheral" organs

such as lymph nodes. This issue has been addressed indirectly by an examination of the recirculation of small lymphocytes isolated from either PN or intestinal lymph of sheep (Reynolds *et al.*, 1982; Hall, 1980; Chin and Hay, 1980; Cahill *et al.*, 1979; Scollay *et al.*, 1976). Small lymphocytes, collected from PN and intestinal lymph ducts, were labeled with ^{51}Cr or ^{111}In and infused intravenously into the same animals. The consistent finding is that lymphocytes obtained from intestinal lymph or peripheral lymph node lymph will recirculate back to their respective lymphoid compartments twofold to sixfold better than to the other compartment. The preferential recirculation exhibited by these cell populations may be due to the large number of memory cells that they contain (Butcher *et al.*, 1982a), since fetal lymphocytes (presumably deficient in memory cell populations due to lack of antigen exposure), isolated from intestinal or peripheral lymph, do not exhibit organ-selective recirculation (Cahill *et al.*, 1979).

2. *Homing of Effector Cells versus Recirculation of Memory Cells*

Pierce and Cray (1982) also described the dynamics with which antibody-producing blast cells, or their immediate precursors, enter the circulation following immune challenge and home to distant mucosal sites. Unlike memory cells, these terminally differentiated lymphocytes do not recirculate to a significant degree; once they enter the target organ they remain tissue associated, secrete antibody, and probably die within a few days (Husband and Gowans, 1978). Pierce and Cray primed and challenged animals by intraintestinal injection of cholera toxin. Specific antibody-containing plasmablasts were found 3 to 5 days later within the thoracic duct lymph, from where they would enter the blood and populate the entire small intestine. The number of plasmablasts within the thoracic duct lymph of the challenged animal quickly diminished after 5 days, whereas the number of memory cells (those that would transfer specific immunity to naive rats) did not peak in the thoracic duct lymph until day 14, and persisted thereafter. These results illustrate the different migratory characteristics of plasmablasts and memory cells; plasmablasts are generated quickly in response to immune challenge, enter the circulation, and home to the site where they will perform their effector function and die; in contrast, memory cells are released to the circulation more slowly in response to antigen stimulation, home to the appropriate tissue sites, and then reenter the lymph and continue to recirculate.

It should be pointed out that lymphocyte homing is not influenced by the presence of antigen in the target tissue. This point has been established by a number of investigators (Weisz-Carrington *et al.*, 1979;

Husband and Gowans, 1978; Hall *et al.*, 1977; Parrott and Ferguson, 1974), but is elegantly shown in the studies of Pierce and Cray (1982). Rats were primed with cholera toxin by intracolonic injection and then were challenged 14 days later by injecting the antigen into a surgically isolated loop of jejunum (with lymph and blood supply intact). As expected, blast cells appeared 5 days later in the stimulated jejunal loop, but they also appeared in comparable numbers in the nonstimulated jejunum. Thus, antigen stimulation of memory cells in the isolated jejunal loop appeared to produce plasmablasts that entered the lymph and circulated to populate the entire jejunum. This was confirmed by cannulating and draining the thoracic lymph duct at the time of antigen challenge in the jejunal loop, thus preventing lymph-borne plasmablasts from entering the circulation. In this case, the number of blast cells found in the nonstimulated jejunum remained essentially at background levels, while the number in the stimulated jejunal loop was reduced only partially. Thus, some of the plasmablasts generated by antigen challenge in the jejunal loop remained at the site of stimulation to become antibody-producing cells, while many others entered the circulation from where they would have normally populated other parts of the intestine had the thoracic duct not been cannulated.

3. Potentially Distinct Lymphocyte Homing Specificities for the Large and Small Intestine

The Pierce and Cray studies further suggest that the colon is represented by a homing specificity different from that of the small intestine. As described above, antigen priming in the colon produced memory cells in the thoracic duct lymph that could transfer specific immunity to the entire intestine, as well as to the trachea of naive recipients. This result is consistent with a common mucosal line of defense. However, general mucosal dissemination of effector cells was not seen following secondary challenge; that is, when animals were both primed and challenged by intracolonic injection of cholera toxin (Pierce and Cray, 1982). Three days after the challenge, plasmablasts were collected from the thoracic duct lymph and injected intravenously into naive recipients. There was virtually no localization of these cells in the jejunum, whereas large numbers of specific antibody-producing cells populated the colon. In a reciprocal study, it was found that plasmablasts isolated from donors primed and challenged intraduodenally failed to localize in the colon of naive recipients, but homed in large numbers to the jejunum or to an isolated jejunal loop. Thus, it appears that plasmablasts can distinguish between the colon and the small intestine, suggesting that the colon may be represented by its own homing specificity. This

possibility could be addressed by application of the *in vitro* HEV-binding assay to the small lymphoid aggregates within the wall of the colon. It would also be of interest to examine the inferior mesenteric lymph node, which might also manifest a "colon specificity" since it receives its lymphatic input from the descending and sigmoid colon (Tilney, 1971).

4. Homing Specificity of Lymphocytes: Step-Wise Restriction with Differentiation?

The fact that plasmablasts can distinguish between the colon and jejunum is in contrast to the relatively broad "mucosal" specificity demonstrated by memory cells, which can populate the colon, jejunum, duodenum, and the trachea following colonic immunization. These findings, as well as the work of other investigators reviewed above, suggest that the range of homing specificities demonstrated by lymphocytes may be narrowed with each step of antigen-directed differentiation. Mature, virgin lymphocytes would exhibit little or no homing specificity, memory cells produced by initial antigen encounter would recirculate among a select group of related tissue sites, and, finally, terminally differentiated blast cells would home specifically to the site most similar to that of antigen encounter. Thus, the strategy of the immune system may be to allow virgin lymphocytes to recirculate promiscuously in order to expose sequestered antigen to the full repertoire of immune specificities. Once lymphocytes have been stimulated by cognate antigen, the resulting memory cells would be restricted to recirculate among tissues similar to the site of antigen encounter, i.e., the tissues where the same antigen is most likely to be reencountered. However, upon subsequent or continued antigen exposure, terminally differentiated, short-lived, and nonrecirculating effector cells would not be wasted with diffuse distribution, but would instead be directed to populate the very organ containing the site of antigen deposition, thus providing rapid and highly localized immune protection.

The sequential narrowing of homing specificity appears to vary depending upon the initial site of antigen encounter. For example, Pierce and Cray (1981) found that while intracolonic priming resulted in distribution of memory cells throughout the colon, jejunum, and trachea, the reverse strategy did not yield reciprocal results; intratracheal priming resulted in memory cell distribution within the trachea, to a lesser degree within the jejunum, and not at all within the colon. Similar results were obtained by Spencer *et al.* (1983), who found that injection of antigen into Peyer's patches resulted in antigen-specific cells within the trachea; however, stimulation of the trachea produced antigen-specific cells within the lung, but not the intestine.

5. Developmental Predetermination of Homing Specificity among Lymphoid Lineages

As discussed above, the homing specificities of at least some lymphocytes appear to be regulated by their history of antigen exposure as well as by the anatomic site in which antigen was encountered. These considerations raise an interesting question alluded to before; namely, to what degree are the homing specificities of different lymphoid lineages predetermined during development (prior to antigen exposure)? Stevens *et al.* (1982) proposed that "virgin" B- and T-cells are predetermined to bind preferentially to PP and PN HEV, respectively. This proposal is based on the observation that small T- and B-cells, regardless of their organ source, exhibited this selective pattern of HEV binding (e.g., B-cells bind better than T-cells to PP HEV, regardless of the source of the cells). However, in view of the results described above, it seems possible that the selective homing and HEV-binding characteristics exhibited by B- and T-cells could be attributed to memory cells, rather than to the intrinsic homing tendencies of virgin lymphocytes. Since Stevens *et al.* (1982) used adult mice that had encountered environmental antigens, it is likely that there were significant numbers of B- and T-memory cells within the cell populations obtained from these animals. Most B-memory cells, regardless of the organ from which they were isolated, would have been generated in response to antigen within mucosal lymphoid tissues, and hence would have been instructed to exhibit preferential binding to mucosal (PP) HEV. In other words, virgin B-cells may localize equally well within peripheral lymph nodes and Peyer's patches, but Peyer's patches may be specialized for B-cell activation in the presence of antigen; hence, more B- than T-memory cells would be generated within Peyer's patches and these cells would exhibit preferential PP HEV binding upon emergence into the recirculating pool. Likewise, a preferential response of T cells to antigen within peripheral lymph nodes would provide a disseminated memory T-cell population with the reverse bias.

Thus, the question becomes, do preferential HEV interactions control the influx of virgin lymphocyte subsets into a lymphoid organ, thereby regulating the character of immune response (as proposed by Stevens *et al.*), or does the lymphoid organ itself regulate the type of immune response, and hence instruct the responding lymphocytes, among the resident population, to demonstrate subsequent binding preference for HEV? Both proposals seem possible; however, studies in sheep would favor the latter. In apparent contrast to the findings of Stevens *et al.* (1982), antigen-naive fetal T-cells demonstrate no preferential localization within fetal sheep (Cahill *et al.*, 1979; see above), whereas, blast cells isolated from adult intestinal lymph and infused into fetal animals

localized preferentially within the small intestine (threefold to fivefold better than blasts isolated from adult PN lymph) (Hall *et al.*, 1977). This result suggests that while expression of HEV-specific ligands is predetermined by the lymphoid organ and occurs during development independent of antigen, lymphocytes demonstrate no migratory preference prior to antigen exposure. Therefore, virgin B- and T-cells may express equivalent levels of several homing receptor classes, and as a result of stimulation with antigen *in the context of a particular lymphoid compartment*, may suppress cell-surface expression of certain receptor subsets.

The fact that the same set of data can support such disparate hypotheses points to the need for further examination of the issue. The HEV-binding characteristics of B- and T-cell populations, derived from neonatal or antigen-free adult mice, should be compared to determine if "mucosal" or "peripheral" homing preferences actually accompany subset differentiation, independent of antigen. This issue is important because it relates to the developmental and environmental signals that regulate homing receptor expression.

It should be noted that no matter what the result is for virgin B- and T-cells, there may be other lymphocyte populations in which homing receptor expression is predetermined by antigen-independent developmental factors.

6. *Lymphocyte Isotype and the Specificity of Recirculation*

Weisz-Carrington *et al.* (1979) immunized mice by oral administration of ferritin and then examined the distribution and antibody isotype of ferritin-specific plasma cells in tissue sections. Approximately the same density of antiferritin plasma cells was found in all lymphoid tissues examined. But within mucosal organs, including the lactating mammary gland, parotid gland, small intestine, respiratory tract, mesenteric lymph node, and Peyer's patch, the ratio of IgA to either IgG or IgM plasma cells was 3:1. In peripheral lymph nodes and the spleen this ratio was reversed. MN and PN cells were then isolated from the immunized mice and injected intravenously into naive recipients. Plasma cells derived from MN cells were found in the highest numbers within the mammary gland and small intestine; in these tissues the ratio of IgA to IgM and IgG plasma cells was 20:5:1. Large numbers of plasma cells were also seen in the mesenteric lymph nodes, but with equal representation by the three isotypes. Smaller but substantial numbers of plasma cells were found in peripheral lymph nodes, and here the cells were almost exclusively of the IgG and IgM isotypes. When cells isolated from peripheral lymph nodes were injected intravenously, almost no IgA plasma cells were found in any of the tissues. Few plasma cells were found

within the breast or small intestine, intermediate numbers were within the mesenteric lymph node (mostly IgG), while large numbers of PN-derived plasma cells were found in peripheral lymph nodes (almost equivalent numbers of IgG and IgM). Thus, it can be concluded that IgA-specific plasmablasts home preferentially to mucosal and mucosal-associated tissues (intestine, breast, and MN), while IgG cells, regardless of the organ source, demonstrate preferential homing to peripheral lymph nodes.

Studies by Roux *et al.* (1977) corroborate these conclusions. When MN blast cells (only 5–10% of which expressed IgA) were injected intravenously into mice, approximately 90% of those that localized in the breast were IgA positive. Furthermore, while treatment of MN cell populations with anti-IgA plus complement prior to injection killed only a small percentage of the cell population, cell localization within the breast and gut was reduced by as much as 75%. In contrast, treatment with either anti-IgG plus complement or IgM plus complement killed 20–40% of the cells prior to injection, but had only minimal effect on the localization of cells within the breast or gut.

Thus, these studies indicate that local environmental signals, impinging on lymphocytes during antigen stimulation, may coregulate homing receptor specificity and isotype differentiation.

