ADVANCES IN
Immunology

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VOLUME 59

ACADEMIC PRESS

San Diego New York Boston
London Sydney Tokyo Toronto

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Academic Press, Inc.
A Division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495

United Kingdom Edition published by
Academic Press Limited
24-28 Oval Road, London NW1 7DX

International Standard Serial Number: 0065-2776

International Standard Book Number: 0-12-022459-3

PRINTED IN THE UNITED STATES OF AMERICA
95 96 97 98 99 00 BB 9 8 7 6 5 4 3 2 1

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ADVANCES IN IMMUNOLOGY, VOL. 59

The CD1 Family: A Third Lineage of Antigen-Presenting Molecules

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I. Introduction

The class I and class II molecules of the major histocompatibility complex (MHC) have been and continue to be a central focus for the modern study of cell-mediated immunity. Several of the most important milestones in advancing our understanding of the immune system relate to the resolution of the structure and function of these molecules. Studies of the MHC-encoded antigen-presenting molecules led to the initial identification of these proteins as the products of immune response genes and identified them as critical structures involved in the interactions between T cells and antigen-presenting cells. More recently, the application of sophisticated biochemical and biophysical approaches has identified these proteins as peptide-binding molecules that capture peptide antigens and display them in an immunogenic form to the receptors of specific T cells. Thus, the application of a wide variety of technical approaches, representing the contributions of a large number of individual laboratories, have answered in molecular detail many of the major questions concerning the basic mechanisms by which MHC-encoded class I and class II molecules bind peptide antigens and present them to mature $CD4^+$ and $CD8^+$ T cells (reviewed by Margulies and Germain, 1993).

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Given the central role of the classical MHC-encoded antigen-presenting molecules in immune recognition events in vertebrates, it is not surprising that other molecules of unknown function showing structural similarity to those encoded by the MHC are also of great interest to immunologists. In general, this interest arises from the suspicion that such molecules may eventually be proven to have a role in the all-important process of antigen presentation, a suspicion that has now been justified in several cases (Fischer-Lindahl *et al.*, 1991; Pamer *et al.*, 1992; Morris *et al.*, 1994; Fling *et al.*, 1994). The CD1 family of cell surface glycoproteins is a well-defined group of MHC-related molecules that currently occupies such a position. Since their first description over 15 years ago, it has become increasingly clear that CD1 proteins are related to MHC-encoded antigen-presenting molecules in terms of their structure, evolution, and presumably in their immunological function. Ironically, despite their relatively early identification as human leukocyte antigens, it is only very recently that direct evidence for the cellular function of CD1 proteins has begun to emerge. The prediction that the structural similarity between CD1 and MHC-encoded proteins reflects a function for CD1 proteins in antigen recognition by T cells is now being borne out by recent and ongoing studies from several laboratories. These studies provide strong direct support for the hypothesis that CD1 proteins are a third distinct lineage of antigen-presenting molecules for specific T cell responses, and suggest some important ways in which the CD1 system may be functionally distinct from and complementary to the classical MHC-dependent systems of antigen presentation.

The demonstration of a third family of antigen-presenting molecules separate from those of the MHC is likely to be important to our overall understanding of the biology and evolution of the immune system and may also have a significant impact on efforts to manipulate the immune response for the treatment and prevention of disease. CD1 has been studied at the serologic, protein, and molecular levels in humans and in several other species including rodents, rabbit, and sheep. Although functional studies of CD1 have so far only been reported using human cells, the stage is now set for the development of animal models for the *in vivo* exploration of CD1 function. This chapter summarizes the information that contributes to our current understanding of CD1 structure and function in humans and other mammals, and will also indulge in some cautious speculation about the manner in which the CD1 family may contribute to the overall maintenance of specific immunity.

II. Genetics and Molecular Biology of the CD1 Family

Although CD1 was originally identified as a serologic entity using monoclonal antibodies, it is the application of techniques of molecular biology that has most dramatically advanced our insight into the structure, evolution, and potential function of these molecules. Therefore, this discussion begins with a summary of the molecular genetics of CD1, including the analysis of CD1 genes and of the features of CD1 proteins deduced from their nucleotide sequences. This will bring the overall structure of CD1 proteins into sharper focus and should facilitate the subsequent discussions of their biochemistry, tissue expression, and proposed function in cell-mediated immunity.

A. The Cloning of Human CD1 Genes

The first and still the most extensive analyses of CD1 genes are the studies of the human CD1 family, carried out principally by the laboratory of C. Milstein (for previous reviews, see Calabi and Bradbury, 1991; Calabi *et al.*, 1991). In 1986, Calabi and Milstein reported the first cloning of a CD1-specific cDNA (Calabi and Milstein, 1986). These investigators used an antiserum raised against purified CD1a protein to immunoprecipitate polysomes from a strongly CD1a-expressing human T cell leukemia line (NH17, a variant of the MOLT-4 cell line). RNA isolated from the purified polysomes was used to construct cDNA clones, which were screened for their ability to hybridize to mRNA that encoded the CD1a polypeptide in an *in vitro* translation system. A cDNA clone derived in this way was then used to screen a cDNA library from NH17 cells. Two clones, FCB6 and FCB1, were sequenced fully and found to represent truncated cDNAs for CD1a, including the complete coding sequence for two out of three proposed extracellular domains (the $\alpha2$ and $\alpha3$ domains) and putative transmembrane and cytoplasmic sequences. The predicted amino acid sequence of the protein encoded by these cDNA clones confirmed the previously proposed suggestion that CD1 proteins have structural similarity to MHC-encoded class I molecules (discussed in detail below). Southern blot analysis of genomic DNA with probes derived from these cDNAs revealed multiple bands, suggesting the existence of multiple cross-hybidizing genes.

The conclusion that CD1 represents a small multigene family was directly confirmed by the isolation and characterization of genomic clones from a human genomic library (Martin *et al.*, 1986). Genomic clones hybridizing with the FCB6 cDNA were found to segregate into five groups, corresponding to the five bands observed on Southern

blots of *Eco*R1-digested human genomic DNA hybridized with an FCB6 probe. Sequencing of the segment of each clone corresponding to the putative $\alpha3$ domains of the coding sequence revealed that they do indeed represent five distinct but closely related genes. The original nomenclature for these clones was based on the size in kilobases of the genomic *Eco*R1 fragment to which they hybridized (e.g., clone R7L4 hybridized to the approximately 7 kb genomic *Eco*R1 fragment, designated as the R7 gene). Shortly after the isolation of human genomic CD1 clones, transfection experiments were carried out in which the R4, R1, and R7 genes were shown to encode the proteins identified by antibodies against CD1a, CD1b, and CD1c, respectively (Martin *et al.*, 1987). As a result, the nomenclature for CD1 genes has more recently been replaced by one which directly identifies each gene on the basis of the protein that it encodes (e.g., the R7 gene which encodes the human CD1c protein is now designated CD1C, and so on as shown in Fig. 1).

B. Genetic and Molecular Mapping of the Human CD1 Locus

Southern blot analyses of DNA from mouse/human hybridoma cell lines demonstrated that CD1 genes did not segregate with human chromosome six, which contains the MHC in humans, and suggested

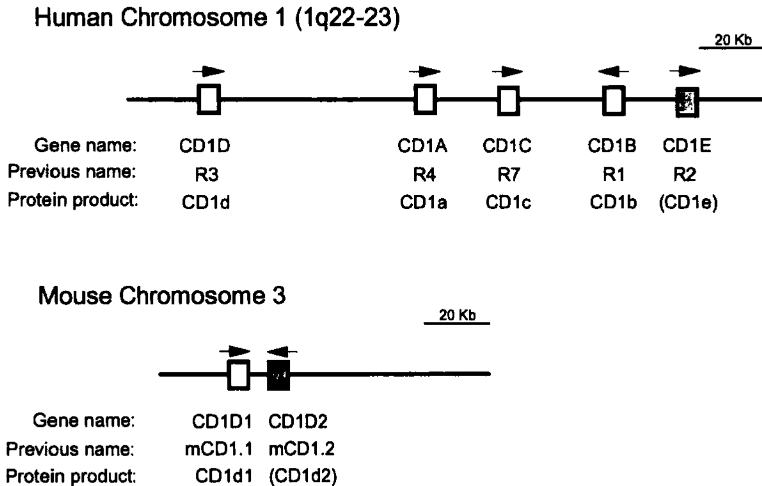

Fig. 1. Genomic maps of the human and mouse CD1 loci. Open boxes indicate CD1 genes for which the protein product has been demonstrated. Shaded boxes are genes that are known to be transcribed, but for which the protein product has not yet been identified. The direction of transcription of each gene is shown by the arrows.

chromosome 1 as the location of the locus encoding these genes (Calabi and Milstein, 1986; Calabi *et al.*, 1987). This result was subsequently confirmed by high-resolution *in situ* hybridization of biotin-labeled CD1 probes to human metaphase chromosomes (Albertson *et al.*, 1988). Alignment of the hybridization signals of probes derived from all five human CD1 genes with chromosome bands visualized by staining with Hoechst dye allowed localization of these genes to human chromosome 1q22–23. Other genes of immunological interest have also been mapped to this area of human chromosome 1 [reviewed in Bruns and Sherman (1989) and Collins *et al.* (1992)], including three others belonging to the immunoglobulin superfamily [BCM1/Blast-1, FcγRII (CD32), and FcγRIII (CD16)].

Construction of a molecular map of the human CD1 locus on chromosome 1 has been accomplished by the use of pulsed field gel electrophoresis, cosmid cloning, and chromosome walking techniques (Yu and Milstein, 1989). Using this approach, all five of the human CD1 genes have been linked with 14 overlapping cosmids spanning approximately 190 kb of DNA. The resulting molecular map of the human CD1 locus is shown schematically in Fig. 1. Intergenic distances are relatively short, ranging between 20 and 73 kb. The tight linkage of these genes is obviously consistent with the cytogenetic data that localized them all within or near a single chromosomal band. All of the human CD1 genes are transcribed in the same direction except for CD1B, consistent with the occurrence of tandem duplication and at least one chromosomal inversion event during the evolution of this gene family.

C. The CD1 Locus in Other Mammalian Species

There have been no reports to date of any species that entirely lacks CD1 genes, although studies have so far been restricted only to a limited number of mammals and have not been extended at all to birds or lower vertebrates. Nevertheless, the unequivocal demonstration of CD1 genes or their products in mice, rats, rabbits, sheep, and humans is consistent with the idea that the maintenance of CD1 genes is a conserved feature of mammalian evolution. However, already it has become clear that the number and complexity of members within the CD1 family may vary substantially between species. Aside from the work summarized previously on human CD1, only in mice have fairly complete cloning and mapping studies of CD1 genes been reported. Initial studies using the human CD1a cDNA to probe Southern blots of mouse genomic DNA revealed two distinct bands, indicating the existence of two related mouse CD1 genes (Calabi and Milstein, 1986).

Subsequently, the two mouse genes were isolated as overlapping genomic DNA clones and characterized by intron/exon mapping and sequencing. These genes were originally designated mCD1.1 and mCD1.2, although more recently they have been renamed mouse CD1D1 and CD1D2 (Bradbury *et al.*, 1990), indicating their demonstrable relationship to the human CD1D gene (see below). The two mouse genes are arranged in opposite transcriptional orientations with a tail to tail orientation and an intergenic distance of approximately 6 kb (Fig. 1) (Bradbury *et al.*, 1988). A 2.1-kb segment within each gene spanning all mapped exons and intervening introns consists of an inverted repeat (>95% identity), consistent with a relatively recent gene duplication or gene conversion event. Based on standard assumptions concerning rates of genetic drift (Li, 1983), this event is postulated to have occurred as recently as 6 million years ago (Bradbury *et al.*, 1988). Analysis of a restriction fragment length polymorphism (RFLP) associated with mouse CD1 genes suggested that they were not linked to the murine MHC on chromosome 17 (Bradbury *et al.*, 1988), a conclusion that is now confirmed by direct analysis using pulsed-field gel electrophoresis. This latter analysis localizes the mouse CD1 locus (also known as *Ly-38*) to murine chromosome 3, and identifies it as an additional member of a large group of genes that is conserved as a linked group in both the pericentromeric region of human chromosome 1 and the distal region of mouse chromosome 3 (Mosely *et al.*, 1989).