E. Regulation of HEV Specificity

1. *Potential Involvement of Lymph-Borne Factors*

It has been suggested that lymph draining from a particular anatomical region carries regional-specific factors that dictate the homing specificity of the HEV within the draining node. For example, mesenteric lymph node HEV exhibit a hybrid character, expressing approximately an equal mixture of PN- and PP-like HEV ligands (Stevens *et al.*, 1982; Rosen *et al.*, 1985; Streeter *et al.*, 1988; see Fig. 1). The PP-like properties of mesenteric nodes presumably reflect the fact that these nodes receive considerable lymphatic input from the gut (Butcher *et al.*, 1980). Fluid from the peritoneal cavity also drains, to a moderate extent, through mesenteric nodes (Oghiso and Matsuoka, 1979). The partial PN-like character of MN HEV may be attributable to the fact that lymphocyte as well as neutrophil migration into the peritoneal cavity involves a PN-like homing specificity (Rose *et al.*, 1978; Lewinsohn *et al.*, 1987).

As reviewed above, the specificity of lymphocyte binding to HEV within the lung hilar lymph node appears to resemble that in lymphoid

aggregates present within the lung. A common specificity is not surprising in view of the fact that the hilar node receives most, if not all, of its lymphatic input from the lung. Interestingly, HEV within this node have characteristics completely distinct from either PN or PP HEV, while HEV within paratracheal lymph nodes share characteristics of HEV in both the lung and peripheral lymph nodes (Geoffroy *et al.*, 1988; see Fig. 1). A possible explanation of this finding is the fact that paratracheal nodes receive combined lymphatic input from the lung and peritoneal cavity (Oghiso and Matsuoka, 1979). Further study is needed to compare the drainage fields of the hilar and paratracheal lymph nodes in the mouse, but the differential representation of an apparent lung-like HEV specificity within these nodes is compatible with the notion of regulatory factors carried by the lymph.[6]

Lymph-borne factors, by dictating HEV specificity, could be critical in defining spheres of regional immunity. One can envision a series of distinct homing specificities associated with different organs and their draining lymph nodes. Antigens that invade a particular organ would be carried by the lymph to the nearest node, where an immune response could be established. The node would instruct responding lymphocytes to express the appropriate organ-specific homing receptors, and resulting effector blast cells, which extravasate efficiently through flat-walled vessels, would then recirculate selectively back to the organ containing antigen. Memory cells produced during this response would continue to recirculate through this and functionally related organs, as discussed above. The existence of region-specific lymph-borne factors would also result in nodes expressing hybrid HEV-binding specificity as a result of receiving lymphatic input from more than one region of the body,[7] as proposed for the mesenteric and paratracheal lymph nodes. Such nodes could provide overlapping spheres of regional immunity; antigen encounter within a hybrid node would result in simultaneous protection of multiple organ systems.

[6] Given the different HEV specificities exhibited by lymph nodes associated with distinct anatomical sites, the descriptive terms "mucosal" and "peripheral" seem wholly inadequate. The designations utilized by Tilney (1971), "visceral" versus "somatic" (or cutaneous) lymph nodes, appear to reflect more accurately the refined specificity of this system.

[7] A testable prediction of this hypothesis is that axillary lymph nodes, which are normally represented exclusively by a PN HEV specificity, would exhibit PP HEV-like ligands at the time of lactation, owing to the fact that they receive lymphatic drainage from the breast (the breast at the time of lactation exhibits an "intestinal" homing specificity; see Section III,B,2).

2. *How Many Homing Specificities Exist?*

Thus far, we have reviewed evidence, either direct or strongly suggestive, for the existence of several distinct homing specificities (e.g., PN, PP, lung, colon). A central, unanswered question is how many specificities exist; that is, what is the level of anatomical refinement in this immune protection system? Freemont (1983) observed HEV-like vessels within lymphocytic infiltrates associated with 24 different human diseases. Transmigrating lymphocytes could be discerned within the wall of many of these structures. In general, the vessels were detectable when the lymphocyte density exceeded 150 lymphocytes/mm^2, supporting the notion that HEV represent a morphological adaptation of postcapillary venules to increase an otherwise low, constitutive influx of lymphocytes. Included among the tissues containing HEV-like vessels were the colon, parotid gland, muscle, kidney, esophagus, thyroid, stomach, breast, testicle, skin, bladder, prostate, and liver, as well as synovium from arthritic patients. What is the lymphocyte-binding specificity of these HEV-like structures? Do lymphocytes trafficking through these sites of inflammation use the same homing mechanisms employed for localization within secondary lymphoid tissues, such as lymph nodes and Peyer's patches,[8] and, if so, which homing receptor specificities do they choose? As discussed above, narrow homing specificities allow the immune system to efficiently protect a particular organ with antigen-specific effector cells. Thus, it may be advantageous for even very limited anatomical sites to exhibit their own specificities for lymphocyte homing.

Pertinent to the question of anatomical refinement is the study of lymphocyte attachment to HEV-like vessels within the synovium of patients with rheumatoid arthritis (Jalkanen *et al.*, 1986b). PN HEV-specific lymphoma cell lines do not bind to HEV in the synovium, and lymphocyte binding to synovial HEV is not affected by the monoclonal antibody MEL-14, which inhibits lymphocyte binding to PN HEV. These results argue that the synovial interaction is distinct from the interaction of lymphocytes with PN HEV. In addition, lymphoma cell lines that bind well to PP HEV fail to bind to HEV in synovium, indicating that the PP and synovial HEV interactions are also distinct. Thus, lymphocyte attachment to synovial HEV appears to involve a separate specificity, which might be restricted to the synovium of joints, or perhaps is shared by a limited number of other organ systems. Jalkanen *et al.* (1986b) raise the additional possibility that binding of lymphocytes to HEV

[8] In addition to the secondary lymphoid tissues discussed previously, evidence of Pals *et al.* (1986) suggests that tonsils may have a unique HEV specificity as well.

in inflamed synovium may reflect a general "inflammatory" specificity. The next section addresses the general issue of lymphocyte migration into sites of chronic inflammation and discusses the possible interplay between region-specific homing and inflammation.

F. Lymphocyte Homing and Inflammation: Site Specificity within a Regional Sphere?

In a site of tissue damage or infection, there is an increased influx of neutrophils, monocytes, and lymphocytes through flat-walled post capillary venules outside of lymphoid tissues (Dvorak *et al.*, 1976). The extravasation of these cell types during an inflammatory response differs temporally; neutrophils are the first to enter, within minutes of the original tissue insult, followed by monocytes, and then several hours later by lymphocytes. Chronic inflammation can lead to the development of a semiorganized lymphoid tissue, replete with T- and B-cell domains, as well as HEV-like vessels within regions of dense lymphocyte aggregates. Presumably, the influx of leukocytes into inflammatory lesions involves multiple types of leukocyte/endothelial adhesive interactions, depending upon the cell type and time after initial tissue injury.

With respect to lymphocytes, entry into sites of inflammation appears to involve endothelial interactions that retain a certain degree of regional specificity. Rose *et al.* (1976) found that cutaneous inflammation produced by painting mouse ears with oxazolone increased the localization of both MN and PN blasts to the site; however, the PN blasts migrated to the inflamed ear sevenfold better than did the MN blasts. Similarly, inflammation of the intestine produced by parasitic infection increased PN and MN blast localization within the gut, but MN blasts entered this site better than did PN blasts. Furthermore, if PN blasts were given the choice between inflammatory sites in the ear or small intestine of the same animal, they chose the ear site in preference to the gut.

Although a general regional specificity ("cutaneous" versus "intestinal") appears to be maintained for lymphocyte migration into sites of inflammation, additional adhesive mechanisms, other than region-specific homing receptors, may be involved. Chin and Hay (1980) compared the recirculation (in sheep) of small lymphocytes isolated from lymph draining the small intestine, a peripheral lymph node, and a cutaneous site of inflammation. While gut cells preferentially returned to gut lymph and PN cells to PN lymph, the inflammatory cells were recovered in highest number within inflammatory lymph. Other examples (Issekutz *et al.*, 1980, 1986) have been reported of lymphocyte populations, both small lymphocytes and blast cells, that migrate to sites of cutaneous inflammation in preference to peripheral lymph nodes. Although these

populations appear to exhibit an "inflammatory specificity," a PN homing tendency is still discernible. For example, in the Chin and Hay study described above, lymphocytes derived from inflammatory lymph were able to migrate to PN lymph, albeit sixfold less well than to inflammatory lymph. However, recovery of these cells within intestinal lymph was virtually undetectable. A possible, if somewhat speculative, explanation of these findings is that lymphocyte migration into cutaneous inflammatory sites involves a region-specific homing receptor (i.e., the PN homing receptor) and a general accessory adhesion molecule. Flat-walled vessels in the skin would express the same ligand as is present on PN HEV, in addition to a ligand for the general adhesion receptor. Up regulation of the general adhesion ligand on the flat-walled endothelial cells during inflammation would select for the attachment of circulating cells that express the specific homing receptor in addition to the general cell adhesion receptor.

For illustrative purposes, consider the class II MHC molecules as possible "general ligands" for cell adhesion; this is a relevant example, since expression of class II MHC can be induced on endothelial cells by inflammatory mediators (Bevilacqua *et al.*, 1985). Nonpolymorphic regions of class II MHC are thought to interact directly with the T-lymphocyte cell-surface antigen, CD4 (Littman, 1987), and class II MHC on B-cells has been shown to support an adhesive interaction with CD4-transfected cells (Doyle and Strominger, 1987). During an inflammatory response, endothelial cells may be induced to express high levels of class II MHC and the PN HEV-like ligand, thus creating a bias for the adhesion of cells expressing both the PN homing receptor and the CD4 antigen. In fact, sites of chronic inflammation have been found to be populated largely by CD4+ T-lymphocytes (Duijvestijn *et al.*, 1988). Thus, the expression of MHC class II at inflammatory sites, in conjunction with the regional homing ligand, could produce site specificity within the regional sphere. According to this model, the small cells isolated from inflammatory lymph by Chin and Hay would be enriched for a subset of CD4+ cells that express the PN homing receptor, and thus these cells, upon intravenous injection, would migrate preferentially to the cutaneous site of inflammation.[9]

[9] It should be noted that lymph draining from noninflamed skin, or from any organ, contains low levels of small lymphocytes, blast cells, and monocytes (Smith *et al.*, 1970b). Issekutz *et al.* (1980) isolated small lymphocytes from normal cutaneous lymph and found these cells to recirculate to cutaneous lymph better than to peripheral lymph nodes. Thus, populations of lymphocytes exist that can establish a functional adhesive interaction with flat-walled vessels in noninflamed sites. According to the model presented

The notion that the migration of lymphocytes through flat-walled endothelium involves tissue-specific homing receptors acting in concert with more general cell adhesion molecules is supported by studies with neutrophils. The membrane glycoproteins LFA-1/Mac-1, members of a leukocyte integrin family, are involved in the attachment of neutrophils to endothelium at sites of inflammation (Arfors *et al.*, 1987). Interestingly, almost all neutrophils (96%) also express high levels of the MEL-14 antigen (Lewinsohn *et al.*, 1987), a molecule strongly implicated in the selective attachment of lymphocytes to PN HEV (see Section IV,A). Treatment of neutrophils with the antibody MEL-14 inhibits their migration into a site of acute, cutaneous inflammation by approximately 65% and into the inflamed peritoneal cavity by 50% (consistent with the suspected PN-like specificity of this site; see above). This result suggests that the MEL-14 antigen on neutrophils functions in parallel with members of LFA-1/Mac-1 family to mediate an endothelial interaction. Unfortunately, the tissue specificity of the antibody blockade was not addressed, since localization within inflamed intestinal sites was not examined. However, MEL-14 was found to inhibit the *in vitro* attachment of neutrophils to PN HEV, but not to PP HEV, demonstrating that the MEL-14 antigen expressed by neutrophils retains tissue specificity. Thus, it appears that dual adhesive mechanisms, one general (LFA-1/Mac-1) and one tissue-specific (the MEL-14 antigen), can function in parallel to support the neutrophil adhesive interaction.

In summary, regional specificity appears to be maintained during an inflammatory response. As argued above, a general adhesive mechanism that is subject to regulation by inflammatory mediators could operate in parallel with tissue-specific homing receptors to give a site of inflammation its own subsite specificity within the regional sphere. Regulated expression of homing receptors, accessory cell adhesion molecules, and endothelial ligands offers a number of ways to generate regional and subsite-specific leukocyte homing.

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in this section, these cells may express exceptionally high levels of one or more general cell adhesion receptors, whose ligands are present on noninflamed endothelium. Inflammation may involve the upregulation of these "normal" ligands. Consistent with this idea, Issekutz *et al.* found the cutaneous lymph cells would migrate exceptionally well to sites of cutaneous inflammation. Blast cells, which normally migrate through flat-walled vessels in noninflamed tissues, may express a complement of cell adhesion molecules similar to the cutaneous cells isolated by Issekutz *et al.* (1980) since they also migrate exceptionally well to sites of inflammation (Issekutz *et al.*, 1986; see Fig. 1).

IV. Molecules Involved in Lymphocyte Attachment to HEV

A. Lymphocyte-Associated Molecules

1. *The MEL-14 Antigen as a Putative PN-Specific Homing Receptor*

The monoclonal antibody MEL-14 was selected for its ability to react with the cell surface of lymphoma cell lines that attach selectively to PN HEV (Gallatin *et al.*, 1983). To date, the ability of lymphocytes or lymphoma cell lines to bind to PN HEV is strictly correlated with expression of the MEL-14 epitope, although expression of the epitope on certain cell lines and perhaps some leukocytes does not assure PN HEV-binding activity (Lewinsohn *et al.*, 1987; M. A. Jutila and E. C. Butcher, personal communication; and personal observations). MEL-14 reacts with the majority of mouse peripheral lymphocytes and inhibits lymphocyte and lymphoma cell line binding to PN HEV, but not to PP HEV, *in vitro*. Furthermore, treatment of lymphocytes with MEL-14 prior to intravenous infusion inhibits their localization within peripheral lymph nodes, but not within Peyer's patches. Thus, the MEL-14 antigen is thought to either mediate lymphocyte attachment to PN HEV directly, or to be involved intimately in this interaction. The MEL-14 epitope does not function in lymphocyte attachment to PP HEV since it is not expressed by a number of PP HEV-specific lymphoma cell lines and since the antibody does not inhibit the attachment of MEL-14-positive cells to PP HEV. As discussed in the next section, the MEL-14 antigen is functionally associated with a carbohydrate-binding activity that is required for lymphocyte attachment to PN HEV.

MEL-14 precipitates an 85- to 95-kDa glycoprotein from detergent lysates of cell-surface-radiolabeled mouse and human lymphocytes (Jalkanen *et al.*, 1987). The apparent molecular weight of this antigen increases with reduction, suggesting the presence of intrachain disulfide bonds, a common feature of may cell-surface receptors. The antigen is heavily glycosylated, with carbohydrate constituting up to 50% of its mass (Siegelman *et al.*, 1986). The protein core of the antigen is apparently modified further by the addition of one or more chains of the 80-amino acid polypeptide, ubiquitin; thus, the antigen is thought to be a branched-chain protein with at least two amino termini (Siegelman *et al.*, 1986).

2. *The MEL-14 Antigen as a Member of a Family of Related Cell Adhesion Molecules*

A molecule has been identified on human lymphocytes that appears to be the human equivalent of the MEL-14 antigen. This antigen was

first identified by a monoclonal antibody, Hermes-1, and has been shown subsequently to cross-react weakly with the MEL-14 antibody (Jalkanen *et al.*, 1986, 1987). Hermes-1 and MEL-14 both precipitate a 90-kDa protein from the surface of human lymphocytes. When a detergent lysate of human lymphocytes is precleared with Hermes-1 linked to Sepharose, MEL-14 no longer precipitates the 90-kDa protein, indicating that Hermes-1 cross-reacts with the MEL-14 antigen. Hermes-1, however, recognizes an epitope on the 90-kDa protein distinct from MEL-14, since Hermes-1 reacts with lymphoma cell lines that bind to either PN or PP HEV. Furthermore, Hermes-1 has no blocking activity, whereas MEL-14 inhibits human lymphocyte attachment to PN HEV. As in the mouse system, MEL-14 has no effect on human lymphocyte attachment to mouse PP HEV or to HEV in human appendix.

A polyclonal immune serum and additional monoclonal antibodies were prepared against purified Hermes-1 antigen, isolated from a PP HEV-specific human lymphoma. One of the resulting monoclonal antibodies, Hermes-3, recognizes the same 90-kDa antigen as Hermes-1, based on reciprocal preclearing experiments. *In vitro*, Hermes-3 inhibits human lymphocyte attachment to HEV in either mouse PP or human appendix, but not to mouse or human PN HEV. Thus Hermes-3 appears to recognize an epitope on the 90-kDa glycoprotein that is involved selectively in lymphocyte attachment to PP HEV. Preclearing experiments established that the polyclonal serum (anti-gp90) recognizes the same antigen as Hermes-1 and Hermes-3. Interestingly, despite the fact that this polyclonal serum was prepared against Hermes-1 antigen isolated from a PP HEV-specific lymphoma, it blocks lymphocyte attachment to both PN and PP HEV. Jalkanen *et al.* suggest that gp90 actually represents a family of closely related but distinct molecules; one member of which would mediate lymphocyte attachment to PN HEV, and another would mediate attachment to HEV in Peyer's patches. Since the molecules are closely related they share common epitopes, such as those recognized by Hermes-1 and Hermes-3, but since they have different HEV-binding specificities, unique epitopes of each are predicted. For example, MEL-14 apparently recognizes a particular epitope on a subset of 90-kDa molecules that is involved in lymphocyte attachment to PN HEV. As for the ability of Hermes-3 to recognize all members of the 90-kDa family, yet inhibit only lymphocyte attachment to PP HEV, the investigators suggest that the Hermes-3 epitope does not represent the active binding site of the PP HEV homing receptor, but interferes indirectly with the activity of this site.

3. Involvement of the MEL-14/Hermes gp90 Family in Lymphocyte Attachment to HEV in Inflamed Synovium

As described in the previous section, lymphocyte attachment to HEV in the inflamed synovium associated with rheumatoid arthritis is based on a binding specificity that is different from that for either PN or PP HEV. However, Jalkanen *et al.* (1987) have demonstrated that this interaction appears to involve the same 90-kDa family of lymphocyte molecules described above. Although the synovial interaction is not affected by Hermes-1 or Hermes-3, it is inhibited by the polyclonal anti-gp90 serum. These workers also demonstrate that a serum raised against whole lymphocytes blocks lymphocyte attachment to PN, PP, and synovial HEV; yet, if this serum is preabsorbed with purified 90-kDa molecules, it no longer affects any of the three HEV interactions. Thus, the authors speculate that a distinct subset of the 90-kDa molecules participates in the interaction of lymphocytes with a unique synovial HEV ligand, or perhaps with an inflammation-specific HEV ligand.

4. Soluble HEV-Binding Factors

A series of studies by Chin, Woodruff, and colleagues (Chin *et al.*, 1980a,b, 1982, 1983, 1984, 1986; Rasmussen *et al.*, 1985; Woodruff *et al.*, 1987) have described the isolation of two factors from rat thoracic duct lymph that interact with HEV *in vitro* and inhibit lymphocyte attachment. One of these high endothelial binding factors ($HEBF_{PP}$) inhibits selectively lymphocyte attachment to HEV in Peyer's patches, and the other ($HEBF_{LN}$) inhibits lymphocyte attachment to HEV in peripheral lymph nodes. A monoclonal antibody (1B.2) against $HEBF_{PP}$ reacts with the surface of 50–60% of rat thoracic duct lymphocytes and blocks *in vitro* lymphocyte adherence to PP HEV, but not to PN HEV. Another monoclonal antibody (A.11) is directed against $HEBF_{LN}$ and reacts to a similar extent with thoracic duct lymphocytes, but exhibits the converse blocking specificity, i.e., inhibiting the PN and not the PP interaction. Each antibody is also able to prevent lymphocyte homing *in vivo* to the appropriate lymphoid organ without affecting localization to the other organ type, the spleen, or nonlymphoid organs.

To determine if the differential expression of these molecules by lymphocytes regulates their HEV-binding and homing preferences, thoracic duct lymphocytes were fractionated by panning on antibody-coated dishes. Two types of populations were obtained, one enriched for cells that express $HEBF_{LN}$ and the other for cells that express $HEBF_{PP}$, and these demonstrated the predicted HEV-binding preferences *in vitro*. Furthermore, a population depleted in $HEBF_{PP}$

expression exhibited poor localization within Peyer's patches *in vivo*, but migrated normally to peripheral lymph nodes. Consistent with the model depicted in Fig. 1, a substantial number of lymphocytes express both $HEBF_{PP}$ and $HEBF_{LN}$, and thus are able to bind well to both PN and PP HEV, *in vitro*, and to migrate to both tissue types, *in vivo* (Woodruff *et al.*, 1987).

These results suggest that the HEBFs are lymphocyte homing receptors, or fragments thereof, that have been shed from the cell surface in an active, soluble form. To test this hypothesis, the cell-associated antigens recognized by the monoclonal antibodies were isolated from detergent lysates of rat lymphocytes and tested for HEBF activity. The isolated antigens were dialyzed into PBS and incubated with sections of lymphoid tissues prior to the HEV-binding assay. Antigen purified with anti-$HEBF_{PP}$ inhibited lymphocyte attachment to PP HEV, but not to PN HEV; while antigen isolated with anti-$HEBF_{LN}$ inhibited lymphocyte binding to PN HEV and not to PP HEV. No HEBF activity would be isolated from cells that had been trypsinized prior to detergent lysis, indicating that this activity is associated with cell-surface molecules (Chin *et al.*, 1983).

5. Relationship between the HEBFs and the 90-kDa Family of Putative Homing Receptors

The HEBFs and the 90-kDa family of receptors share many characteristics, and yet appear to be different molecules. For example, the MEL-14 antigen and $HEBF_{LN}$ are both expressed on 50–70% of peripheral lymphocytes, are poorly expressed on thymocytes and bone marrow lymphocytes, and are involved in the selective attachment of lymphocytes to PN HEV (both *in vitro* and *in vivo*). Both molecules are also found on lymphocytes that can bind to PP HEV, and thus can evidently be coexpressed with the PP-specific homing receptor. The biochemical properties of the MEL-14 antigen and $HEBF_{LN}$ differ dramatically, however; the MEL-14 antigen has an apparent molecular weight of 85,000–95,000 Da, which increases with reduction; $HEBF_{LN}$, when precipitated with the monoclonal antibody A.11 from surface-radioiodinated cells, is represented by three proteins with apparent molecular weights of 135,000, 60,000, and 40,000 Da, none of which change with reduction (Rasmussen *et al.*, 1985); finally, the isoelectric points of the $HEBF_{LN}$ proteins range from 8.3 to 8.6 (Chin *et al.*, 1983), while the p*I* of the MEL-14 antigen is 4.0–4.5 (Siegelman *et al.*, 1986). It seems implausible that the differences between the MEL-14 antigen and $HEBF_{LN}$ represent mere species variance (mouse versus rat), since the antigens isolated with MEL-14 from mouse and human lymphocytes are

indistinguishable by SDS-PAGE analysis (Jalkanen *et al.*, 1987), and since the biological activity of $HEBF_{LN}$ is not species restricted between the human and rat (Woodruff *et al.*, 1987).

It is possible that the monoclonal antibody used to isolate $HEBF_{LN}$ (A.11) cross-reacts with several unrelated but highly represented cell-surface proteins, thus confounding the biochemical analysis. In contrast to rat $HEBF_{LN}$, the analysis of human $HEBF_{LN}$ and rat $HEBF_{PP}$ yield simple and similar molecular profiles. A monoclonal antibody (3.A.7), raised against human $HEBF_{LN}$ derived from serum, blocks selectively the attachment of human lymphocytes to PN HEV (Woodruff *et al.*, 1987). This antibody precipitates a single protein of 80 kDa from the surface of radioiodinated human lymphocytes (L. M. Clarke and J. J. Woodruff, personal communication). Unlike the 90-kDa molecules defined by MEL-14 and the Hermes antibodies, the molecular weight of this protein does not change with reduction (L. M. Clarke and J. J. Woodruff, personal communication). Similar to human $HEBF_{LN}$, $HEBF_{PP}$ isolated from rat lymphocytes by the monoclonal antibody 1B.2 is a single 80-kDa cell-surface protein that does not change in apparent molecular weight with reduction (Chin *et al.*, 1986). Thus rat $HEBF_{PP}$ and human $HEBF_{LN}$ appear to be related molecules with distinct HEV-binding activities.