Partial characterization of the CD1 family in the rat and rabbit has also been reported. In the former species, a cDNA clone encoding rat CD1 was isolated, and sequence analysis reveals it to be closely related to the mouse CD1 sequences, especially mouse CD1D1 (Ichimiya *et al.*, 1994). Probes derived from this cDNA hybridize to only one band in Southern blots of restriction enzyme digests of genomic rat DNA, indicating that only a single CD1 gene may exist in the rat. Interestingly, this would be consistent with the predicted time for the duplication event that gave rise to the second CD1 gene in the mouse, since the divergence of the mouse and rat species is estimated to have occurred more than 10 million years ago (Bradbury *et al.*, 1988). An RFLP associated with rat CD1 also indicates that it is not linked to the MHC in this species, although direct mapping studies of the rat CD1 locus have not yet been reported. In contrast to the unexpanded or contracted CD1 families of mice and rats, partial characterization of rabbit CD1 reveals a more expanded multigene family with a level of complexity equaling or exceeding that of human CD1. The number of CD1 genes in the domestic rabbit genome was estimated by probing genomic Southern blots with a cross-hybridizing human CD1 $\alpha3$ do-

main probe. This reveals as many as eight cross-hybridizing bands using multiple different restriction enzymes, indicating the existence of up to eight rabbit CD1 genes. The chromosomal location of these genes is not yet known. However, isolation of genomic DNA fragments encoding portions of two different rabbit CD1 genes has been reported. Based on their nucleotide and deduced amino acid sequences, these genes have been identified as the rabbit homologues of the human CD1B and CD1D genes (Calabi *et al.*, 1989a).

D. The Intron/Exon Structure and Organization of CD1 Genes

Nucleotide sequencing and intron/exon mapping have revealed a similar structure for CD1 genes in humans, mice, and rabbits (Martin *et al.*, 1987; Calabi *et al.* 1989b; Balk *et al.*, 1989; Bradbury *et al.*, 1988; Calabi *et al.*, 1989a). All CD1 genes characterized to date consist of multiple exons separated by introns of less than 1 kb in size. The intron/exon organization of all CD1 genes so far analyzed has been presented in detail previously (Calabi *et al.*, 1991), and the essential features are summarized in Fig. 2. All CD1 genes consist of six functional domains. The first domain encodes the 5′-untranslated and hydrophobic leader sequence. The second, third, and fourth domains encode the three extracellular domains of mature CD1 heavy chains, designated $\alpha 1$, $\alpha 2$, and $\alpha 3$. The fifth exon encodes the transmembrane domain and a short stretch of charged membrane anchoring residues. Finally, the sixth exon encodes a short cytoplasmic tail and the 3′-untranslated sequence. In general, CD1 genes show a pattern of intron/exon size, number, and organization that is similar to that of MHC class I genes, and this has been recognized as one important level at which significant similarity between CD1 and MHC molecules can be discerned. The major difference between CD1 and MHC class I genes at this organizational level relates to the manner in which the cytoplasmic domains are encoded, with the longer cytoplasmic tails of class I molecules being assembled from three additional exons as opposed to the single exon in CD1 genes (Fig. 2).

E. Upstream Regions and Transcriptional Regulation of CD1 Genes

The structure of the 5′ ends of CD1 genes and the manner in which they regulate the transcription of CD1 genes has only been partially examined. Preliminary mapping of the transcriptional start sites by RNAse protection studies has been reported for human CD1A, -B, and -C genes (Calabi *et al.*, 1989c; Calabi *et al.*, 1991). These studies

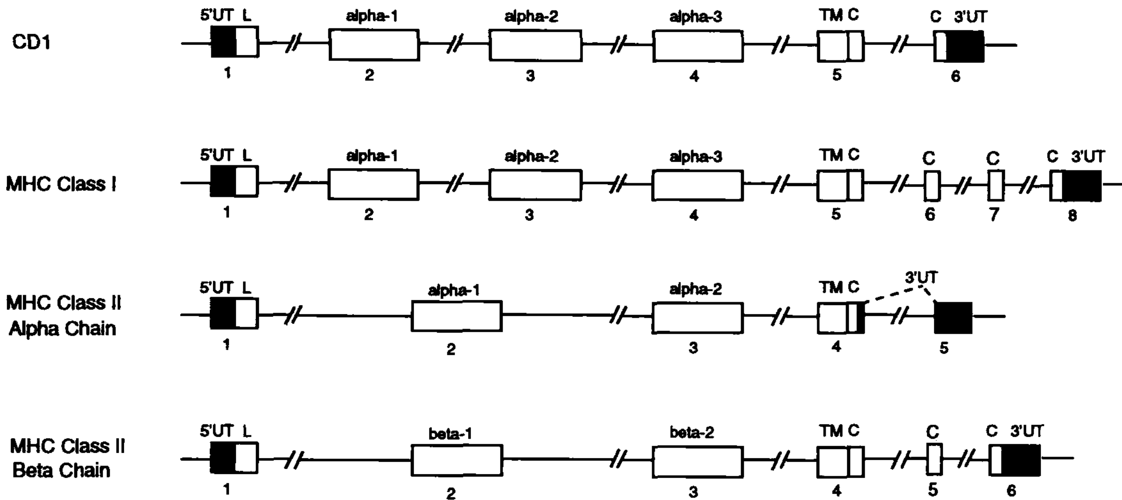

FIG. 2. Intron/exon organization of CD1 and MHC class I and II genes. Open boxes are coding region exons. Filled boxes indicate 5′ and 3′ untranslated regions.

were apparently carried out using poly(A)$^+$ mRNA from MOLT-4 thymic leukemia line cells and, thus, can be presumed to be most directly relevant to the transcription of CD1 genes in thymocytes. This analysis indicates the existence of potential cap sites located roughly 550, 110, and 140 base pairs (bp) upstream of the translation initiation site in the CD1A, -B, and -C genes, respectively. Interestingly, no obvious promoter sequence elements (e.g., TATA, CCAAT, or CCGCCC boxes) have been found in the 5'-untranslated regions of these genes. However, approximately 260 nucleotides of the 5'-untranslated sequence are significantly conserved between CD1A, -B, and -C genes (Calabi *et al.*, 1989b). This area is also conserved in the rabbit CD1B gene, and to a lesser extent in human CD1E. Specific blocks of completely conserved untranslated sequence are seen in this region for all of these genes, including two heptamer sequences located approximately 200 (GGGAAAT) and 100 (GAAGTCA) nucleotides upstream of the proposed translational start site. These sequences have been proposed as candidate binding sites for nuclear factors that regulate CD1 gene transcription. Interestingly, these conserved motifs are not found in the 5' untranslated sequences of human CD1D or mouse CD1 genes, suggesting that genes in the CD1D homology group may be regulated differently at the transcriptional level (see Section III,C). Other features of possible significance to regulation of the CD1A gene include a 49-nucleotide long poly-T sequence in the sense strand immediately upstream of the proposed cap site, and a $(TG)_{21}$ intronic sequence immediately downstream of the leader exon. It has been pointed out that this latter sequence could in theory be predisposed to assume a Z-DNA conformation and could function in this manner as a transcriptional control element (Calabi *et al.*, 1991). Unfortunately, no detailed dissection of regulatory elements of any CD1 gene using reporter constructs or by mapping of DNAse hypersensitivity sites has yet been reported, and this entire area remains primarily one of speculation.

F. Complexity of CD1 Transcripts and CD1 mRNA Splicing

All of the five human CD1 genes have been shown to be transcribed in the MOLT-4 thymic leukemia cell line, and transcription of various human and rodent CD1 genes has also been demonstrated in a variety of other tissues (see Section VI). Thus, all CD1 genes so far described appear to be transcriptionally active and lack obvious features of pseudogenes. This includes the human CD1E and mouse CD1D2 genes, for which protein products have not yet been clearly demonstrated. The complexity of CD1 mRNA transcripts in MOLT-4 cells was ini-

tially suggested by Northern blots using gene-specific probes derived from the 3′-untranslated regions of each of the human CD1 genes. Such analyses reveal multiple mRNA sizes ranging from 1.2 to 3.0 kb for all of the human genes, except for CD1D which shows a single transcript size (Calabi *et al.*, 1989c). Analysis of cDNA sequences supports the idea that this complex array of CD1 transcripts is generated through alternative mRNA splicing, as examples have been described in which apparently cryptic donor splice sites within the α3 domain sequences of CD1B or CD1E are joined to the splice acceptor site of the transmembrane/cytoplasmic domain exon. These alternative splicing events would be predicted to result in transcripts encoding a truncated and presumably nonfunctional form of the protein. However, more recently it has been shown that alternative splicing at the 3′ ends of CD1 mRNAs may be responsible for generating secretory isoforms of certain CD1 proteins. Examination by reverse-transcription polymerase chain reaction (PCR) analysis of CD1 transcripts from human thymus reveals a pronounced pattern of alternative splicing at the 3′ ends for CD1C and -E, but not CD1A, -B, or -D. In the case of CD1C, sequence analysis demonstrates that one of the alternatively spliced transcripts is formed by the joining of the 3′ splice donor site of the α3 domain directly to the 5′ splice acceptor site of the cytoplasmic/3′-untranslated domain exon. This transcript thus deletes the transmembrane domain and is predicted to encode a secretory form of CD1c. In support of the possible relevance of such alternative splicing, a soluble form of CD1c is found to be present in the supernatant of cultured cells transfected with the CD1C gene. A related by slightly different pattern of alternative splicing is seen for CD1A when transfected into mouse myeloma cells, producing both a secreted product and a retained intracellular component in addition to the expected membrane-bound form of the protein (Woolfson and Milstein, 1994). The functional significance (if any) of these proposed secreted isoforms of CD1 is currently unknown, although this may be another noteworthy parallel with MHC class I and II molecules, both of which have also been found to have secreted isoforms (Krangel, 1986; Briata *et al.*, 1989).

III. The CD1 Proteins: Characterization of a Family of MHC-Related Molecules

Initial studies describing CD1-specific monoclonal antibodies revealed two remarkable features of the proteins that they identified. One is their pattern of cellular expression, which was initially found

to include thymocytes and some thymic leukemias but not mature medullary thymocytes or normal peripheral T cells (Reinherz *et al.*, 1980). The other notable feature came from biochemical analyses, which revealed a structure reminiscent of class I MHC antigens. Thus, it was recognized that all classic CD1 proteins are hererodimers of an approximately 45-kDa heavy-chain associated with a 12-kDa light-chain subunit, subsequently identified as β-microglobulin. These features led to the hypothesis that CD1 proteins are probably related at a structural level to classical transplantation antigens (i.e., MHC class I molecules), and ultimately pointed the way to studies of CD1 as the key component of a separate system of antigen recognition by T cells. Thus, a particular emphasis of the section that follows will be the structural parallels that exist between CD1 proteins and the MHC-encoded class I and II antigen-presenting molecules.