It remains to be determined whether the 80-kDa HEBFs are, in fact, different from the 90-kDa proteins, or whether the molecular differences are attributable to technical matters. The question of the relatedness of these molecules poses a key issue in the homing receptor field and warrants special attention. If the 80- and 90-kDa proteins are not homologous, the possibilities exist that they represent separate and parallel adhesive mechanisms for lymphocyte attachment to HEV, or, alternatively, separate subunits of a single HEV-binding complex.[10]

6. *Lymphocyte Homing Receptors as Calcium-Dependent, Protease-Resistant Cell Adhesion Molecules*

Stamper and Woodruff (1977) were the first to demonstrate that lymphocyte attachment to rat PN HEV *in vitro* requires calcium. Recent quantitative measurements have revealed that maximal lymphocyte attachment to both PN and PP HEV (mouse) occurs at calcium

[10] One possibility is that HEBF is a peripheral membrane protein that depends upon a particular member of the 90-kDa family for association with the cell surface. This suggestion is consistent with the known HEV-binding activity of the HEBFs and their presence as soluble factors in the lymph and serum. In such a model, MEL-14 might inhibit lymphocyte attachment to HEV by dissociating $HEBF_{LN}$ from the cell surface, whereas Hermes-3 would preferentially dissociate $HEBF_{PP}$.

concentrations of 10^{-4} M or greater (Yednock *et al.*, 1988a). Remarkably, 1 mM magnesium will supplement concentrations of calcium as low as 10^{-6} M to allow control-level lymphocyte binding to PP HEV, but will not, at any concentration of calcium, contribute to the PN HEV interaction. Below 10^{-6} M calcium, there is no lymphocyte binding to either type of HEV, regardless of the presence of magnesium. Thus, while the attachment of lymphocytes to both PN and PP HEV requires calcium, these interactions differ absolutely in the ability of magnesium to synergize low calcium concentrations.[11]

Further experiments provide evidence that both the PN and PP lymphocyte homing receptors interact directly with calcium (Yednock *et al.*, 1988a). The principal observation is that calcium protects the biological activity of both receptors from the serum protease plasmin; lymphocytes treated with plasmin in the presence of calcium bind at near-control levels to PN and PP HEV, while binding is eliminated by treatment with plasmin in the absence of calcium. Furthermore, plasmin-calcium treatment of the isolated MEL-14 antigen has no effect on the protein's apparent molecular weight, as determined by SDS-PAGE analysis, while plasmin treatment in the absence of calcium results in extensive degradation. Thus, an additional correlation is established between the PN homing receptor, assayed by functional activity, and the MEL-14 antigen; both appear to interact with calcium to assume a conformation that is plasmin resistant.

With respect to the PN homing receptor, the serum protease, plasmin, is unusual. Treatment of lymphocytes, in the presence or absence of calcium, with all other proteases examined (including trypsin,[12] chymotrypsin, papain, V8 protease, proteinase K, and pronase) eliminates binding to PN HEV. A close correlation exists between the

[11] There is a striking similarity in divalent cation requirements for the activities of the PP homing receptor (Yednock *et al.*, 1988a) and the platelet receptor IIb/IIIa (Phillips and Baughan, 1983): both molecules appear to contain two divalent cation binding sites; one with high affinity (1 μM) that requires calcium, and another with lower affinity (1 mM) that can accommodate either calcium or magnesium. LFA-1 has similar but opposite characteristics; the activity of this molecule has an absolute requirement for magnesium, but in the presence of low magnesium can be supported by calcium. The PN homing receptor is distinct from all of these molecules since its activity depends entirely upon calcium; magnesium cannot support activity at any calcium concentration. Thus, the divalent cation requirements for the activities of the lymphocyte homing receptors present interesting comparisons to those of LFA-1 and gpIIb/IIIa (LFA-1 and gpIIb/IIIa are members of the integrin superfamily of cell adhesion molecules; Hynes, 1987).

[12] Stamper and Woodruff (1977) were the first to demonstrate that trypsin treatment of rat lymphocytes eliminates PN HEV-binding activity *in vitro*.

sensitivity of the MEL-14 epitope on intact lymphocytes to the various proteases and the ability of the protease-treated cells to bind to PN HEV, strengthening further the case for an involvement of the MEL-14 antigen in PN HEV binding. Thus, plasmin-calcium has no effect on the epitope or on PN HEV binding, whereas both are eliminated by plasmin-EDTA, as well as by all of the other proteases (independent of calcium).

When the isolated MEL-14 antigen is exposed to trypsin in the presence of calcium, an approximate 8-kDa reduction in molecular weight is observed. However, in the absence of calcium, trypsin produces extensive degradation; again, suggesting a direct interaction of calcium with the protein (Yednock *et al.*, 1988a). As noted above, the MEL-14 antigen is modified covalently by ubiquitin, which is thought to form an important constituent of the epitope recognized by the MEL-14 monoclonal antibody (Siegelman *et al.*, 1986). Since ubiquitin has a molecular weight of 8000 Da and contains a trypsin-cleavage site near its carboxy terminus (ubiquitin links to proteins via its carboxy terminus), it is tempting to speculate that trypsin-calcium treatment selectively removes ubiquitin from the core protein of the MEL-14 antigen. If this suggestion is true, removal of ubiquitin renders the MEL-14 molecule both serologically and functionally inactive. Thus, trypsin-calcium treatment may provide a means to examine the role that ubiquitin, in its unusual association with a cell-surface protein, plays in the activity of the PN homing receptor.

A striking observation is that, while trypsin-calcium treatment eliminates the ability of lymphocytes to bind to PN HEV, this treatment has no effect on lymphocyte binding to PP HEV. Furthermore, none of the other proteases examined, with the exception of high concentrations of pronase, affects PP HEV-binding activity when lymphocytes are treated in the presence of calcium. In contrast, exposure of lymphocytes to trypsin in the absence of calcium completely eliminates their ability to bind to PP HEV. These findings establish a convenient method of preparing a population of lymphocytes that can bind to PP HEV, but not to PN HEV (i.e., trypsin-calcium treatment). As described in Section III,C,2, this tool was employed for the analysis of the homing receptor specificity involved in lymphocyte attachment to HEV within lung-associated lymphoid tissues.

A number of cell-cell adhesive interactions exhibit a requirement for calcium, and in several examples the presence of calcium renders the cell adhesion molecules protease resistant (Hatta and Takeichi, 1986; Damsky *et al.*, 1983; Brackenbury *et al.*, 1983). A key question for the homing receptors, as well as for other calcium-dependent adhesion molecules, concerns the possible regulatory role for calcium. In general, the concentration of extracellular calcium (approximately 1 m*M*)

exceeds greatly the concentration required for receptor activity (usually < 0.1 m*M*), thus precluding a role for calcium in modulating adhesive receptor function once the receptor has been expressed on the cell surface. However, calcium regulation may be exerted during the transit of the receptor molecules from intracellular sites to the surface of the cell. This possibility is illustrated by the cytolytic protein, perforin (Young and Cohn, 1987). Perforin is stored by cytotoxic T cells and natural killer cells within secretory granules that are exocytosed following conjugate formation with the target cell. During storage in the presence of low intracellular calcium, perforin exists as an inactive soluble monomer; however, when secreted and exposed to the relatively high levels of extracellular calcium, perforin undergoes a conformation change that allows the monomer to intercolate into the target cell membrane and then to self-aggregate, forming lethal pores. Obviously, it would be disadvantageous for the cytotoxic T-cell to store perforin in an active state, and regulation by calcium provides a mechanism for activation at the time of secretion. Likewise, activation of the calcium-dependent cell adhesion molecules may be appropriate only after their arrival at the cell surface. The "activation" process may involve acquisition of the receptor's ligand-binding activity, regulation of turnover rate, self-aggregation to form a multivalent adhesive complex, association with accessory molecules, or the interaction of transmembrane proteins with cytoskeletal elements. In principle, a calcium-induced conformational change in the molecule could trigger any of these hypothetical activation processes. Understanding the possible functional significance for the calcium dependency exhibited by many cell adhesion molecules may require careful examination of the events that occur during the transit of these molecules to the cell surface.

7. *Possible Role of Accessory Molecules in HEV Binding*

If the interactions of lymphocytes with antigen-presenting cells or target cells serve as examples of adhesive systems (Goverman *et al.*, 1986), then the mechanisms underlying lymphocyte attachment to the different types of HEV and flat-walled vessels can be expected to be complex, indeed. Multiple adhesive interactions may be required to strengthen lymphocyte binding to HEV, and as discussed in Section IV,F, differential expression of accessory molecules at sites of inflammation may serve to regulate the degree and selectivity of lymphocyte influx. The evidence reviewed above indicates that the 90-kDa proteins and the HEBF family (in an as-yet unknown interrelationship) are involved in organ-specific HEV binding. Below, several general cell adhesion molecules will be discussed that may work in conjunction with the organ-specific receptors to mediate lymphocyte homing.

LFA-1 (lymphocyte function-associated antigen-1) is a member of an integrin subfamily (comprised of LFA-1, Mac-1, and p150,95),[13] which is found exclusively on leukocytes (Hynes, 1987). This molecule consists of a 180-kDa α chain and a 95-kDa β chain arranged in a heterodimer complex. Most lymphocytes express LFA-1, where it can function as an accessory molecule in a number of lymphocyte adhesive interactions, including: T-cell- and natural killer cell-mediated lysis of target cells, antibody-dependent cytotoxicity, and the binding of lymphocytes to cultured endothelium (Springer *et al.*, 1987; Marlin and Springer, 1987). In the case of T-cell killing, LFA-1 is thought to work in concert with CD2, CD8, and the T-cell receptor on cytotoxic T-cells to form a stable adhesive interaction with the target cell molecules ICAM-1, LFA-3, and class I MHC plus antigen, respectively. Inhibition of either LFA-1 or CD2 weakens this interaction and may prevent target cell lysis (Shaw *et al.*, 1986). As discussed in Section III,F, the leukocyte integrin subfamily of which LFA-1 is a member, is involved in the attachment of neutrophils to vascular endothelium during an inflammatory reaction, and, in the case of cutaneous sites, these molecules appear to work in parallel with the MEL-14 antigen.

ICAM-1 (intercellular adhesion molecule-1) has been demonstrated to be a ligand for LFA-1 (Marlin and Springer, 1987), although additional ligands are thought to exist (Rothlein *et al.*, 1986). ICAM-1 is a 90- to 114-kDa glycoprotein expressed by a variety of cells, including vascular endothelium (Marlin and Springer, 1987; Dustin *et al.*, 1986). From the data of Dustin *et al.* (1986) ICAM-1 appears to be prominently represented on the HEV of various lymphoid organs.

Hamann *et al.* (1988) examined the potential role of LFA-1 in lymphocyte attachment to HEV. Treatment of lymphocytes with monoclonal antibodies against LFA-1 inhibited binding to mouse PN HEV *in vitro* by 40–50%, while MEL-14 inhibited lymphocyte binding by 80–90%.

[13] Although LFA-1 is the only member of the integrin superfamily to be discussed in this section, others are worth noting. Mac-1 and p150,95 have also been implicated in leukocyte-endothelial interactions (TeVelde *et al.*, 1987; Arfors *et al.*, 1987). Mac-1 is expressed in high levels by monocytes. Neutrophils express lower levels of Mac-1, but cell-surface representation of this molecule, in conjunction with p150,95, increases with activation (Anderson *et al.*, 1986). These molecules could contribute to an adhesive interaction specified by the MEL-14 antigen, since 50% of monocytes and all neutrophils express high levels of the MEL-14 antigen (Lewinsohn *et al.*, 1987). VLA-4 is unique among the VLA integrin molecules in that it is expressed mainly by circulating, rather than tissue-associated, cells. This molecule is present on blood lymphocytes and monocytes, where it may be associated with a soluble fibronectin-like molecule, but it is not expressed by neutrophils or platelets (Hemler, 1988; Sundqvist and Otteskog, 1987).

Several T-cell lines, which varied in their cell-surface expression of the MEL-14 antigen, were also examined. In general, it was found that cell lines expressing the highest levels of the MEL-14 antigen bound well to PN HEV, and were affected minimally by anti-LFA-1. For example, anti-LFA-1 inhibited the binding of one MEL-14-high cell line by only 9%, while inhibiting the binding of a MEL-14-low cell line by 97%. In all cases, MEL-14 inhibited the binding of the cell lines to PN HEV by at least 90%. *In vivo* relevance was established by the demonstration that localization of lymphocytes treated with anti-LFA-1 prior to intravenous infusion was inhibited within peripheral lymph nodes by approximately 80% and within Peyer's patches by 50%. These results indicate that LFA-1 can function in parallel with the MEL-14 antigen to strengthen the adhesion of mouse lymphocytes to PN HEV, and presumably with different homing receptors for interactions with other HEV. Furthermore, the adhesive contribution of LFA-1 can vary depending upon the strength of the homing receptor (e.g., the MEL-14 antigen) interaction.

A further indication that LFA-1 plays no more than an accessory role in lymphocyte–HEV binding can be inferred from divalent cation requirements. The ligand-binding (i.e., ICAM-1-binding) activity of LFA-1 requires magnesium (Marlin and Springer, 1987). In contrast, as described above, lymphocyte binding to HEV requires calcium and can occur in the absence of magnesium. Clearly, then, LFA-1 is not necessary for the HEV interaction, at least *in vitro*.[14] The involvement of general accessory molecules, such as LFA-1, in lymphocyte–HEV binding is depicted in the model of Fig. 1.