The structure of a modest but growing number of CD1 proteins has now been well defined by application of conventional techniques of serology and protein biochemistry. However, perhaps even more revealing has been the information obtained from the study of the predicted amino acid sequences of these proteins, as determined from CD1 genes and cDNA clones. This discussion begins with the predicted features of CD1 protein structure based on deduced amino acid sequences, which should bring the overall picture into the sharpest possible focus. Subsequent sections procede to summarize the direct observations relevant to CD1 protein structure that have been made using techniques of protein biochemistry and serologic analysis.

A. Deduced Amino Acid Sequences and Predicted Structural Features of CD1 Proteins

The primary amino acid sequences of CD1 α-chains can be deduced in their entirety from cDNA and genomic DNA sequences for the 4 currently demonstrated human CD1 proteins, as well as for the potential product of the human CD1E gene (Martin *et al.*, 1987; Aruffo and Seed, 1989; Longley *et al.*, 1989; Calabi *et al.*, 1989b). In addition, full-length cDNA sequences have been reported for mouse CD1 (Balk *et al.*, 1991b; Bradbury *et al.*, 1988), 1 rat CD1 protein (Ichimiya *et al.*, 1994), and 1 sheep CD1 protein (D. Ferguson, unpublished gene bank entry). Finally, partial sequences can also be deduced from 2 rabbit genomic DNA clones (Calabi *et al.*, 1989a). Alignment of the predicted amino acid sequences for these 11 different CD1 proteins from five species is shown in Fig. 3. The protein sequences have been divided into six domains based mainly on the organization of coding regions in CD1 genes, as outlined in the preceding section. It is note-

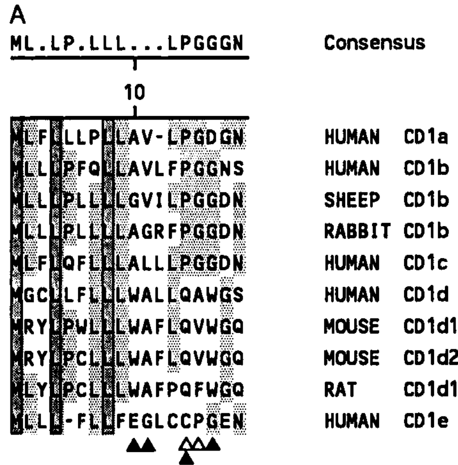

FIG. 3. Alignment of deduced amino acid sequences of CD1 proteins. (A) Leader domains, (B) α1 domains, (C) α2 domains, (D) α3 domains, and (E) transmembrane plus cytoplasmic domains. Residues matching the consensus are shaded in light gray, and residues conserved in all CD1 sequences are boxed. Open triangles indicate positions at which group 1-specific residues are found, and solid triangles indicate positions at which group 2-specific residues are found (see Section III,C for explanation of group 1 versus group 2 CD1 proteins). Dashes represent gaps that have been introduced to maximize alignment, and asterisks denote cysteines conserved in all or nearly all CD1 sequences that are presumed to be involved in intrachain disulfide bond formation. Alignments were performed using the MegaAlign module of the Lasergene software package (DNASTAR Inc., Madison, WI) by the clustal method using a PAM250 residue weight table. A limited amount of manual adjustment has been done to maximize alignment, particularly in the cytoplasmic domains. References for sequences are given in the text. Consensus residues are indicated for positions at which 50% or more of the sequences share the same residue; a period indicates that no consensus exists at that position. Numbers at the start of each line denote the position of the first residue in each domain in the mature CD1 heavy chain, with residue 1 being the first residue predicted for the α1 domains. Note that for rabbit CD1d, three residues are likely to precede the first N-terminal amino acid shown for α1, but the cDNA or gene sequence corresponding to this region is not yet available.

worthy that complete predicted amino acid sequences of human CD1a, -b, and -c can be compared from two separate sources, the cell lines MOLT-4 (Martin *et al.*, 1987; Longley *et al.*, 1989) and HPB-ALL (Aruffo and Seed, 1989). These sequences are nearly identical in each case (two amino acid substitutions in CD1a, none for CD1b, and one for CD1c). Furthermore, the two mouse CD1 sequences have been derived for two separate strains, (C57 × CBA)F1 (Balk *et al.*,

B

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     ....Q...SFH.LQISSF.N.SW.RT.GSGWLGDLQTH.W..DS.TI.FLKPWS.GNFS.QEW..LQ..FRVY..SFTR.IQ..V.....KE-   Consensus
              10        20        30        40        50        60        70        80        90

1    ADGLKEPLSFHVIWIASFYNHSWKQNLVSGWLSDLQTHTWDSNSSTIVFLWPWSRGNFSNEEWKELETLFRIRTIRSFEGIRRYAHELQF-E    HUMAN  CD1a
1    EHAFQGPTSFHVIQTSSFTNSTWAQTQGSGWLDDLQIHGWDSDSGTAIFLKPWSKGNFSDKEVAELEEIFRVYIFGFAREVQDFAGDFQMK     HUMAN  CD1b
1    EDVFQGPTSFHLKQISTFVNSTWAQNLGSGWLDDLQIHGWESDSGTAIFLKPWSKGNFSDEEITELVDLFRVYLIGFIREVQDRVNEFQL-E    SHEEP  CD1b
1    EDALQGPTSYHVMQISSFTNSTWTENRGSGWLEDLQIHRWDSETGTAIFLKPWSRGNLSDEEITELVELFRVYFFGLVRELRDHVTEFQMK     RABBIT CD1b
1    ADASQEHVSFHVIQIFSFVNQSWARGQGSGWLDELQTHGWDSESGTIIFLHNWSKGNFSNEELSDLELLFRFYLFGLTREIQDHASQDYSK     HUMAN  CD1c
1    AEVPQRLFPLRCLQISSFANSSWTRTDGLAWLGELQTHSWSNDSDTVRSLKPWSQGTFSDQQWETLQHIFRVYRSSFTRDVKEFAKMLRLS     HUMAN  CD1d
1    ---LQRSFPFHGLQISSFVNSSQTRTDCLAWLGELQTHSWSNDSDTIHFLKPWSQGTFNFQQWEQVQNELWVYRLSVTRDIHDFVKLLKLT     RABBIT CD1d
1    SEAQQKNYTFRCLQMSSFANRSWSRTDSVVWLGDLQTHRWSNDSATISFTKPWSQGKLSNQQWEKLQHMFQVYRVSFTRDIQELVKMMSPKED   MOUSE  CD1d1
1    SEVQQKNYTFRCLQTSSFANISWSRTDSLILLGDLQTHRWSNDSATISFTKPWSQGKLSNQQWEKLQHMFQVYRVSFTRDIQELVKMMSPKED   MOUSE  CD1d2
1    SEVQQ-NYTFCCLQISSFANRSWSRTDSVVWLGDLQTHRWSNDSDTISFTKPWSQGKFSNQQWEKLQHMFQVHRTSFTRDIKEIVKMMSPKED   RAT    CD1d1
1    TAAAEEQLSFRMLQTSSFANHSWAHSEGSGWLGDLQTHGWDTVLGTIRFLKPWSHGNFSKQELKNLQSLFQLYFHSFIQIVQASAGQFQL-E    HUMAN  CD1e
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C

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     YPFEIQV.AGCEMHPGNASESFLHVAFQG.D.LSFQGTSW.P.PGAGS...L.CKVLN-QDQGT.ETVQ.LLN.TCPRF..GLLEAGKS.L.KQ   Consensus
              10        20        30        40        50        60        70        80        90

92   YPFEIQVTGGCELHSGKVSGSFLQLAYQGSDFVSFQNNSWLPYPVAGNMAKHFCKVLN-QNQHENDITHNLLSDTCPRFILGLLDAGKAHLQRQ   HUMAN  CD1a
92   YPFEIQGIAGCELHSGGAIVSFLRGALGGLDFLSVKNASCVPSPEGGSRAQKFCALII-QYQGIMETVRILLYETCPRYLLGVLNAGKADLQRQ   HUMAN  CD1b
92   YPFVIQVIEGCELHSGEAIESSLRGALGGLDVLRIQNHSCMPAPDSGNRGQKLCALLS-QYQGTSDIIERLVSETCPRYLLGVLDAGKAELQRQ   SHEEP  CD1b
92   YPFEVQVKAGCELHSGKSPEGFFQVAFNGLDLLSFQNTTWVPSPQCGSLAQSVCHLLNHQYEGVTETVYNLIRSTCPRFLLGLLDAGKMYVHRQ   HUMAN  CD1c
92   YPLELQVSAGCEVHPGNASNNFFHVAFQGKDILSFQGTSWEPTQEAPLWVNLAIQVLN-QDKWTRETVQWLLNGTCPQFVSGLLESGKSELKKQ   HUMAN  CD1d
89   YPIELQVFAGCEMHPGNTSESFFHVAYQGMHVLSFRGTLWETAPGTPPFVKLVVKELN-LDHGTREMIQELLNNTCPQFVSGLIEAGRSELEKQ   RABBIT CD1d
94   YPIEIQLSAGCEMYPGNASESFLHVAFQGKYVVRFWGTSWQTVPGAPSWLDLPIKVLN-ADQGTSATVQMLLNDTCPLFVRGLLEAGKSDLEKQ   MOUSE  CD1d1
94   YPIEIQLSTGCEMYPGNASESFFHVAFQGKYAVRFRGTSWQRVLGAPSWLDLPIKVLN-ADQGTSATVQTLLNDTWPQFARGLLEAGKSDLEKQ   MOUSE  CD1d2
93   YPIEVQLSAGCEMYPGNASESFLHVAFQGEYVVRFHGTSWQKVPEAPSWLDLPIKMLN-ADEGTRETVQILLNDTCPQFVRGLLEAGKPDLEKQ   RAT    CD1d1
92   YPFEIQILAGCRM---NAPQIFLNMAYQGSDFLSFQGISWEPSPGAGIRAQNICKVLN-RYLDIKEILQSLLGHTCPRFLAGLMEAGESELKRK   HUMAN  CD1e
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FIG. 3.—*Continued*

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D
      VKPEAWLSSGPSPGPGRLQLVCHVSGFYPKPVWVMWMRGEQEQQGTQRGDILPNADETWYLRATLDVAAGEAAGLSCRVKHSSLGGQDIILYW   Consensus
               10        20        30        40        50        60        70        80        90
185   VKPEAWLSHGPSPGPGHLQLVCHVSGFYPKPVWVMWMRGEQEQQGTQRGDILPSADGTWYLRATLEVAAGEAADLSCRVKHSSLEGQDIVLYW   HUMAN  CD1a
185   VKPEAWLSSGPSPGPGRLQLVCHVSGFYPKPVWVMWMRGEQEQQGTQLGDILPNANWTWYLRATLDVADGEAAGLSCRVKHSSLEGQDIILYW   HUMAN  CD1b
185   VKPEAWLSSGPTPGPGRLLLVCHVSGFYPKPVQVIWMRGKQEQPGTQQGDIMPNADWTWYLRVTLNVAAGEAAGLSCRVKHSSLGDQDIILYW   SHEEP  CD1b
186   VRPEAWLSSRPSLGSGQLLLVCHASGFYPKPVWVTWMRNEQEQLGTKHGDILPNADGTWYLQVILEVASEEPAGLSCRVRHSSLGGQDIILYW   HUMAN  CD1c
185   VKPKAWLSRGPSPGPGRLLLVCHVSGFYPKPVWVKWMRGEQEQQGTQPGDILPNADETWYLRATLDVVAGEAAGLSCRVKHSSLEGQDIVLYW   HUMAN  CD1d
182   VKPEAWLSSGPSPGPGRLLLVCRVSGFYPKPVQVMWMRGDQEQPHTRQGDFLPNADGTWYLRVTLDVAAGDAAGLSCRVKHSSLGGQDIILYW   RABBIT CD1d
187   EKPVAWLSSVPSSADGHRQLVCHVSGFYPKPVWVMCMRGDQEQQGTHRGDFLPNADETWYLQATLDVEAGEEAGLACRVKHSSLGGQDIILYW   MOUSE  CD1d1
187   EKPVAWLSSVPSSAHGHLQLVCHVSGFYPKPVWVMWMRGDQEQQGTHRGDFLPNADETWYLQATLDVEAGEEAGLACRVKHSSLGGQDIILYW   MOUSE  CD1d2
186   EKPVAWLSRGPNPAHGHLQLVCHVSGFHPKPVWVMWMRGDQEQGGTHRGDILPNADETWYLQATLDVEAGDEAGLACRVKHSSLEGQDIILYW   RAT    CD1d1
182   VKPEAWLSCGPSPGPGRLQLVCHVSGFYPKPVWVMWMRGEQEQRGTQRGDVLPNADETWYLRATLDVAAGEAAGLSCRVKHSSLGGHDLIIHW   HUMAN  CD1e
                           *                                                      *

E
      -G...S.GLI.LAVIVPLLVLLIV-L.LWF-RRR-------------SYQDI.-------------   Consensus
               10        20        30        40        50        60
278   -EHHSSVGFIILAVIVP-LLLLIG-LALWF-RKR-------------CFC                    HUMAN  CD1a
278   -RNPTSIGSIVLAIIVPSLLLLLC-LALWYMRRR-------------SYQNIP                 HUMAN  CD1b
278   -GHPTSIGLILVAIIVPSLILSIC-LALWFWRRW-------------SYQNIL                 SHEEP  CD1b
279   -GHHSSMNWIALVVIVP-LVILIV-LVLWF-KKHC------------SYQDIL                 HUMAN  CD1c
278   GGSYTSMGLIALAVLACLLFLLIVGFTSRF-KRQT------------SYQGVL                 HUMAN  CD1d
280   DARQAPVGLIVFIVLIMLVVVGAVVYYIW--RRRS------------AYQDIR                 MOUSE  CD1d1
280   DARQAPVGLIVFIVLIMLVVVGAVVYYIW--RRRS------------AYQDIR                 MOUSE  CD1d2
279   GGRQVSPVLIFLIVGVLVLVVCAVAYYIIRKRRR-------------SYQDIM                 RAT    CD1d1
275   GGYSIFLILICLTVIVTLVILVVVDSRL---KKQSPAFSWEPTLRTPRIQDISSAWHKYRGSKTEY    HUMAN  CD1e
                    Δ Δ          Δ Δ
```

FIG. 3.—*Continued*

1991b) and BALB/cJ (Bradbury *et al.*, 1988). These sequences are identical except for a single nucleotide substitution resulting in an isoleucine to threonine conversion in the α1 domain of mouse CD1d2. This supports the notion also borne out by biochemical and serologic analyses that CD1 proteins are essentially monomorphic, lacking the highly significant allelic polymorphism that is a hallmark of classical MHC-encoded antigen-presenting molecules. The possible significance of this lack of polymorphism in CD1 is discussed under subsequent sections (see Section V,B).

The precursors of all CD1 polypeptides begin with a hydrophobic leader or signal sequence of 17 or 18 amino acids at their N termini, which is required for translocation across the membrane of the endoplasmic reticulum. Thus, CD1 proteins are type I transmembrane glycoproteins, oriented with their N termini in the lumenal space of intracellular compartments or in the extracellular space at the plasma membrane, and their C termini located in the cytosol. The point of cleavage of the signal peptide to generate the first residue of the α1 domain of the mature protein has been difficult to determine with certainty because blocked N termini have been encountered in several cases in which N-terminal sequencing has been attempted (Lerch *et al.*, 1986). However, an 18-residue N-terminal sequence was derived by direct protein sequencing of the rabbit CD1b protein (Wang *et al.*, 1988), and this has been used as the basis for the selection of the first residue of the α1 domains shown in Fig. 3. Recently, a second successful effort at N-terminal sequencing was reported which showed that the mature human CD1a protein expressed in mouse myeloma cells begins with the sequence DGLKEPLS (Woolfson and Milstein, 1994). This suggests that the cleavage point for generation of the N terminus of the mature CD1a protein may be one residue downstream from that shown in Fig. 3. Further revisions in the predicted N termini of the mature protein may thus be necessary as more information becomes available.

The three extracellular domains of CD1 proteins (α1, α2, α3) show a highly conserved length of approximately 90 amino acids (range 90–94). Between three and five potential sites for N-linked glycosylation can be identified on the extracellular portions (i.e., α1, α2, and α3 combined) of all CD1 proteins. This is shown schematically in Fig. 4. Only one of these sites (N19 or 20) is conserved in all CD1 proteins. The CD1 transmembrane domains consist of a short connecting peptide (approximately 4 to 6 residues) followed by a hydrophobic stretch of approximately 20 residues that corresponds to the membrane-spanning portion of the protein. This is typically followed by 3 basic

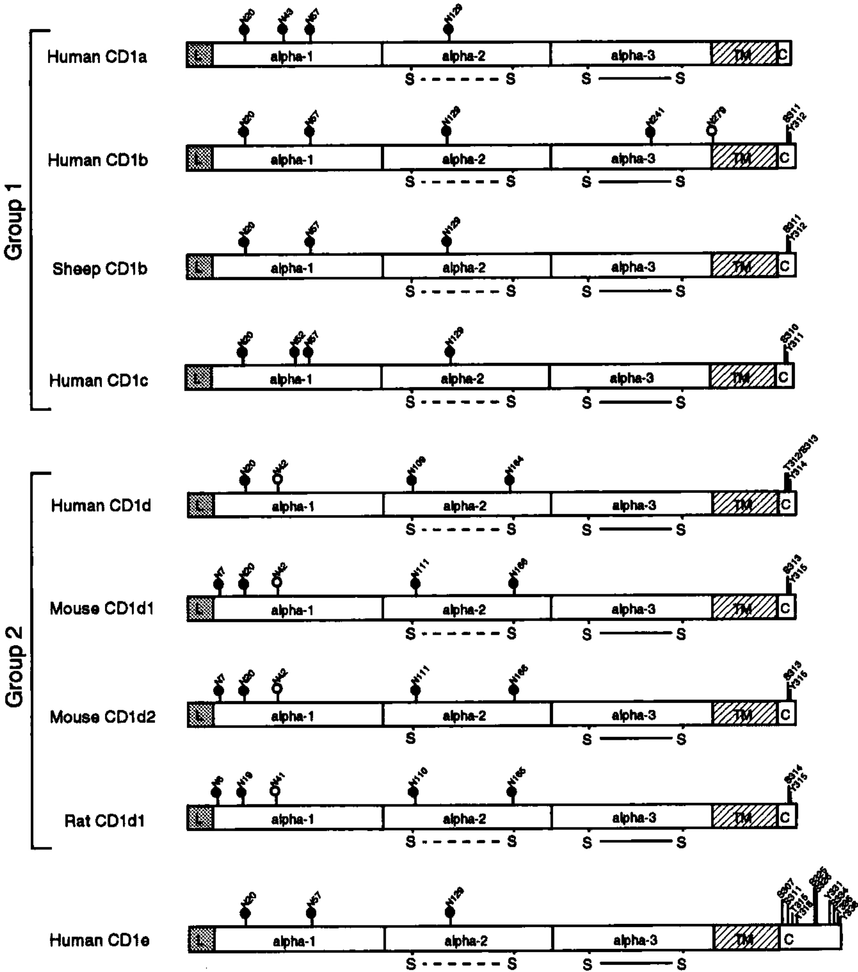


FIG. 4. Structure and post-translational modifications of CD1 heavy chains. Potential asparagine-linked glycosylation sites (N-X-S or N-X-T) are indicated by the circles above the schematized protein. Open circles indicate sites that are less likely to be used because of sequence considerations [i.e., asparagine followed by aspartate or proline (Kornfeld and Kornfeld, 1985)] or because of close proximity to the plasma membrane (i.e., for human CD1b). Potential phosphorylation sites (i.e., serine, threonine, and tyrosine residues) in the cytoplasmic tails are indicated. Probable intradomain disulfide bonds in the $\alpha3$ domains of all CD1 heavy chains are indicated by solid lines, and possible intrachain disulfide bonds in the $\alpha2$ domains of most CD1 heavy chains are shown as dashed lines. Classification of CD1 proteins as group 1 versus group 2 is detailed in the text (see Section III,C).

residues which probably comprise a charged membrane anchor that is postulated to interact with negative charges present on membrane phospholipids. CD1 membrane-spanning segments lack charged residues which may be involved in subunit interactions between components of multisubunit complexes. However, approximately half of the CD1 membrane-spanning segments contain cysteine residues that could participate in covalent interactions with other proteins (see Section V,E).

With the exception of human CD1e, the cytoplasmic tails predicted for CD1 proteins are extremely short (6–10 amino acids). Nevertheless, these can be divided into three distinct types. The first is the CD1a tail, which contains two cysteine residues that have been postulated to be involved in disulfide-linked interactions with other proteins (see Section V,E). The second type is represented by CD1e, which is substantially longer than the others. The third type of tail is found in all of the other reported CD1 sequences and consists of a short (6–7 residues not including the membrane anchor) sequence containing a conserved YQXI or YQXV sequence. This sequence is generally not found in the cytoplasmic tails of MHC class I or II proteins or in those of other MHC-related proteins. Although the function of CD1 cytoplasmic tails has not yet been directly studied, it is interesting that the sequences corresponding to the motif YXXZ (where Z is a hydrophobic residue) have been associated with endocytosis of plasma membrane molecules (e.g., transferrin receptor) and with endosomal or lysosomal localization of certain cellular proteins (Sandoval and Bakke, 1994). A related sequence also conforming to this YXXZ motif (i.e., YPTL) is present in the cytoplasmic tail of the HLA-DMβ chain (Kelly *et al.*, 1991; Cho *et al.*, 1991) and may be involved in the colocalization of HLA-DM with MHC class II molecules within the endocytic system. These observations raise the interesting possibility that the short cytoplasmic tails of most CD1 proteins may significantly influence their trafficking and localization within cells.

B. Intraspecies and Interspecies Conservation of CD1 Protein Sequences

Inspection of the sequence alignments in Fig. 3 reveals a high level of conservation for CD1 proteins both within and between different mammalian species. A summary of pairwise comparisons for amino acid sequences of the leader, α1, and α2 domains is shown in Table I, and similar analyses at both the nucleotide and amino acid levels have been presented previously for most of the CD1 sequences analyzed here (Calabi *et al.*, 1989c; Calabi and Bradbury, 1991a; Calabi

TABLE I
AMINO ACID SEQUENCE SIMILARITY AND DIVERGENCE OF MAMMALIAN CD1 PROTEINS[a]

A. CD1 leader domains

Percent Similarity

Percent Divergence

Human CD1a	Human CD1b	Sheep CD1b	Rabbit CD1b	Human CD1c	Human CD1d	Mouse CD1d1	Mouse CD1d2	Rat CD1d1	Human CD1e	
	58.8	47.1	47.1	52.9	41.2	29.4	29.4	35.3	29.4	Human CD1a
47.1		61.1	66.7	61.1	38.9	27.8	27.8	33.3	38.9	Human CD1b
35.3	38.9		77.8	66.7	33.3	38.9	38.9	38.9	50	Sheep CD1b
41.2	33.3	22.2		66.7	38.9	33.3	33.3	38.9	44.4	Rabbit CD1b
35.3	38.9	33.3	33.3		44.4	33.3	33.3	33.3	38.9	Human CD1c
58.8	61.1	66.7	72.2	55.6		61.1	61.1	55.6	33.3	Human CD1d
64.7	72.2	61.1	66.7	66.7	38.9		94.4	77.8	27.8	Mouse CD1d1
64.7	72.2	61.1	66.7	66.7	38.9	5.6		83.3	27.8	Mouse CD1d2
64.7	66.7	61.1	61.1	66.7	44.4	22.2	16.7		22.2	Rat CD1d1
64.7	61.1	50	55.6	61.1	83.3	83.3	83.3	77.8		Human CD1e

B. CD1 alpha-1 domains

Percent Similarity

Percent Divergence

Human CD1a	Human CD1b	Sheep CD1b	Rabbit CD1b	Human CD1c	Human CD1d	Rabbit CD1d	Mouse CD1d1	Mouse CD1d2	Rat CD1d1	Human CD1e	
	45.1	48.4	47.3	49.5	30.8	31.9	30.8	29.7	33	44	Human CD1a