Kraal *et al.* (1987) examined a monoclonal antibody, CT4, directed against guinea pig lymphocytes. These investigators found that CT4 inhibited lymphocyte binding to both PN and PP HEV *in vitro* by 60–80%. Treatment of lymphocytes with this antibody prior to intravenous infusion inhibited their localization within Peyer's patches, peripheral lymph nodes, tracheal lymph nodes, and mesenteric lymph nodes by 40–75%, and within the spleen by 40%. The CT4 antigen exhibits a wide range of expression on peripheral lymphocytes and is also expressed by thymocytes, although to a slightly lesser degree. CT4 precipitates a protein of 32–36 kDa, which does not change under reducing conditions. This molecule is dissimilar to the MEL-14 and HEBF

[14] In the case of LFA-1, use of the *in vitro* HEV-binding assay may be somewhat misleading; this assay requires low temperature (7°C) and the activity of LFA-1 is temperature dependent. Marlin and Springer (1987) found LFA-1 to exhibit 20% binding activity at 14°C and no activity at 4°C (for LFA-1-mediated attachment of T cells to purified ICAM-1). Thus, the contribution of LFA-1 to the interaction of lymphocytes with HEV *in vitro* may be minimized by the conditions of the assay.

molecules not only in molecular weight, but also in that blocking by the CT4 antibody is never complete and is not organ selective. However, the CT4 antigen demonstrates a similar cellular distribution as the MEL-14 antigen, being expressed by peripheral lymphocytes, to a lesser extent by thymocytes, and not at all by the majority of germinal center cells. These results are consistent with the possibility that the CT4 antigen may serve as a general (i.e., not tissue specific) accessory molecule in the attachment of lymphocytes to HEV.

B. Molecules Associated with HEV

1. *Antibodies Against Mouse and Human Endothelial Cell Antigens: MECA-325 and HECA-425*

MECA-325 and HECA-425 are monoclonal antibodies that stain HEV in all lymphoid organs of mouse and human, respectively (Duijvestijn *et al.*, 1987, 1988; and see Section II,A). MECA-325 also recognizes flat-walled vessels within the lamina propria of the small intestine, which are presumed to be sites of blast cell extravasation.

Both of these antigens appear to be modulated during inflammation. The MECA-325 antigen is induced at late stages of an inflammatory reaction and its expression parallels the appearance of lymphocyte infiltrates. HECA-425 stains vessels in tissue specimens taken from patients with autoimmune thyroiditis (Grave's and Hashimoto's disease) and Crohn's ileocolitis. These vessels are similar to HEV, but the height of the endothelium is more irregular. Within the diseased tissues, HECA-425 staining is found exclusively on vessels associated with lymphocyte infiltrates (always within T-cell areas, which are usually CD4+) and the intensity of antibody staining appears to correlate with the extent of lymphocyte accumulation. Positive vessels were not observed in sites of cutaneous acute inflammation or within peptic ulcer lesions, which lacked dense lymphocyte aggregates, yet contained large numbers of monocytic cells. The investigators conclude that the antigen is apparently not required for low-level lymphocyte influx, but is associated with extreme examples of lymphocyte traffic, comparable to that occurring within organized lymphoid tissues. These results argue that the MECA-325 and HECA-425 antibodies identify vessels that are specialized for lymphocyte traffic. However, since neither of these antibodies block lymphocyte attachment to HEV *in vitro*, the relationship of the antigens to HEV ligands is not evident.

2. *MECA-89/MECA-367*

Streeter *et al.* (1988) have subsequently described two monoclonal antibodies, MECA-89 and MECA-367, that recognize HEV within gut-associated mouse lymphoid tissues. These antibodies stain all HEV in

Peyer's patches, but fail to stain the majority of HEV within peripheral lymph nodes (axillary, brachial, popliteal, and inguinal). Consistent with the proposed hybrid nature of HEV within mesenteric lymph nodes (Stevens *et al.*, 1982), these vessels exhibit a mixed pattern of staining with MECA-89 and -367. These antibodies, like MECA-325, recognize small vessels within the lamina propria of both the small and large intestine. Small vessels within the mammary gland are also recognized, supporting the gut-like homing specificity of this organ (see Section III,B,2). Positive vessels are not observed within a cutaneous granuloma, which would be predicted to have a PN-like rather than gut homing specificity. MECA-367 precipitates a 58- to 66-kDa protein from detergent lysates of mesenteric lymph node stroma. MECA-89 appears to recognize a separate epitope on the same antigen, based on Western blot and preabsorption analyses.

When tissue sections were preexposed to MECA-367, lymphocyte attachment to PP HEV was completely inhibited (92%); attachment to MN HEV was inhibited by 52%, and attachment to PN HEV was unaffected. MECA-89 demonstrated no blocking activity. Intravenous injection of MECA-367 inhibited lymphocyte localization within Peyer's patches by 97% and within mesenteric lymph nodes by 37%, but did not affect localization within peripheral lymph nodes or the spleen. The antigen recognized by MECA-367/89 is termed a "vascular addressin" because of its predicted role as an organ-specific HEV ligand in the binding of lymphocytes to gut-associated HEV.

V. Involvement of Carbohydrates in Lymphocyte–HEV Interactions

A. Carbohydrates as Recognition Determinants in Cell Adhesion

It has long been appreciated that carbohydrates are ideal candidates for recognition structures or as informational molecules because of their enormous potential for structural diversity. The oligosaccharide chains of glycoconjugates are composed of a sequence of monosaccharides (neutral hexoses, *N*-acetyl amino sugars, and acidic sugars) that can be linked together in an α or β configuration in any of several positions on each sugar ring (Sharon, 1975). The variation in sugar sequence, anomerity, and linkage position, together with the potential for modifications of the individual monosaccharides with sulfate, phosphate, or acetyl moieties, make possible an almost limitless number of oligosaccharide structures. This potential for diversity appears to be realized to a large degree. For example, in the case of glycosphingolipids, 130 species have been identified and structured thus far (Hakomori, 1984, 1986).

Moreover, the use of carbohydrate-directed monoclonal antibodies and lectins has revealed an impressive diversity of carbohydrate structures, some of which show interesting patterns of tissue distribution or variations with differentiation and transformation of cells (Hakomori, 1984; Slack *et al.*, 1985; Pennington *et al.*, 1985; Feizi, 1985).

It is, of course, naive to assume that all cell-surface carbohydrates serve as targets for receptors or have some other informational role. In fact, the basic similarity in structure of many oligosaccharides—in particular, protein-linked carbohydrate chains—argues that they have a nonspecific role, which is based on their general physiocochemical properties (West, 1986). Protection against proteolysis, stabilization against denaturation, and enhancement of solubility are examples of such nonspecific functions (Olden *et al.*, 1982; West, 1986). Yet, mundane functions for probably the bulk of cell-surface carbohydrates do not preclude the possibility of specific functions for a subset of surface carbohydrates, in particular those having rare structures. In fact, with respect to several cell adhesion systems, there is suggestive, and in some cases compelling evidence for a direct role of carbohydrates as recognition determinants. These examples include the binding of bacteria or viruses to host cells (Paulson, 1985; Sharon, 1987), symbiosis between nitrogen-fixing bacteria and plants (Ho *et al.*, 1986), fertilization in sea urchin (Glabe and Lennarz, 1981; Glabe *et al.*, 1982) and in mouse (Shur and Hall, 1982; Wassarman, 1987), adhesion between embryonal carcinoma cells (Grabel *et al.*, 1983), adhesion between mouse preimplantation embryo cells (Fenderson *et al.*, 1984), aggregation of cellular slime molds (Loomis *et al.*, 1985; Ziska and Henderson, 1988), and neuron-glial and glial-glial adhesions (Künnemunde *et al.*, 1988). The microbe-host cell interactions comprise the best understood systems in terms of our knowledge about the participating receptors and their carbohydrate-binding specificities. For example, some enterobacteria attach to host cells via mannose-binding receptors (Firon *et al.*, 1987), others employ Galα1,4 Gal-binding receptors (Bock *et al.*, 1985) and still others appear to utilize receptors with specificity for sialyloligosaccharides (Parkkinen *et al.*, 1983).

Undoubtedly, the study of influenza virus attachment to erythrocytes represents the most thorough and convincing analysis of a carbohydrate-directed cell adhesion event (Paulson, 1985; Wiley and Skehel, 1987). The hemagglutinin of the influenza viruses mediates viral attachment to host cells by recognizing sialic acid-containing glycoconjugates on the host cell membrane.[15] Removal of sialic acid from the host cell by

[15] The term sialic acid refers to a family of more than 20 naturally occurring derivatives of neuraminic acid, an acid amino sugar in pyranose form with a three-carbon side chain. The most common form is *N*-acetylneuraminic acid (NeuAc), which has an *N*-acetyl group substituted on the fifth carbon.

treatment with broad-spectrum sialidases prevents virus attachment. By restoring sialic acid using purified sialyltransferases and a sugar donor (CMP-sialic acid), Paulson and co-workers have been able to define the exact linkage and form of sialic acid required for viral recognition. Some strains of virus prefer *N*-acetylneuraminic acid (NeuAc) linked α2,3 to Galβ1,4GlcNAc while others prefer NeuAc linked α2,6 to Galβ1,3GalNAc. Remarkably, a single amino acid substitution in the binding pocket of the hemagglutinin (residue 226 from leucine to glutamine) converts its specificity from NeuAcα2,6Gal to NeuAcα2,3Gal (Rogers *et al.*, 1983). The exact form of sialic acid can also be critical for recognition. Some influenza strains prefer the *N*-acetyl form to the *N*-glycolyl form (Susuki *et al.*, 1985), whereas other viruses exhibit preference for sialic acid containing both *N*-acetyl and 9-*O*-acetyl moieties to the *N*-acetyl form (Herrler *et al.*, 1985). This range of recognition specificities, based upon subtle changes in the form and linkage of a single terminal sugar (i.e., sialic acid), provides the most dramatic illustration to date of the potentiality of carbohydrates as specific recognition ligands in cell adhesion events. As will be described below, this example may be particularly pertinent, in view of the evidence that sialic acid is critical to the function of HEV ligands.

B. Inhibition of Lymphocyte–HEV Interaction by Specific Carbohydrates

1. *Neutral and Phosphorylated Monosaccharides*

The initial attempts to determine whether carbohydrate-directed receptors were important in lymphocyte attachment to HEV involved monosaccharide competition experiments employing the *in vitro* adherence assay of Stamper and Woodruff (1976). The neutral monosaccharides commonly found in cell-surface glycoconjugates were tested for their ability to competitively inhibit the attachment of lymphocytes to PN HEV in the rat system (Stoolman and Rosen, 1983). At 150 m*M*, L-fucose and D-mannose significantly inhibit binding (50%) relative to equimolar concentrations of D-fucose, D-galactose, D-glucose, *N*-acetyl-D-glucosamine, and *N*-acetylgalactosamine. The inhibitory activity of either L-fucose or D-mannose could be substantially potentiated by increasing the ionic strength of the buffer used in the *in vitro* assay. Increasing the salt concentration by 60 m*M*, 40% above physiological levels, reduces lymphocyte attachment directly (50% reduction relative to binding in physiological buffer) and enhances the inhibitory activity of L-fucose or D-mannose by at least twofold. These initial observations stimulated interest because there are several known carbohydrate-binding receptors that recognize common structural features of L-fucose and D-mannose (Kameyama *et al.*, 1979; Townsend and Stahl, 1981; Stahl and

Gordon, 1982). However, the findings with the neutral monosaccharides were not entirely satisfying because of the high concentrations that were required for activity. There was no indication of selective toxicity to the lymphocytes by either active sugar, based on trypan blue exclusion or on measurements of protein synthetic rates; yet, before the possibility of a carbohydrate-specific receptor could be invoked in this system, it was necessary to identify more potent carbohydrate inhibitors.

This objective was realized with the discovery that either mannose-6-phosphate (M6P) or its structural analog, fructose-1-phosphate, produces 80-90% inhibition of lymphocyte binding to PN HEV at a concentration of 10 m*M* (Stoolman *et al.*, 1984). In contrast, mannose-1-phosphate, fructose-6-phosphate, galactose-1- or 6-phosphate, and glucose-1- or 6-phosphate have little or no activity at this concentration. The selective effects of the phosphorylated monosaccharides are achieved in a physiological buffer without supplementary salt. The I_{50} for M6P is 2-3 m*M*. Mannose-1-phosphate is about fivefold less potent than M6P (Yednock *et al.*, 1987a).

2. *Polysaccharides*

Two polysaccharides, PPME and fucoidin, proved to be considerably more potent inhibitors of *in vitro* lymphocyte binding to PN HEV than the most active phosphorylated monosaccharides. Of the two, PPME is the best structurally defined, consisting of a M6P-rich core mannan derived from the yeast *Hansenula holstii*. This mannan is 2.5×10^6 kDa in mass and is made up exclusively of mannose and phosphate, with one out of every six mannose residues phosphorylated at the sixth position. The molecule is a highly branched ("brushlike") structure with an α1,6-linked backbone that is substituted at various carbon-2 positions with side chains containing α1,2 and α1,3 linkages (M. E. Slodki *et al.*, 1973, personal communication). The nonreducing termini of these side chains are the probable sites of phosphorylation. PPME inhibits lymphocyte binding to PN HEV by 50% at 10-20 μg/ml, which is equivalent to 4-8 n*M* polysaccharide or 8-16 μM M6P equivalents. In this same assay, fucoidin, a polysaccharide containing a high concentration of fucose-4-sulfate, is at least 10-fold more active ($I_{50} \approx 1$ μg/ml) than PPME on a mass basis.[16] An *in vivo* homing study has confirmed the ability of fucoidin to prevent entry of lymphocytes into peripheral lymph nodes (Spangrude

[16] It is noteworthy that fucoidin has been reported to interact with several known and putative cell-cell adhesion molecules in a variety of systems, including sea urchin fertilization, mammalian fertilization, embryonal carcinoma cell adhesion, and endothelial monolayer organization (Glabe *et al.*, 1982, 1983; Huang *et al.*, 1982; Töpfer-Petersen *et al.*, 1985; Grabel *et al.*, 1983). In addition, fucoidin is known to bind to the matrix adhesion proteins, thrombospondin and von Willebrand factor (Roberts *et al.*, 1985, 1986).

et al., 1984). The efficacy of PPME and fucoidin as inhibitors cannot be explained on the basis of trivial physicochemical properties, since a wide range of charged polysaccharides and glycosaminoglycans are largely inactive in blocking *in vitro* lymphocyte attachment to PN HEV. Furthermore, specific inhibition by carbohydrates is not restricted to the rat system as M6P, fructose-1-phosphate, PPME, and fucoidin selectively prevent lymphocyte attachment to PN HEV in mouse (Yednock *et al.*, 1987a) and in human (Stoolman *et al.*, 1987a).

As described above, increasing the ionic strength of the assay buffer reduces lymphocyte attachment to PN HEV *in vitro* and augments the inhibitory activity of D-mannose. In addition, phosphorylation of mannose increases its inhibitory activity directly, while fucoidin and PPME are inactivated by desulfation and dephosphorylation, respectively. Taken together, these observations strongly suggest the importance of negative charge in lymphocyte-HEV binding. The nature of the sugar is also critical, as clearly established by the selective effects of mannose and fucose among the neutral sugars and M6P and fructose-1-phosphate among the phosphorylated sugars. Direct surface-charge measurements on intact lymphocytes demonstrate further that negative charge imparted to the cell surface by the various charged monosaccharides or polysaccharides cannot alone account for the inhibitory potency of the active carbohydrates (Stoolman *et al.*, 1984).

C. PPME-Binding Receptor on Lymphocytes

1. *Detection with Solid-Phase PPME Probes*

The studies with soluble inhibitors indicated the involvement of carbohydrate-directed receptors in lymphocyte attachment to PN HEV. However, since the inhibitors were present continuously throughout the assays, it was not clear which of the interacting cell types (i.e., lymphocytes, endothelial cells, or both) expressed the putative receptors. This issue was first addressed with simple preincubation studies (Stoolman and Rosen, 1983; Stoolman *et al.*, 1984). When lymphocytes (rat) were exposed to either fucoidin or PPME and then washed, their binding to PN HEV *in vitro* was inhibited relative to that of control lymphocytes. In contrast, there was no persistent inhibition when cryostat-cut sections of lymph nodes were exposed to the polysaccharides and washed prior to the assay. Thus, it was evident that PPME and fucoidin were exerting their inhibitory effects at the level of the lymphocyte.

The fast off-rates of PPME and fucoidin precluded the use of these soluble polysaccharides as the basis for labeled probes for receptors on the lymphocyte surface. However, covalent derivatization of small fluorescent beads with PPME results in a solid-phase cytochemical probe that permits direct detection of a carbohydrate-specific receptor on the

lymphocyte surface (Yednock *et al.*, 1987a). The binding of PPME-beads to intact, viable lymphocytes can be visualized by fluorescence microscopy and is readily quantified by flow cytometry. PPME-beads bind extensively and avidly to the surface of peripheral lymphocytes from mouse, rat, and human. In the mouse, a majority ($\approx 60\%$) of the cells bind an average of 18 beads/cell, while the remaining 40% bind an average of fewer than 1 bead/cell, which is the same level of binding exhibited by nonderivatized beads or by beads derivatized with control carbohydrates (heparin, chondroitin sulfate, or a sulfated galactan). As predicted, the binding of PPME-beads to lymphocytes (rat, mouse, human) is inhibited selectively by M6P and fructose-1-phosphate among the phosphorylated monosaccharides, and by PPME and fucoidin among an extensive series of polysaccharides and glycosaminoglycans (Yednock *et al.*, 1987a; Stoolman *et al.*, 1987a). These findings established that lymphocytes from three different species have a surface receptor that can recognize M6P and related structures. Subsequently, Brandley *et al.* (1987) have employed PPME immobilized to an inert polyacrylamide surface to demonstrate what is presumably the same receptor on rat lymphocytes.

2. *Correlations with PN Homing Receptor*

The binding of peripheral lymphocytes to PPME-beads (as monitored by flow cytometry) mimics the *in vitro* interaction of lymphocytes with PN HEV. The same battery of carbohydrates inhibit lymphocyte attachment to PPME and to PN HEV. The bead interaction (Yednock *et al.*, 1987a) and the HEV interaction (see Section IV,A,6) both require calcium. Moreover, the same quantitative dependence on extracellular calcium is observed for both adhesive interactions (Yednock *et al.*, 1988a); the transition from nonbinding to binding occurs between 10^{-5} and 10^{-4} *M* of calcium. This concentration requirement is readily satisfied by plasma.

Protease experiments provided further correspondences. As discussed in Section IV,A,6, brief exposure of lymphocytes to trypsin or to other proteases inactivates the PN homing receptor (monitored by the *in vitro* attachment assay), and this inactivation occurs whether or not calcium is present during the protease treatment (Yednock *et al.*, 1988a). The same proteases inactivate the receptor for PPME-beads, again irrespective of calcium. However, the serum protease, plasmin, does not affect either the PN homing receptor or the PPME-bead receptor when lymphocytes are treated in the presence of calcium, whereas both receptors are inactivated by plasmin treatment in the absence of calcium.

The binding of lymphocytes to PN HEV in the *in vitro* assay shows an unusual and, as yet, unexplained dependency on temperature. Lymphocyte attachment is maximal at 7°C. At lower (1°C) and higher

temperatures (24 and 37°C), it is markedly reduced or largely eliminated (Stamper and Woodruff, 1977; Braaten *et al.*, 1984). In correspondence, lymphocytes bind well to PPME-polyacrylamide at 8°C while binding at 37°C is reduced substantially (Brandley *et al.*, 1987). In both instances, reduced binding at temperatures >7°C may reflect the fact that exothermal interactions (e.g., hydrogen bonds) are favored at lower temperatures.[17] Another interpretation suggests that the reduced adhesion at 37°C reflects a physiological mechanism for breaking contacts between adherent lymphocytes and endothelial cells, which may be necessary for migration of lymphocytes across the HEV (Stamper and Woodruff, 1977). Consistent with this possibility, Brandley *et al.* (1987) have reported that lymphocytes appear to inactivate attachment sites on the PPME-polyacrylamide gel during coincubation at 37°C. If the temperature effect proves to be the result of a biologically significant deadhesion mechanism, the artificial carbohydrate-derivatized surfaces may prove to be very useful for examining the underlying mechanisms.

The most impressive correlations between the expression of the PPME receptor and the PN homing receptor have come from an examination of various lymphocyte and cultured lymphoma cell populations (Yednock *et al.*, 1987a,b). Thymocytes bind 10-15% as well as peripheral lymphocytes to PN HEV (Stamper and Woodruff, 1976; Butcher *et al.*, 1979a). In correspondence, thymocytes bind equally poorly to PPME-beads. When six lymphoma lines were investigated, their degree of binding to PN HEV corresponded closely to the degree to which they could bind to PPME-beads in an EDTA-sensitive manner. One of the lines exhibiting a low level of PPME-bead and PN HEV binding (S49 lymphoma) was subjected to fluorescence-activated cell sorting (FACS) selection with fluorescent PPME-beads. A stable cell-line isolate (S49-PB^{HI}), obtained after seven rounds of sequential selection and cell culture, showed a sixfold to eightfold increase (relative to the parental cell) in its ability to bind PPME-beads and to attach to PN HEV (Yednock *et al.*, 1987a). When S49-PB^{HI} was negatively selected for PPME-bead binding, the back-selected cell line isolate (S49-PB^{LO}) exhibited the original low parental values in both PPME-bead binding and PN HEV attachment. Neither positive nor negative selection altered binding to several other bead types (nonderivatized beads, heparin beads, galactan beads), indicating that the selection procedure affected specifically the PPME receptor rather than producing a generalized physicochemical alteration of the entire cell surface. Further analysis (see below) strongly supports this contention.

[17] Perhaps the reduced binding at 1°C versus 7°C is due to a restriction in the mobility of membrane receptors at the lower temperature.

3. *Relationship to Lysosomal Sorting Receptors*

A 215-kDa M6P-binding receptor has been detected in a wide variety of cells. This receptor is involved in the targeting of newly synthesized lysosomal enzymes from the Golgi to lysosomes (Sly and Fischer, 1982; von Figura and Hasilik, 1986; Kornfeld, 1986). Accordingly, the receptor is found to be concentrated within the Golgi and associated structures (Sahagian *et al.*, 1981; Brown and Farquhar, 1984a; Geuze *et al.*, 1984). A few cell types also express the receptor on the cell surface (Kaplan *et al.*, 1977; Brown and Farquhar, 1984a,b). Furthermore, PPME is known to be a high-affinity ligand for the receptor (Kaplan *et al.*, 1978). However, the interaction of PPME-beads with peripheral lymphocytes differs from the ligand-receptor interactions described for the 215-kDa molecule in several important respects. The latter activity is not inhibited by fucoidin (Shepherd *et al.*, 1984), is much more selective (>100-fold) for M6P than mannose-1-phosphate (Kaplan *et al.*, 1977), and does not require divalent cations (Rome *et al.*, 1979). A more recently discovered M6P receptor, apparently also involved in the sorting of lysosomal enzymes, does have a requirement for divalent cations, but shows no preference for calcium relative to magnesium (Hoflack and Kornfeld, 1985a,b). For several human and mouse lymphoma cell lines (but not normal lymphocytes), an EDTA-resistant component of PPME-bead binding is observed. There is evidence for human lines (Stoolman *et al.*, 1987b) that the 215-kDa receptor mediates this divalent cation-independent interaction.[18] However, this activity is not observed on the surface of normal lymphocytes. Taken together, the evidence clearly establishes that the PPME receptor associated with lymphocyte attachment to PN HEV is distinct from the lysosomal sorting receptors.

4. *Relationship to MEL-14 Antigen*

An important question concerns the relationship of the carbohydrate-binding receptor detected by the PPME beads[19] to the other lymphocyte

[18] It should be stressed that PPME-beads represent a novel cytochemical reagent in that it is a ligand-based probe, detecting cell-surface receptors (i.e., lysosomal sorting receptors and lymphocyte homing receptor) on the basis of their ligand-binding (i.e., M6P-binding) activities. This feature distinguishes this class of probe from those based upon antibodies.

[19] This surface receptor is referred to as a carbohydrate-binding receptor because of its selective interactions with monosaccharides and polysaccharides. This designation should be regarded as provisional until an actual biological ligand for this receptor is shown to be carbohydrate in nature. Furthermore, the fact that M6P competes with this receptor implies that the biological ligand may resemble, but does not necessarily contain, this structure.

surface molecules implicated in lymphocyte homing (see Section IV,A). The studies carried out thus far have focused on the relationship of the PPME receptor to the MEL-14 antigen. A series of striking correlations, reviewed briefly below, suggests that the two are inextricably linked and are perhaps identical (Yednock *et al.*, 1987b): (1) the MEL-14 monoclonal antibody inhibits PPME-bead binding to mouse lymphocytes in exact reciprocal correspondence to the degree of antibody bound to the cell surface; (2) the antibody has no effect on the binding of other types of beads (underivatized or derivatized with control carbohydrates) to either normal lymphocytes or lymphoma cells; (3) a series of control antibodies that react with lymphocyte surface antigens (including two that block accessory adhesion activities of LFA-1) have no effect on PPME-bead binding, despite the fact that some bind to a higher level than MEL-14; (4) among six lymphoma cell lines, the presence of the MEL-14 antigen correlates with the expression of EDTA-inhibitable PPME-bead binding; (5) treatment of intact cells with proteases (with or without calcium present) always affects both MEL-14 antigen expression and PPME receptor activity in parallel; (6) both appear to interact with calcium in preference to magnesium (Yednock *et al.*, 1988a); (7) analysis of 14 independent cell-line isolates, obtained during the positive and negative selections of the S49 lymphoma for PPME binding, shows a highly significant ($r = 0.97$, $p < 0.001$) correlation between PPME receptor activity and expression of cell-surface MEL-14 antigen; (8) the positively selected S49-PB^{HI} line, which exhibits sevenfold to eightfold higher levels of PPME receptor and PN HEV binding activities relative to the parental S49 cells (see above), displays a fivefold higher level of the MEL-14 antigen on its surface; (9) the back-selected S49-PB^{LO} line does not show enhanced PPME-bead binding or PN HEV attachment, and expresses the low parental levels of the MEL-14 antigen; and (10) neither positive nor negative selection affects the cell-surface levels of several control antigens, including LFA-1.