54.4		72.5	68.1	59.3	42.9	35.2	38.5	37.4	36.3	50.5	Human CD1b
51.1	27.5		69.2	54.9	40.7	36.3	35.2	35.2	36.3	48.4	Sheep CD1b
52.2	31.9	30.8		53.8	35.2	33	35.2	33	33	41.8	Rabbit CD1b
50	40.7	45.1	46.2		37.4	36.3	37.4	35.2	36.3	48.4	Human CD1c
68.9	57.1	59.3	64.8	62.6		65.9	60.4	61.5	63.7	44	Human CD1d
68.2	63.6	62.5	65.9	62.5	31.8		52.7	52.7	54.9	34.1	Rabbit CD1d
68.9	62.6	64.8	65.9	63.7	39.6	44.3		93.5	89.1	42.9	Mouse CD1d1
70	63.7	64.8	68.1	65.9	38.5	44.3	6.5		85.9	41.8	Mouse CD1d2
65.9	64	62.9	67.4	64	34.8	40.7	9.9	13.2		39.6	Rat CD1d1
55.1	48.9	51.1	57.8	51.1	55.6	64.4	56.7	57.8	59.1		Human CD1e

C. CD1 alpha-2 domains

Percent Similarity

Percent Divergence

	Human CD1a	Human CD1b	Sheep CD1b	Human CD1c	Human CD1d	Rabbit CD1d	Mouse CD1d1	Mouse CD1d2	Rat CD1d1	Human CD1e
Human CD1a		51.6	52.7	49.5	43	39.8	45.2	41.9	41.9	47.8
Human CD1b	48.4		66.7	51.6	37.6	31.2	38.7	34.4	39.8	43.3
Sheep CD1b	47.3	33.3		45.2	33.3	33.3	36.6	32.3	33.3	36.7
Human CD1c	47.3	45.2	51.6		41.9	38.7	37.6	35.5	39.8	40
Human CD1d	57.6	63	67.4	55.9		62.4	62.4	61.3	63.4	44.4
Rabbit Cd1d	59.8	68.5	66.3	58.1	37.6		61.3	58.1	61.3	42.2
Mouse CD1d1	54.3	60.9	63	59.1	37.6	38.7		89.2	87.1	42.2
Mouse CD1d2	57.6	65.2	67.4	61.3	38.7	41.9	10.8		81.7	37.8
Rat CD1d1	57.6	59.8	66.3	57	36.6	38.7	12.9	18.3		38.9
Human CD1e	49.4	52.8	59.6	54.4	53.9	56.2	56.2	60.7	59.6	

[a] Shaded boxes indicate pairs with greater than 45% similarity. Pairwise comparisons for the individual domains were performed after alignment by the clustal method using the PAM250 residue weight table. Alignments were made using the MegaAlign module of the LaserGene software package (DNASTAR Inc., Madison, WI). Similarity and divergence are defined as follows: for any two sequences i and j, similarity (i,j) = [100 × sum of matches]/[length - gap residues (i) - gap residues (j)]. Divergence (i,j) = [100 × distance (i,j)]/total distance, where total distance refers to the sum of all branch lengths of the phylogenetic tree constructed from the multiple alignment of all sequences in the analysis, and distance (i,j) is the sum of the branch lengths between two particular sequences. See text for references for individual sequences.

et al., 1991). These analyses reveal a very high level of conservation between all CD1 sequences in the α3 domains, amounting to at least 70% identity for both the nucleic acid and the amino acid sequences. This high level of conservation for α3 domains is apparent from the number of completely conserved residues present in the aligned sequences of the 10 CD1 α3 domains (Fig. 3D) amounting to 45 of 93 residues. As a comparison, it is useful to note that in a similar analysis of MHC class I sequences from a variety of mammalian species, only 24 out of 90 positions in α3 were found to be completely conserved (Hughes, 1991). Thus, the α3 domains of CD1 proteins appear to be even more highly constrained than the corresponding domains of MHC class I proteins, strongly pointing to an important conserved function for this region of CD1.

The sequences in α1 and α2 domains of CD1 proteins show greater variability than that in α3, although a number of completely conserved residues are again evident (10 in α1 and 12 in α2). The pattern of variability in CD1 α1 and α2 domains was analyzed in one study revealing a lack of evidence for positive selection of nonsynonymous nucleotide substitutions such as one typically finds in regions corresponding to the peptide antigen binding site of MHC class I and class II proteins (Hughes, 1991). This seems to indicate that the α1 and α2 domains of CD1 are not undergoing the relatively rapid evolution characteristic of this region in MHC-encoded antigen-presenting molecules. When pairwise alignments of the amino acid sequences of the available CD1 α1 and α2 domains are examined, it is noteworthy that interspecies homologues (orthologues) can readily be identified. Thus, the mouse, rat, and one of the rabbit proteins can be identified as homologues of human CD1d. These proteins are all related to one another by greater than 50% amino acid sequence identity in α1 and α2. In contrast, human CD1d is only 31 to 43% identical to human CD1a, -b, or -c in these domains (see Tables 1B and 1C). A similar relationship exists between the CD1b sequences from human, sheep, and rabbits. It is convenient to refer to these homologous groups as isotypes, with all of the currently known CD1 proteins being identifiable as one of five known isotypes (i.e., CD1a, -b, -c, -d, or -e). Thus, the similarity of CD1 proteins of given isotype *between* different species (e.g., human CD1b vs rabbit or sheep CD1b) is greater than the similarity of different CD1 isotypes *within* a given species (e.g., human CD1b vs human CD1a, -c, -d, or -e). This orthologous relationship among CD1 proteins from different species suggests that the α1 and α2 domains of different CD1 isotypes evolved structural features corresponding to particular conserved functions prior to the radiation of mammalian species (i.e., approximately 80 million years ago).

Phylogenetic trees based on alignments of full-length CD1 protein sequences (Fig. 5A) or of the $\alpha1$ or $\alpha2$ domains (Figs. 5B and 5C) illustrate this interspecies relationship of CD1 proteins. This relationship is unlike that observed among nonclassical MHC class I genes, which appear to have evolved by much more recent independent duplications of classical class I genes after the divergence of the mammalian orders (Hughes and Nei, 1989). Thus, whereas a particular human gene (e.g., CD1D) can be identified as the homologue of the mouse CD1 genes, it is not possible to identify pairs of class I genes that represent clear interspecies homologues between such distantly related mammals. Interestingly, interspecies homologues of MHC class II genes can be readily identified in some cases (e.g., HLA-DR and I-E genes), suggesting that CD1 is more like MHC class II than MHC class I in terms of its pattern of evolutionary diversification. It should be noted that this relationship between CD1 isotypes of different species is not seen when comparisons are made only between $\alpha3$ domains (for example, see Fig. 7). This has been postulated to be due to interlocus exchanges (e.g., gene conversion events) that have homogenized this domain in human CD1 genes, resulting in unexpectedly high levels of similarity between all human CD1 $\alpha3$ sequences except for that of CD1c (Hughes, 1991).

C. Two Distinct Groups of CD1 Genes and Proteins

Analysis of DNA and protein sequences of human and mouse CD1 first led Calabi and colleagues to propose the existence of two structurally (and at least in theory functionally) distinct classes of CD1 genes and proteins (Calabi *et al.*, 1989b). These investigators noted that sequences of the leader, $\alpha1$, and $\alpha2$ domains contain conserved features that allow the categorization of most CD1 genes and their protein products into two distinct classes. As originally defined, one class consisted of human CD1A, -B, and -C genes. This group has often been called "classic CD1," since it contains the members of the human CD1 family that have been serologically identified as the classic thymic differentiation/thymus leukemia antigens. The other class consisted of the human CD1D and both mouse CD1 genes. The human CD1E gene was considered to be intermediate, with some features characteristic of each class. This categorization of CD1 into two classes appears to have held up and in fact has been strengthened by the analysis of additional CD1 genes and cDNA clones (seen Table I and Fig. 3) and may prove to be a valuable concept in the search for function of different members of the CD1 family. In further discussions of this concept, these two classes will be referred to as "groups" (i.e.,

"group 1" and "group 2") in order to avoid potential confusion with class I and class II MHC proteins.

One line of evidence supporting the existence of two groups of CD1 proteins is the simple analysis of sequence homology between CD1 genes in the 5'-untranslated regions and coding regions of the leader, $\alpha1$, and $\alpha2$ domains. Thus, pairwise alignment scores of sequences extending 330 bp upstream of the 3' end of the leader exon are significantly higher for comparisons between human group 1 members (CD1A, -B, and -C) than for comparisons of group 1 members with human CD1D, and comparisons with CD1E give intermediate values. Furthermore, the nucleotide and predicted amino acid sequence homologies across the leader, $\alpha1$, and $\alpha2$ coding regions are consistently higher for intragroup sequence comparisons (i.e., group 1 members with other group 1 members or group 2 members with other group 2 members) than for intergroup comparisons (Calabi *et al.*, 1989b). This relationship is still seen when the analysis is extended to include the available CD1 sequences from rabbit, sheep, rat, and mouse (Calabi *et al.*, 1991; Table 1). All of the sequences can be categorized as either group 1 (human CD1a, -b, and -c; sheep and rabbit CD1b) or group 2 (human CD1d; mouse CD1d1 and -d2; rat CD1d1; rabbit CD1d), except for human CD1e which seems to be equally related to both groups. This grouping based on amino acid sequence similarity in $\alpha1$ or $\alpha2$ domains is illustrated by phylogenetic trees generated from multiple sequence alignments (Fig. 5) and by the levels of similarity determined in pairwise alignments (Table I). The categorization of CD1 proteins into groups 1 or 2 is further facilitated by the identification of group-specific residues (Calabi *et al.*, 1989b, 1991). These are identical residues that occur in the same sequence position in all members of one group, but none of the members of the other group. The majority of group-specific residues are present in the $\alpha1$ and $\alpha2$ domains, although they can be identified in all domains of the proteins except for $\alpha3$ (see Fig. 3). A number of these group-specific residues are potential N-linked glycosylation sites, and this leads to the prediction of group-specific patterns of glycosylation (Fig. 4).

The importance of this distinction on structural grounds between two groups of CD1 proteins will obviously depend on the ability to show that group 1 and group 2 proteins perform different functions in the immune system. The function of the group 1 CD1 proteins in immune recognition is only just beginning to become clear, and even less is known with regard to the function of group 2 CD1 (see Section VII). It would thus be premature to speculate very far on the meaning of this apparent structural dichotomy. Nonetheless, it is perhaps signifi-

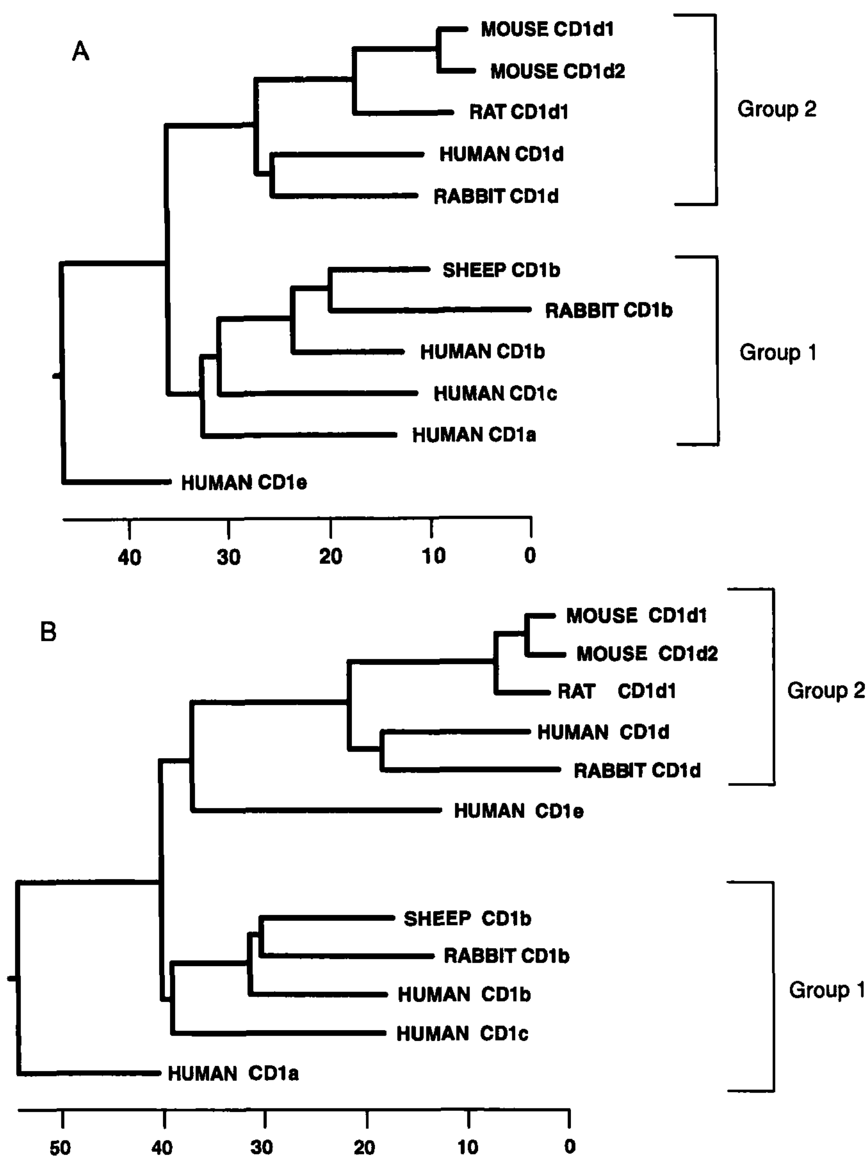


FIG. 5. Phylogenetic trees showing level of similarity and proposed evolutionary relationships between known CD1 proteins. (A) Phylogeny constructed by alignments of full-length amino acid sequences or maximum available sequence data. Full-length sequences (leader + α1,2,3 + TM + CYT domains) were used in all cases except for rabbit CD1d (only α1,2,3 reported) and rabbit CD1b (only leader + α1 reported). (B) Phylogeny based on α1 domain amino acid sequences. (C) Phylogeny based on α2 domain amino acid sequences. The x axes show calculated distance based on amino acid sequence divergence. Branch lengths in this unbalanced tree are proportional to the extent of sequence divergence. Alignments were performed as described in the legend to Fig. 3.

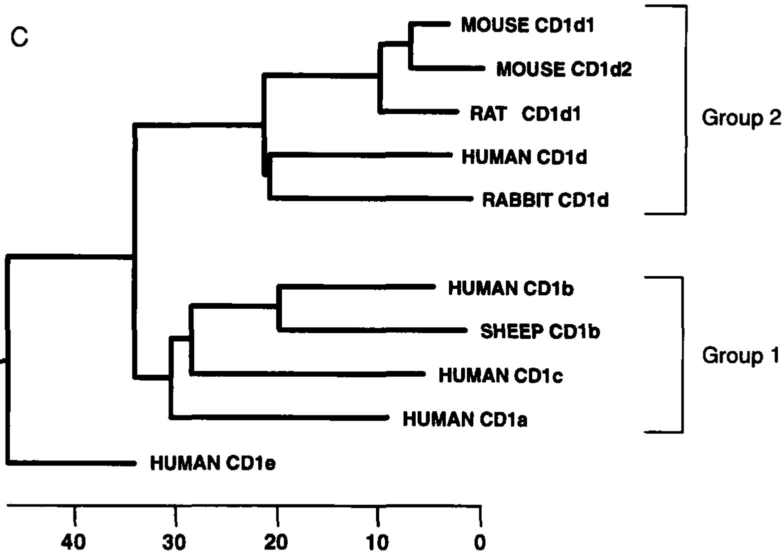


FIG. 5.—*Continued*

cant that the pattern of cellular expression for group 2 CD1 molecules appears to be quite different from that of the group 1 proteins (see Section VI). It seems likely that this reflects differences in transcriptional regulation of the two groups, which may be related to some of the differences that have been observed in their 5'-untranslated sequences as described earlier (see Section II,E). Furthermore, this difference in cellular expression is supportive of the hypothesis that group 1 and 2 CD1 proteins have functions that are probably related but slightly different.

D. RELATION OF CD1 TO MHC-ENCODED PROTEINS

Analyses of the deduced amino acid sequences of CD1 proteins have contributed significantly to our understanding of their evolution and their relationship to other molecules of importance to the immune system, particularly the MHC-encoded class I and II proteins. Based on the widely accepted criteria of Williams and Barclay, the membrane proximal $\alpha3$ domain of CD1 proteins has been identified as a member of the immunoglobulin (Ig) superfamily. Like the membrane proximal domains of both MHC class I and II proteins and $\beta2$-microglobulin, CD1 falls within the C1 set of Ig superfamily members, which are likely to have an immunoglobulin C domain folding pattern with the two conserved cysteine residues forming an intradomain disulfide bond (Williams and Barclay, 1988) (Figs. 4 and 8). The $\alpha1$ and $\alpha2$

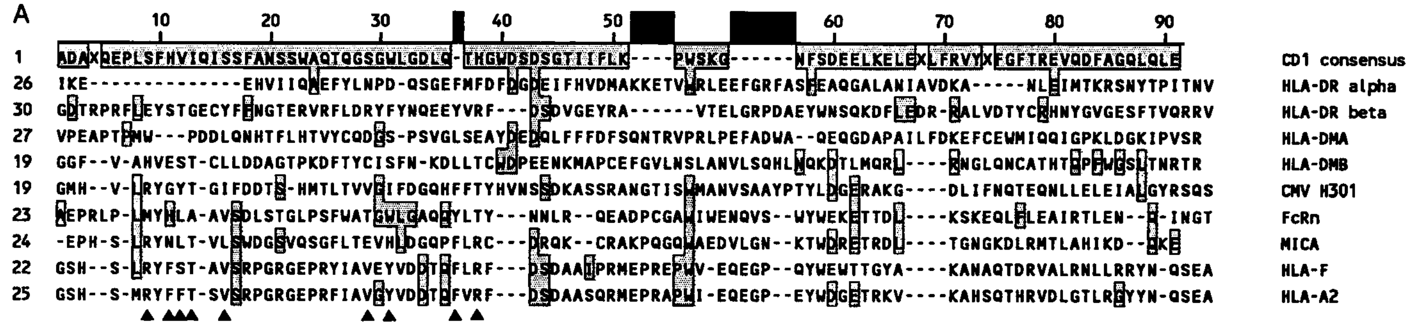
domains of CD1, like the corresponding domains in MHC-encoded proteins, do not satisfy the criteria required for inclusion into the Ig superfamily. As is the case for MHC class I and II, the evolutionary origin of these non-Ig-like domains is uncertain. Like all MHC class I $\alpha2$ domains and class II $\beta1$ domains, most CD1 $\alpha2$ domains also contain a pair of similarly spaced cysteine residues (shown at $\alpha2$ consensus positions 11 and 76 in Fig. 3c), the one exception being mouse CD1d2. These cysteines participate in intradomain disulfide bonds in the MHC-encoded proteins and may also do so in CD1 proteins. In contrast, the $\alpha1$ domains of CD1 proteins, like the corresponding domains of MHC proteins (i.e., the $\alpha1$ domains of MHC class I and II), lack such regularly spaced cysteines and are not predicted to contain intradomain disulfide bonds.

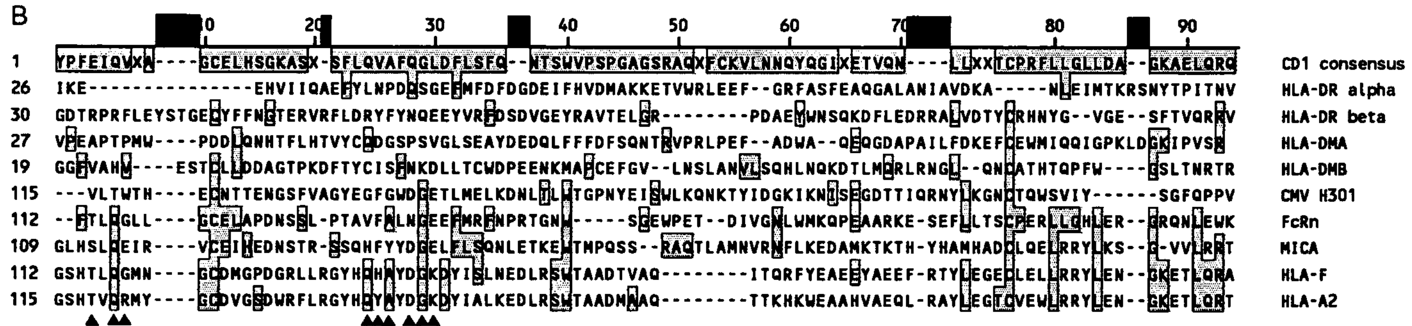
A consensus sequence for the three extracellular domains of the five human CD1 polypeptides is shown aligned with the corresponding domains of a representative group of MHC or MHC-related proteins in Fig. 6. Included in this analysis are a classical MHC class I α-chain (HLA-A2), a nonclassical MHC class I α chain (HLA-F), and MHC class II α (HLA-DRα) and β (HLA-Drβ/DRw8.2)-chains. These represent examples of known human classical and nonclassical antigen-presenting molecules. In addition, a number of proteins that are clearly related to MHC-encoded antigen-presenting molecules have also been included. The class II related HLA-DMA and -DMB chains (Kelly *et al.*, 1991) are MHC-encoded intracellular proteins involved in antigen presentation by MHC class II. MICA is one member of a family of recently described MHC-encoded molecules identified as distant relatives of classical MHC class I proteins (Bahram *et al.*, 1994). FcRn is an MHC class I-related $\beta2$-microglobulin-binding protein that functions as an Fc receptor for IgG in the rodent intestine (Simister and Mostov, 1989). The gene encoding FcRn in the mouse maps to chromosome 7, proving that it is not linked to either the MHC (chromosome 17) or CD1 (chromosome 3) loci in this species (Ahouse *et al.*, 1993). A cDNA predicted to encode a human homologue with 65% overall amino acid sequence identity to rat FcRn has also been recently cloned from placental tissue (Story *et al.*, 1994), although the sequence has not been included in this analysis. Of note, the three-dimensional structure of the rat FcRn protein has now been determined, revealing a remarkable similarity in overall shape to the structures of MHC class I and II proteins despite the relatively distant relationship between these proteins at the amino acid sequence level (Burmeister *et al.*, 1994). Finally, the cytomegalovirus-encoded protein H301 is a $\beta2$-microglobulin-binding protein that apparently functions as part of a pathway lead-

ing to viral infection (Beck and Barrell, 1988). Together, these proteins are representative of all of the known antigen-presenting molecules and all of the proteins that are known to contain β2-microglobulin as part of their subunit structure.

As can be seen in the multiple alignment shown in Fig. 6 and from the pairwise alignment similiarity values shown in Table II, CD1 shows little or no demonstrable similarity to any of the MHC or MHC-related proteins in α1 domain comparisons. However, in the alignment of α2 domains a low but significant level of homology is apparent, especially with the group of β2-microglobulin-associated proteins. The highest similarity scores in this region are seen with FcRn (37.3%), HLA-F (35.6%), and HLA-A2 (33.1%). In the α3 domain, detectable homology is again seen between CD1 and the other β2-microglobulin-binding proteins and also to an almost equal extent with the MHC class II proteins. The highest similarity scores in this region are seen with HLA-F (34%), HLA-A2 (27.8%), FcRn (25.8%), and HLA-DRβ (25.8%). These alignments also demonstrate that residues involved in β2-microglobulin binding by classical class I molecules are not more highly conserved than are other areas in CD1 (5 of 28 conserved between CD1 consensus and HLA-A2), nor is a loop in the α3 domain of classical class I that is involved in interaction with CD8 on T cells (3 of 10 residues conserved in CD1) (Salter *et al.*, 1990).