These results argue for a close physical and functional relationship between the MEL-14 antigen and the PPME receptor, reinforcing the evidence that the antigen and its associated carbohydrate-binding activity are both involved in lymphocyte attachment to PN HEV. Modeling of the homing receptor is, however, complicated by the finding that excess soluble fucoidin competes with MEL-14 antibody binding to the cell surface, whereas PPME does not. Since the PPME- and PN HEV-binding activities on lymphocytes have remarkably similar characteristics, it is speculated that PPME and, in particular, its constituent M6P, interact at the active site of the homing receptor (Yednock *et al.*, 1987b). It is further suggested that the MEL-14 antibody and fucoidin bind at a

common site (perhaps at the site of ubiquitination, but distant to the receptor's active site) and indirectly influence the molecule's activity by altering its conformation. A related issue is whether the MEL-14 antigen is sufficient, as well as necessary, for PPME receptor activity and PN HEV-binding activity. The simplest model would be that the MEL-14 antigen functions alone to mediate directly PPME/PN HEV binding. Proof of this possibility requires the demonstration that the isolated 90-kDa antigen, in a reconstituted system, can manifest both activities. An alternative possibility is that the MEL-14 antigen is a single component of a receptor complex. Should reconstitution experiments with the isolated 90-kDa molecule fail, it will be necessary to explore this possibility. Cross-linking experiments or carefully controlled membrane solubilization procedures, such as those used effectively in the analysis of the multicomponent T-cell receptor complex, might be employed for this purpose (Brenner *et al.*, 1985; Weiss *et al.*, 1986).

5. Relationship to Other Homing Receptors

As reviewed extensively in Section III, there is at present biochemical evidence for at least four distinct homing receptor specificities (i.e., peripheral lymph nodes, Peyer's patches, lung-associated lymphoid tissue, inflamed synovium), and other studies suggest that additional specificities may exist. Given the demonstration that variation in carbohydrate structure can define multiple cell adhesion ligands within a single system (i.e., influenza virus), a provocative possibility is that different homing receptors may exhibit distinct carbohydrate-binding specificities. The available evidence, reviewed above, indicates that PPME mimics a ligand (presumably, carbohydrate in structure) for the PN homing receptor. Consistent with this proposal, three separate lines of evidence establish that this polysaccharide does not have functional interactions with the PP homing receptor. First, trypsin/calcium (TC) treatment of intact lymphocytes eliminates PPME receptor activity from the surface of lymphocytes while completely sparing PP HEV-binding activity (Yednock *et al.*, 1988a). Second, neither PPME nor fucoidin at high concentration competes with the binding of TC-treated lymphocytes to PP HEV (Yednock *et al.*, 1988a).[20] Third, selection of the S49 cell line for PPME binding increases the ability of the cell lines to interact with PN HEV,

[20] When nonproteolyzed lymphocytes are used, PPME and fucoidin produced partial (50%) and variable inhibition of binding to PP HEV. This result suggested that polysaccharides may bind to the PN homing receptor and nonspecifically interfere (through steric or charge influences) with the PP homing receptor present on the same cells. Thus, a population of lymphocytes lacking the PN homing receptor (i.e., TC-treated cells) was employed.

but binding to PP HEV is unaffected (Yednock *et al.*, 1987a). Based on these results with PPME, it is tempting to speculate that the PP homing receptor may have its own carbohydrate-binding specificity. Thus far, however, carbohydrate-based inhibitors of the PP HEV interaction have not been reported.

A different situation arose when lymphocyte attachment to lung-associated lymph nodes (LuN) was investigated. As reviewed in Section III, the lymphocyte receptor involved in this interaction can be clearly distinguished from the receptors involved in both PN and PP HEV binding. Yet, when PPME was tested in this system, it was found to inhibit lymphocyte attachment to lung lymph node HEV as effectively as the PN HEV interaction (Geoffroy *et al.*, 1988). Examples are well known of different lectins that share specificity for monosaccharides or disaccharides but discriminate among more complex carbohydrate structures. For example, lactose competes with the binding of three rat lung lectins to asialofetuin to a similar extent (Leffler and Barondes, 1986); however, the lectins demonstrate very different affinities for larger oligosaccharides. Relative competition by the extended oligosaccharide inhibitors varies by as much as 100-fold among the three lectins. Thus, the binding sites of these lectins have overlapping but clearly separate specificities. Perhaps a more pertinent example is provided by the hemagglutinins of influenza viruses. As mentioned above, these receptors all recognize some form of sialic acid on target cells, but different viral hemagglutinins show preferences for particular sialyloligosaccharides (e.g., NeuAcα2,3 versus NeuAcα2,6 to galactose). One can speculate that M6P, the presumed active constituent of PPME, is interacting with a common domain found in the binding pockets of both the PN and LuN homing receptors; yet, the actual biological ligand for each of the homing receptors might involve several pyranose residues forming a structure that is highly preferred by its own receptor. The identification of closer analogs to the biological ligands for the LuN and PN homing receptors or, ideally, the identification of the actual biological ligands would permit evaluation of this hypothesis.

D. Role of Carbohydrates in the Activity of HEV Ligands

The *in vitro* cell-binding assays and the homing studies reviewed in Section III suggest a model (Butcher and Weissman, 1984; Woodruff *et al.*, 1987) in which (1) lymphocyte attachment to HEV is mediated through the interaction of homing receptors with complementary attachment sites on HEV, designated as HEV ligands; (2) HEV ligands are organ-specific or region-specific, differing from one anatomical site to the next; and (3) the basis of homing specificity (i.e., selective migration

of lymphocytes to a particular lymphoid organ in the body) resides in the ability of individual homing receptors to preferentially recognize one kind of HEV ligand from among the others. The HEBF studies of Chin, Woodruff, and colleagues provide direct evidence that presumptive lymphocyte homing receptors (shed into lymph in a soluble form) are capable of discriminating between PN and PP HEV ligands. Further biochemical evidence for organ-specific HEV ligands derives from studies with the monoclonal antibody MECA-367, which selectively blocks lymphocyte adherence to PP HEV (Streeter *et al.*, 1988).

1. *Effects of Denaturants and Periodate*

In view of the evidence that homing receptors may be carbohydrate directed, the obvious question that arises is whether the recognition determinants of the HEV ligands contain carbohydrates as essential elements. An involvement of carbohydrates is compatible with the finding that the functional activity of the HEV ligands, as determined in the *in vitro* adherence assay, is substantially preserved after exposure of cryostat-cut sections to various protein denaturants (M. S. Singer and S. D. Rosen, unpublished). These harsh treatments included aldehyde fixation (glutaraldehyde or formaldehyde) and aldehyde fixation followed by exposure to trichloroacetic acid, acetic acid, HCl, or NaOH.

The first direct evidence that carbohydrates are essential for activity is based on periodate oxidation experiments.[21] When sections of lymphoid organs (mouse) are pretreated with periodate under conditions that are relatively, but not absolutely, selective for carbohydrate oxidation (low pH, 4°C, in the dark), lymphocyte attachment to both PN and PP HEV is eliminated. The PP HEV sites are somewhat more susceptible to periodate oxidation than the PN sites.

2. *Effects of Sialidases in Vitro and in Vivo*

Because soluble M6P inhibits lymphocyte attachment to PN HEV, apparently by competing with the PN homing receptor, it was relevant to determine whether M6P moieties are essential for the activity of PN HEV sites. However, no positive evidence for the existence of critical M6P residues on HEV could be established, since treatment of cryostat-cut sections (mouse PN and PP) with high concentrations of alkaline phosphatase (*Escherichia coli*) or with α-mannosidase (jack bean) does not affect subsequent lymphocyte attachment (M. S. Singer and S. D. Rosen,

[21] Periodate oxidizes glycosides containing free adjacent hydroxyls (Sharon, 1975). The three-carbon glycerol side chain on certain forms of sialic acid is known to be particularly sensitive to periodate oxidation (Schauer, 1982).

unpublished observation). These negative findings raise the possibility that the M6P inhibition of lymphocyte attachment to PN HEV may be fortuitous; that is, M6P may be a structural mimic of the actual ligand on PN HEV. Several other glycosidases, including α-fucosidase (rat testes), endo-β-galactosidase (*Escherichia freundii*), *N*-acetylglucosaminidase (jack bean), endoglycosidase F (*Streptomyces plicatus*), and *N*-glycopeptidase F (*Flavobacterium meningosepticum*) were also found to have no effect on the activity of the PP and PN HEV ligands (mouse tissues; M. S. Singer and S. D. Rosen, unpublished).[22]

In contrast to these negative findings, dramatic effects were observed when sections were exposed to sialidases (Rosen *et al.*, 1985). Treatment of PN sections (rat, mouse) with bacterial sialidases (*Vibrio cholera* or *Clostridium perfringens*) or with the sialidase from *Arthrobacter ureafaciens* (unpublished data) completely eliminates subsequent lymphocyte attachment. Strikingly, the effect is organ specific, since the activity of PP HEV ligands is completely resistant to these enzymes. Mesenteric lymph node HEV sites demonstrate partial susceptibility ($\approx 50\%$), consistent with the evidence that MN HEV is a hybrid endothelium expressing both PN and PP HEV ligands (Stevens *et al.*, 1982; Streeter *et al.*, 1988). Cytochemical analysis for sialic acid, using the *Limax flavus* agglutinin (a lectin that is specific for sialic acid; Miller *et al.*, 1982), has confirmed that sialidase is effective at removing sialic acid moieties from the tissue sections. A sialidase treatment that completely eliminates lymphocyte attachment to PN HEV results in the loss of virtually all *Limax* agglutinin-reactive sites associated with the HEV of both PN and PP (D. D. True and S. D. Rosen, 1988).

The three sialidases described above are broad-spectrum enzymes, each showing substantial activity against α2,3, α2,6 and α2,8 linkages of sialic acid, the predominant linkages found in glycoproteins and glycolipids (Schauer, 1982). The use of linkage-restricted sialidases has provided additional information about the glycosidic linkage of the sialic acid required for activity of PN HEV ligands. One of these is endoneuraminidase N (endo-N), which cleaves linear α2,8-linked homopolymers of sialic acid having a chain length of seven to nine or more sialic acid residues (Vimr *et al.*, 1984). The polysialic acid component of the N-CAM molecule is known to be susceptible to this endoglycosidase (Vimr *et al.*, 1984; Rutishauser *et al.*, 1988). Treatment of PN or PP sections with a high concentration of endo-N (provided

[22] Negative experiments with glycosidases must be interpreted cautiously, since these enzymes frequently show limited activity against the complex substrates associated with the cell surface (Flowers and Sharon, 1979).

by Dr. F. A. Troy, University of California, Davis, California), under optimal conditions, does not affect subsequent lymphocyte attachment (D. D. True and S. D. Rosen, unpublished). Thus, $\alpha2,8$-linked polysialic acid does not seem to be involved in HEV ligand function. The sialidase from Newcastle disease virus (NDV) hydrolyzes $\alpha2,3$ and $\alpha2,8$ linkages of sialic acid but exhibits negligible activity against the $\alpha2,6$ linkage (Schauer, 1982; Paulson *et al.*, 1982). Like the broad-spectrum enzymes, NDV sialidase inactivates PN HEV ligands while exhibiting no activity against the PP sites (True and Rosen, 1988). Furthermore, the sialidase from type A influenza virus, which has a linkage specificity closely related to that of the NDV enzyme (Schauer, 1982), also selectively inactivates the PN HEV ligands. These results argue that the linkage of the critical sialic acid associated with the activity of PN HEV ligands is likely to be $\alpha2,3$ or $\alpha2,8$, but is probably not $\alpha2,6$.