Several important points can be made from this comparative analysis. It is apparent that CD1 molecules are significantly but distantly related to MHC class I molecules not only in the membrane proximal domain (which may be primarily involved in β2-microglobulin binding) but also in at least one of the two more distal domains (which form the peptide antigen binding site in class I molecules). Also, it is apparent that nonclassical MHC class I molecules like HLA-F are much more closely related to classical MHC class I molecules than is CD1. It is therefore not accurate to refer to CD1 as "nonclassical class I" or "class Ib" proteins. This underscores the point that human CD1 is not a homologue of the mouse TL nonclassical MHC class I proteins (Bradbury *et al.*, 1988), which was suggested early after the discovery of CD1 because of certain similarities in the tissue distribution of group 1 CD1 and TL proteins. In actuality, CD1 appears to be a separate lineage of MHC-related proteins that is distantly related to both MHC-encoded antigen-presenting molecule families. The nearly equal sequence similarity of CD1 to both MHC class I and class II families is consistent with the idea that the CD1 family may have diverged from an ancestral antigen-presenting molecule gene at a point not very far removed in evolutionary terms from the point at which


A
CD1 consensus
HLA-DR alpha
HLA-DR beta
HLA-DMA
HLA-DMB
CMV H301
FcRn
MICA
HLA-F
HLA-A2


B
CD1 consensus
HLA-DR alpha
HLA-DR beta
HLA-DMA
HLA-DMB
CMV H301
FcRn
MICA
HLA-F
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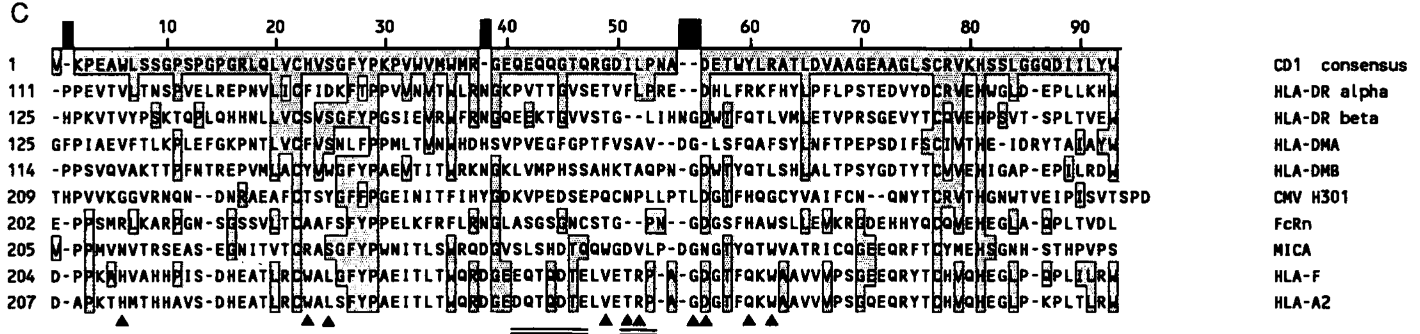

FIG. 6. Amino acid sequence comparison of CD1 domains with homologous domains of MHC and MHC-related proteins. (A) Alignment of human CD1 $\alpha1$ consensus sequence with $\alpha1$ (HLA-DRα, HLA-DMA, CMV H301, FcRn, MICA, HLA-F, and HLA-A2) or $\beta1$ (HLA-DRβ, HLA-DMB) domains of other proteins. (B) Alignment of human CD1 $\alpha2$ consensus sequence with $\alpha1$ (HLA-DRα, HLA-DMA), $\alpha2$ (CMV H301, FcRn, MICA, HLA-F, and HLA-A2), or $\beta1$ (HLA-DRβ, HLA-DMB) domains of other proteins. (C) Alignment of human CD1 $\alpha3$ consensus sequence with $\alpha3$ (CMV H301, FcRn, MICA, HLA-F, and HLA-A2), $\alpha2$ (HLA-DRα, HLA-DMA), or $\beta2$ (HLA-DRβ, HLA-DMB) domains of other proteins. The human CD1 consensus sequences are derived from the alignment of the five human CD1 proteins shown in Fig. 3. Consensus residues indicate the most common amino acid occurring at each position. An X denotes that no residue occurs more than once at that position. Alignments were performed as described in the legend to Fig. 3. Dashes indicate gaps that have been introduced to maximize alignments. Residues that match the CD1 consensus sequence are shaded and boxed. The filled triangles indicate probable $\beta2$-microglobulin contact points in the HLA-A2 crystal structure (Bjorkman *et al.*, 1987). Double horizontal lines beneath the $\alpha3$ domain sequences indicate an area in MHC class I molecules involved in the interaction with CD8. See text for references for individual sequences. Numbers at the start of each line indicate the position of the first residue in the full-length polypeptide (including leader sequence), except for the CD1 consensus sequence for which the number 1 indicates the first residue in each domain.

TABLE II

COMPARISON OF CD1 EXTRACELLULAR DOMAINS WITH HOMOLOGOUS DOMAINS OF MHC AND MHC-RELATED PROTEINS[a]

A. CD1 alpha-1 domain

Percent Similarity

Percent Divergence

CD1 alpha-1	HLA-DR alpha-1	HLA-DR beta-1	HLA-DMA alpha-1	HLA-DMB beta-1	CMV H301 alpha-1	FcRn alpha-1	MICA alpha-1	HLA-F alpha-1	HLA-A2 alpha-1	
	10.6	12.1	11	12.1	12.1	12.4	11.8	10	11.1	CD1 alpha-1
91.4		11.8	18.8	10.6	10.6	10.6	12.9	12.9	10.6	HLA-DR alpha-1
89	88		11.6	11.6	10.5	11.2	12.9	13.3	15.6	HLA-DR beta-1
95.1	74.4	95.5		11.6	11.5	11.2	14.1	11.1	11.1	HLA-DMA alpha-1
87.7	93.7	88.4	93.3		15.8	12.4	12.9	12.2	11.1	HLA-DMB beta-1
90.1	96.2	89.7	91	83		12.4	15.3	15.6	16.7	CMV H301 alpha-1
81.3	88.7	86.7	93.9	92.9	84.9		21.2	21.3	19.1	FcRn alpha-1
86.3	88.2	86.1	96.2	86.7	78.3	76.5		20	21.2	MICA alpha-1
88.3	89.2	77.4	91.6	93.2	79.8	74.4	74.7		71.1	HLA-F alpha-1
85.7	85.1	76.2	92.8	93.2	74.2	74.4	73.5	28.9		HLA-A2 alpha-1

B. CD1 alpha-2 domain

Percent Similarity

Percent Divergence

CD1 alpha-2	HLA-DR alpha-1	HLA-DR beta-1	HLA-DMA alpha-1	HLA-DMB beta-1	CMV H301 alpha-2	FcRn alpha-2	MICA alpha-2	HLA-F alpha-2	HLA-A2 alpha-2	
	16.9	15.3	14.5	21.2	20.3	37.3	28.8	35.6	33.1	CD1 alpha-2
87.2		20.3	29.9	16.1	16.1	21.2	16.1	17.8	18.6	HLA-DR alpha-1
83.9	88.9		11.1	21.2	20.3	21.2	18.6	21.2	21.2	HLA-DR beta-1
87	76.9	87.1		12.8	7.7	14.5	6.8	15.4	15.4	HLA-DMA alpha-1
86.4	84.5	80.9	87.1		21.2	22	20.3	19.5	20.3	HLA-DMB beta-1
80.4	89.7	84.6	91.8	88.9		26.3	26.3	30.5	30.5	CMV H301 alpha-2
68	91.4	84.5	86.2	92.4	83.1		33.9	39	33.9	FcRn alpha-2
77.4	87.1	79.7	83.3	91.2	77.6	77.5		40.7	36.4	MICA alpha-2
73.2	93.1	84.1	84.8	92.3	76.5	74.7	69		77.1	HLA-F alpha-2
71	90.7	80	80.3	90.5	74	79.1	70.3	26.5		HLA-A2 alpha-2

C. CD1 alpha-3 domain

Percent Similarity

Percent Divergence

CD1 alpha-3	HLA-DR alpha-2	HLA-DR beta-2	HLA-DMA alpha-2	HLA-DMB beta-2	CMV H301 alpha-3	FcRn alpha-3	MICA alpha-3	HLA-F alpha-3	HLA-A2 alpha-3	
	24.7	25.8	17.5	20.6	13.4	25.8	20.6	34	27.8	CD1 alpha-3
75.8		35.1	29.9	39.2	18.6	32	14.4	27.8	28.9	HLA-DR alpha-2
71.9	64.8		21.6	34	20.6	33	24.7	27.8	26.8	HLA-DR beta-2
79.5	67	77.3		23.7	12.4	18.6	17.5	18.6	16.5	HLA-DMA alpha-2
79.1	62.4	66.3	74.2		21.6	26.8	22.7	30.9	27.8	HLA-DMB beta-2
87.5	80.7	79.5	87.5	78.7		16.5	18.6	26.8	23.7	CMV H301 alpha-3
77.9	69.3	67.4	80.2	73	81.4		25.8	36.1	35.1	FcRn alpha-3
78.4	83.9	74.7	83.9	75	79.5	75		38.1	35.1	MICA alpha-3
65.6	72.2	70.8	81.8	69.2	73.6	65.1	60.2		88.7	HLA-F alpha-3
72.2	71.1	71.9	83	72.5	74.7	66.3	63.6	12		HLA-A2 alpha-3

[a] The CD1 sequence represents a consensus for all known CD1 sequences, as described in the legend to Fig. 6. Method for pairwise sequence comparisons, calculation of similarity and divergence are as described in the footnote to Table I. References for individual sequences are given in the text.

the precursors of MHC class I and II diverged (Calabi, *et al.*, 1989c, 1991).

As a further illustration of this important point, a phylogenetic tree based on the membrane proximal domain sequences of CD1, MHC class I and II, and MHC-related molecules is shown in Fig. 7. On the basis of this evolutionary scheme, it seems reasonable to consider the possibility that CD1 could represent a family of antigen-presenting molecules that has evolved to perform a function that is distinct and complementary to that performed by MHC class I and II. As discussed

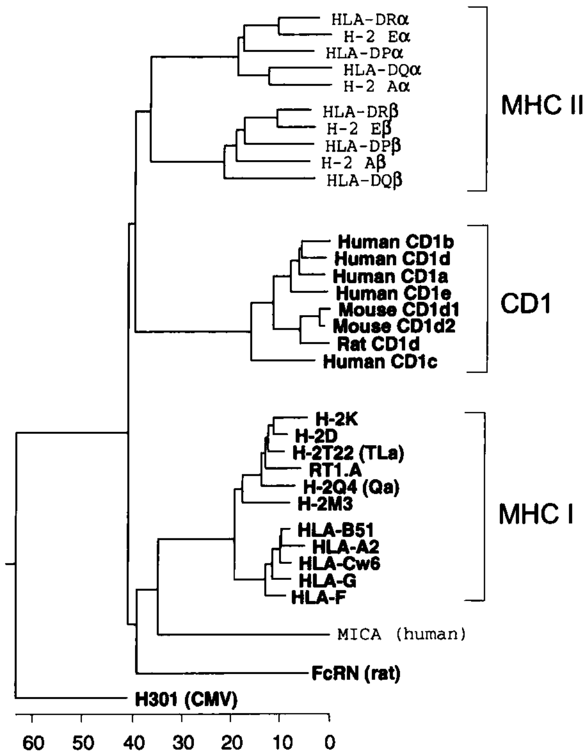

FIG. 7. Phylogenetic tree showing proposed relationship between CD1, MHC, and MHC-related proteins. The tree is based on amino acid sequence alignments of the membrane proximal domains of all of the proteins shown (i.e., $\alpha3$ domains of $\beta2$-microglobulin-associated proteins and MICA, $\alpha2$ domains of class II α chains, and $\beta2$ domains of class II β chains). Proteins known or strongly suspected to bind $\beta2$-microglobulin are indicated by bold print. The x axis shows calculated sequence distance, and branch lengths in this unbalanced tree are proportional to the extent of sequence divergence. Alignments were performed as described in the legend to Fig. 3. References for most sequences are given in the text, with additional sequences from Kaufmann *et al.*, (1984) and Parham *et al.*, (1988).