The sialidase effects, initially established with the *in vitro* adherence assay, have been corroborated *in vivo* (Rosen *et al.*, 1988). *Vibrio cholera* or *C. perfringens* sialidase was injected intravenously into mice. At various intervals, peripheral lymph nodes and Peyer's patches were removed from the animals to be tested in the *in vitro* adherence assay. Three hours after sialidase injection, PN HEV of sialidase-treated animals support $<20\%$ of the lymphocyte binding exhibited by PN HEV of saline-injected animals. In contrast, *in vivo* sialidase treatment has no effect on lymphocyte binding to PP HEV. The inhibitory effect of sialidase on PN HEV is detectable within 1 hour after injection, and almost complete recovery of HEV function occurs within 24 hours. *In vivo* homing studies demonstrate that intravenously injected sialidase selectively impairs lymphocyte recirculation through peripheral lymph nodes. Thus, the accumulation of ^{51}Cr-labeled lymphocytes in peripheral lymph nodes is markedly inhibited in sialidase-injected animals compared to that in control animals, whereas the accumulation of lymphocytes in Peyer's patches and in various nonlymphoid organs is not affected. Presumably, the selective effect on lymphocyte recirculation through peripheral lymph nodes is due to the inability of PN HEV exposed to sialidase to support lymphocyte attachment. These observations are significant for two reasons. First, they provide *in vivo* confirmation of the previous results obtained with tissue sections treated with sialidase *in vitro*. Second, these studies may offer insights as to why sialidases are commonly elaborated by pathogens. A number of bacterial and parasitic infections in animals and humans are known to result in dramatically elevated levels of serum sialidase (Schauer *et al.*, 1984). In fact, the serum sialidase levels measured during infection by such organisms can exceed

the minimal concentration of sialidase necessary for inactivation of PN HEV ligands in the mouse. Schauer (1985) has argued that the common expression of sialidases by pathogens gives these organisms a selective advantage by somehow facilitating the spread of infections. One mechanism for this facilitation might be the reduction of immune defenses as a result of impaired lymphocyte extravasation through HEV of peripheral lymph nodes, as well as other HEV or thin-walled venules that have sialidase-sensitive ligands (including the lung).

3. Effects of Lectins

Additional information about the role of carbohydrate structures in the function of HEV has been derived from lectin blockage experiments. Cryostat-cut sections were exposed to each of several lectins of known specificity, washed, and then treated with glutaraldehyde. The aldehyde fixation step was designed to cross-link the lectin covalently to the structure to which it had bound and to inactivate its unoccupied carbohydrate-binding sites, thus preventing the immobilized lectin from mediating the attachment of lymphocytes. Lectin-treated sections were then tested for their ability to support specific lymphocyte attachment to HEV. *Limax* agglutinin blocks attachment to both PP and PN HEV, whereas another sialic acid-binding lectin (*Limulus polyhemus* agglutinin) as well as lectins having other sugar-binding specificities (concanavalin A, wheat germ agglutinin, *Ricinus communis* agglutinin, *Dolichus biflorus* agglutinin, *Ulex europeus* I) are inactive (D. D. True and S. D. Rosen, 1988).[23] The inhibitory effect of *Limax* agglutinin is prevented if the sections are incubated with the lectin in the presence of bovine submaxillary mucin, a potent sialylated inhibitor of the lectin. This establishes that the *Limax* agglutinin effect is, in fact, due to the sialic acid-binding function of the lectin and not to a contaminant of the preparation. It is noteworthy that treatment of PP HEV with sialidase does not affect lymphocyte binding, yet binding is blocked by this sialic acid-specific lectin. Furthermore, the *Limax* agglutinin will block lymphocyte attachment to sialidase-treated PP sections, even though most of the *Limax* agglutinin-stainable sites (as determined cytochemically) have been removed.

The activity of *Limax* agglutinin on Peyer's patches suggests that, as in the case of peripheral lymph nodes, sialic acid is important for the activity of its HEV ligand. The failure of the sialidases to inactivate the

[23] The specificity of these lectins (Goldstein and Hayes, 1978), defined in terms of the most inhibitory monosaccharides, are as follows: Con A (D-mannose or D-glucose); wheat germ agglutinin (*N*-acetylglucosamine or sialic acid); *R. communis* (D-galactose); *D. biflorus* (*N*-acetylgalactosamine); *U. europeus* I (L-fucose).

PP HEV ligands indicates that the particular form or linkage of sialic acid associated with PP HEV is resistant to these enzymes. Among this very diverse family of sugars,[24] forms of sialic acid exist that are partially or fully resistant to sialidases (Schauer, 1982). For example, complete resistance to sialidases is observed for 4-O-acetylated sialic acids and for sialic acids that are linked to internal galactose residues (e.g., the GMl ganglioside). In addition, sialidases may be sterically hindered when acting on membrane-associated structures (Schauer *et al.*, 1984).

4. Role of Sialic Acid—Direct or Indirect?

Two very different roles for sialic acid in the function of HEV ligands can be envisioned. The first possibility is that sialic acid comprises part of the actual recognition determinant of the ligands. There are many precedents for a function of this kind. As described above, sialic acid is frequently an essential determinant on host cell ligands that are recognized by microbial lectinlike receptors. Recently, Crocker and Gordon (1986) have described a lectinlike receptor on tissue-associated macrophages that interacts with sialylated ligands on erythrocytes. The fact that desialylation inactivates these ligands and that specific gangliosides or sialyloligosaccharides compete with the receptor argues strongly that the binding pocket of the macrophage receptor accommodates sialic acid. Sialic acid is also an essential recognition element for many of the epitopes reactive with carbohydrate-directed antibodies (Magnani, 1987). The alternative model to sialic acid as a recognition determinant is that this sugar somehow modulates the function of HEV ligands; for example, by exerting conformational control over the actual recognition site of the ligand (Sadler *et al.*, 1979; Jennings *et al.*, 1981), by affecting the overall charge density of a molecule involved in binding [as polysialic acid apparently does in N-CAM (Rutishauser *et al.*, 1988)], or by affecting the formation of molecular complexes within the plane of the endothelial plasma membrane.[25]

If sialic acid forms part of the recognition determinant for an HEV ligand, then, in principal, it should be possible to identify sialyloligosaccharides that competitively inhibit lymphocyte attachment to HEV. Free

[24] Members of the sialic acid family can vary in the nature of the N-substitution on carbon-5 (acetyl or glycolyl) and in the number, positions, and kind (methyl, acetyl, lactyl, phosphate, or sulfate) of O-linked substituents (Schauer, 1982, 1985).

[25] For example, certain gangliosides (GD2 and GD3) on melanoma cells appear to associate with integrin molecules to form an active receptor complex. Desialylation of the gangliosides reduces the activity of the receptor, possibly by limiting the extent of complex formation (Cheresh *et al.*, 1986, 1987; Ruoslahti and Pierschbacher, 1987).

sialic acid (NeuAc) has no more inhibitory activity than equimolar concentrations of NaCl (Rosen *et al.*, 1985). This negative finding does not refute the idea of direct recognition; most, if not all, of the sialic acid-directed receptors and antibodies recognize this sugar as part of an extended oligosaccharide structure, and hence, free sialic acid does not compete. A series of readily available glycoproteins and mucins containing multiple representations of defined sialyloligosaccharide structures were also tested in the lymphocyte adherence assay.[26] Each of these glycoconjugates (with the exception of colominic acid) have been shown to inhibit particular examples of microbe-host cell interactions involving sialic acid recognition (Glasgow and Hill, 1980; Loomes *et al.*, 1984; Paulson, 1985). None, however, was found to inhibit lymphocyte attachment to either PN or PP HEV, indicating that the HEV ligands do not involve relatively simple and commonly occurring sialyloligosaccharides (M. S. Singer and S. D. Rosen, unpublished). These findings also do not constitute definitive evidence against the notion of sialic acid as part of a recognition determinant, since one would not expect lymphocyte homing receptors to bind with high affinity to sialyloligosaccharides that are found on serum glycoproteins or erythrocytes. The possibility still exists that relatively rare sialyloligosaccharides, containing unusual linkages or forms of sialic acid, serve as the actual recognition determinants of HEV ligands. Distinguishing between the recognition site model and the modulatory model may ultimately require the isolation and structural analysis of the actual HEV ligands.

E. Future Directions

1. *Homing Receptors*

Based on a large number of studies, it is clear that lymphocyte homing receptors and their complementary HEV ligands represent an extended family of cell-cell adhesion molecules of highly refined specificities. At least four adhesive specificities have been identified, and there may be many others. The Hermes antibodies appear to identify a family of 90-kDa proteins, expressed by lymphocytes and other leukocytes, that function in HEV attachment. Distinct members of this family may be involved in the activity of individual homing receptors (i.e., homing to PP, PN, LuN, or inflamed synovium). As yet, it is not known whether the postulated functional differences among the members of this family

[26] Glycoconjugates that have been tested (and their associated sialyloligosaccharides) are as follows: glycophorin (NeuAcα2,3Galβ1,3[NeuAcα2,6]GalNAc); human α1 acid glycoprotein (NeuAcα2,3Galβ1,4GlcNAc and NeuAcα2,6Galβ1,4GlcNAc); bovine submaxillary mucin (NeuAcα2,6GalNAcThr/Ser); and colominic acid [poly(NeuAcα2,8)].

are due to differences in primary structure, to alternative posttranslational modifications (e.g., glycosylation, ubiquitination, sulfation), or to selective associations with other molecules. Identification of the gene or genes encoding these molecules is essential for addressing this issue.

Thus far, the evidence is very strong for a necessary role of the 90-kDa proteins in homing receptor activity (and, in the case of the MEL-14 antigen, for the activity of the closely associated PPME-binding receptor), but there is no direct evidence that any of the 90-kDa proteins is sufficient to mediate an interaction with the appropriate HEV. To prove sufficiency for a homing receptor candidate requires the demonstration that the isolated molecule, when properly reconstituted (e.g., into a liposome), can bind with appropriate specificity to HEV or to an isolated HEV ligand. If biological activity cannot be reconstituted from a purified 90-kDa component, then it will be necessary to consider the possible involvement of additional subunits that associate with the 90-kDa component to form a functional adhesive complex[27] or the need for supplementary, adhesion-strengthening molecules (e.g., LFA-1, CD4, the CT4 antigen). In the case of the HEBFs, biological activity (i.e., organ-specific blockade of HEV function) has been demonstrated, but biochemical characterization has not yet defined the minimal components sufficient for activity. Furthermore, the relationship between the HEBFs and the 90-kDa family is not currently understood. The reconstitution of a minimal PN homing receptor (containing only the necessary subunits) should allow an unequivocal determination of the role of PPME-binding activity in receptor function. It is reasonable to expect, based upon the current evidence, that the reconstituted receptor will recognize PPME and related carbohydrates, and that calcium will be required for both HEV- and PPME-binding activities.

2. *HEV Ligands*

The 58- to 66-kDa antigen recognized by the MECA-367 monoclonal antibody is an excellent candidate for the PP HEV ligand. Formal proof of this possibility, as in the case of the homing receptor candidates, would require reconstitution of biological activity; that is, a demonstration that the isolated antigen can support lymphocyte attachment via the PP homing receptor. Should the reconstituted molecule exhibit the proper

[27] For example, individual members of an integrin family exhibit distinct ligand-binding activities and consist of heterodimers formed by the association of a common subunit ("familial") with a unique subunit. Antibodies against either protein can block ligand-binding activity (Hynes, 1987). Perhaps a similar model will be relevant to the homing receptors.

specificity, then the MECA-367 antigen must be analyzed in detail to determine the nature of its recognition determinants. Does it contain a periodate-sensitive, sialidase-resistant, *Limax* agglutinin-reactive oligosaccharide? If so, does this oligosaccharide, when isolated from the polypeptide backbone, competitively block lymphocyte attachment to PP HEV, or, alternatively, does this carbohydrate play a modulatory role?

Establishing that a specific oligosaccharide functions as a recognition determinant of the PP HEV ligand would then focus attention on the nature of the carbohydrate associated with the PN HEV ligand. Can the binding specificities of the PN and PP homing receptors (as well as the other homing receptors) be explained in terms of the ability of each to recognize a unique oligosaccharide ligand expressed on the appropriate HEV? In addition, the availability of detailed structural information may reconcile the apparent discrepancy between the sugar-binding specificity of the PN homing receptor for M6P-like sugars and the requirement of sialic acid for the activity of the PN HEV ligand. The key questions are whether a M6P-like moiety serves as the recognition determinant of the HEV ligand and how sialic acid might contribute, directly or through a modulatory role, to this recognition structure.

It is evident that a number of very important basic questions remain to be answered in this relatively young field. Thus, it is anticipated that the study of homing receptors and their HEV ligands will emerge in the next several years as a very active area of research for cell biologists, biochemists and immunologists, alike. One can envision, in the near future, a detailed understanding of the cell adhesion molecules and the mechanisms by which they function in this highly refined and biomedically relevant cell recognition system.

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