in Section VII, there is now direct evidence supporting this view. This scheme also predicts that the ancestor of all extant CD1 proteins emerged early in vertebrate evolution, as did the MHC class I and II families (Klein *et al.*, 1993). Thus, a prediction of this model is that CD1 proteins should be present in most or all species that show separate MHC class I and II gene families. One would therefore anticipate finding CD1 genes in all mammals and in birds, and possibly also in lower vertebrate species including fish, reptiles, and amphibians. However, other comparisons of CD1 and MHC sequences using slightly different methods than those employed here have suggested that CD1 and MHC may have diverged around the time of the bird–mammal divergence approximately 250–300 million years ago (Hughes, 1991). If so, then CD1 genes may not be present in birds or more ancient vertebrate lineages. These alternate possibilities concerning the existence of CD1 in nonmammalian vertebrates have not yet been explored experimentally. Nevertheless, all analyses of CD1 evolution seem to agree on the essential point that the CD1 family represents a third lineage of proteins that should be viewed as separate from the MHC-encoded class I and class II lineages.

E. Predictions of Higher-Order Structure of CD1 Proteins

The three-dimensional structure of CD1 proteins has not yet been determined, and at this point it is only possible to speculate concerning the arrangement and three-dimensional structure of the domains of these proteins. Figure 8 shows a plausible schematic view of how the domains of CD1 are likely to be organized in the native protein compared to the homologous domains of the related MHC class I and II molecules. This arrangement implies that the immunoglobulin-like $\alpha 3$ domain of CD1 functions mainly in binding $\beta 2$-microglobulin and in supporting the more distal $\alpha 1$ and $\alpha 2$ domains. These latter domains might associate to form a structure capable of functioning as an antigen binding site or a target ligand for specific receptor recognition. The question of how the $\alpha 1$ and $\alpha 2$ domains compare to the homologous domains of MHC class I or II is thus an area of great interest.

Unfortunately, there is no direct information addressing this point at present. In general, attempts to superimpose the CD1 sequences on the known spatial corrdinates of MHC class I and II proteins have been avoided because the very low sequence homology of CD1 and MHC proteins in the membrane distal domains makes such modeling essentially arbitrary and potentially misleading. However, some provocative insights have been suggested by computer-based analysis and molecular modeling employing algorithms for secondary structure

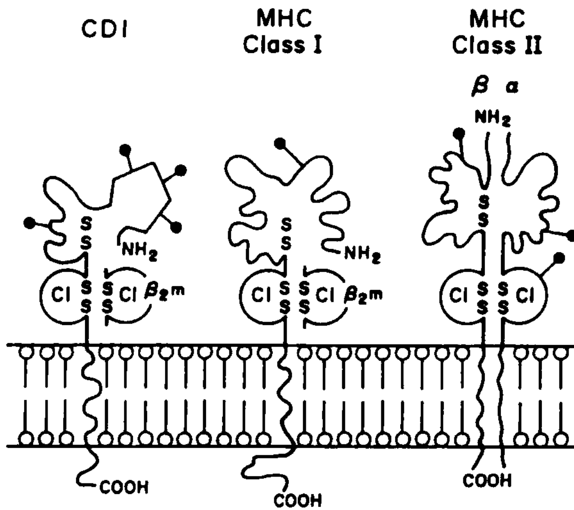

FIG. 8. Drawing showing proposed domain organization of CD1 proteins in comparison to MHC class I and II proteins. N-linked glycosylation sites for MHC class I and II are indicated by stem and circle symbols, and one of several predicted glycosylation patterns for CD1 is shown. "C1" indicates the immunoglobulin C1-like domains. Also shown are intradomain disulfide bonds known to be present in MHC molecules and predicted to be present in most CD1 proteins (reproduced with permission from Porcelli *et al.*, 1991).

prediction. Two analyses of predicted secondary structure in the $\alpha 1$ and $\alpha 2$ regions of CD1 proteins have been reported (Longley *et al.*, 1989; Calabi *et al.*, 1989b). Analyzing only the protein sequence of CD1a, Longley *et al.* concluded that CD1a shared many of the basic features found in class I molecules, including three or four areas of β-pleated sheet struture in the N-terminal portions of both $\alpha 1$ and $\alpha 2$ domains followed by stretches of α helix. In contrast, Calabi *et al.* analyzed a collection of different CD1 sequences and averaged the results to obtain a consensus prediction for the entire group. These authors were most impressed by the predicted tendency for β-strand formation in the second half of the $\alpha 2$ domain, a region that is predominantly α helix and contains no β-sheet structure in MHC class I and class II molecules (Bjorkman *et al.*, 1987; Brown *et al.*, 1993) or in the recently described FcRn structure (Burmeister *et al.*, 1994). This result seems to be generally born out when predictive algorithms based on either Chou–Fasman or Garnier–Robson methods are applied to individual CD1 amino acid sequences using parameters that allow

reasonably correct prediction of major features of known secondary structure in MHC and MHC-related (i.e., FcRn) proteins (Fig. 9 and additional data not shown). Although the accuracy of such methods is questionable in the absence of supportive experimental data, these findings at least raise the possibility that the $\alpha 1$ and $\alpha 2$ domains of CD1 may not fold into a structure that closely resembles the peptide-binding groove of MHC class I or II molecules. In fact, it is suggested that the three-dimensional structure of the CD1 $\alpha 1$ and $\alpha 2$ domains may be less similar to MHC molecules than what has been found for FcRn, which shares the basic arrangement of α helices and β-pleated

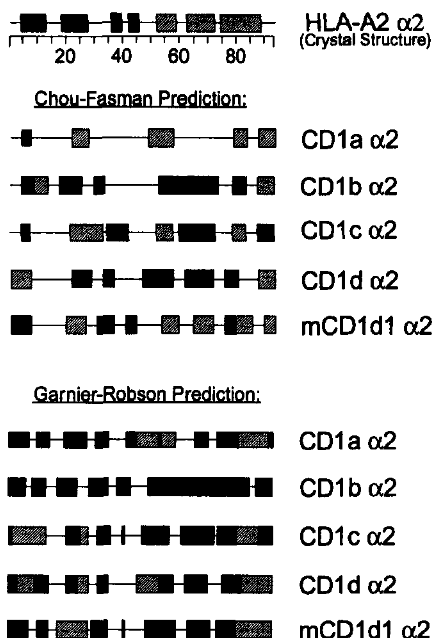

FIG. 9. Secondary structure predictions for the $\alpha 2$ domains of CD1 proteins. The scale indicates the amino acid position, starting from the first N-terminal residue of the $\alpha 2$ domains. Filled boxes indicate predicted β strands of β-pleated sheet structure, and hatched boxes indicate predicted α-helical regions. Predictions were carried out on the CD1 $\alpha 2$ domain amino acid sequences using the Protean module of the Lasergene software package (DNASTAR, Madison, WI). Chou–Fasman predictions were done using the α/β class method (α threshold 103, β threshold 105) (Chou and Fasman, 1978). Garnier–Robson predictions were done using the original method based on observed residue patterns in 25 proteins without specified decision constants (Garnier *et al.*, 1978). The diagram of the HLA-A2 structure is based on X-ray crystallographic data (Bjorkman *et al.*, 1987).

sheets found in MHC proteins but lacks an open groove that could accomodate peptide binding (Burmeister *et al.*, 1994).

Another noteworthy feature of CD1 proteins that may point to structural differences compared to MHC class I and II relates to their relative hydrophobicity. Analysis of the relative hydrophobicity of the $\alpha1$ and $\alpha2$ regions of MHC and CD1 proteins indicates that CD1 proteins are in all cases more hydrophobic (E. Beckman, personal communication). This analysis is summarized in Fig. 10. Note that CD1 proteins contain more hydrophobic regions in their $\alpha1$ and $\alpha2$ domains than do MHC class I or II molecules, and that index of hydrophobicity is generally greater in such regions in CD1 proteins. This is true even when CD1 proteins are compared to H-2M3, which is an unusually hydrophobic mouse nonclassical MHC class I protein that is known to bind a set of hydrophobic N-formyl-modified antigenic peptides (Shawar *et al.*, 1990). The hydrophobicity of CD1 proteins is particularly pronounced on the $\alpha2$ domain, especially in CD1b and -c. This feature of CD1 proteins could indicate the ability to form a binding site for hydrophobic ligands. As discussed later (see Section VI), this would fit well with current proposals for how CD1 functions in antigen presentation.

IV. Serologic Definition of CD1

As has been the case for so many important cell surface molecules expressed by cells of the immune system, CD1 antigens were first discovered in the course of characterizing the antigens recognized by monoclonal antibodies (mAbs) raised in mice against human thymocytes (McMichael *et al.*, 1979; Reinherz *et al.*, 1980). CD1 has the distinction of being the first cluster of differentiation (CD) recognized by the International Workship on Human Leukocyte Differentiation Antigens in 1984 (Bernard *et al.*, 1984). As originally defined, CD1 consisted of antigens recognized by the cluster of monoclonal antibodies that reacted with cortical thymocytes as well as certain T cell malignancies, but not with medullary thymocytes, mature peripheral T cells, or other hematopoietic cell types. The antigens recognized were known or expected to be similar to the heterodimer recognized by the prototype mAb NA1/34. Thus, CD1 molecules were first introduced to immunologists as thymocyte differentiation antigens, and the initial interest in defining their structure and function related to their putative role in the process of T cell development. It should be noted that with the molecular definition of the CD1 gene family, it now seems reasonable to broaden the definition of serologically de-

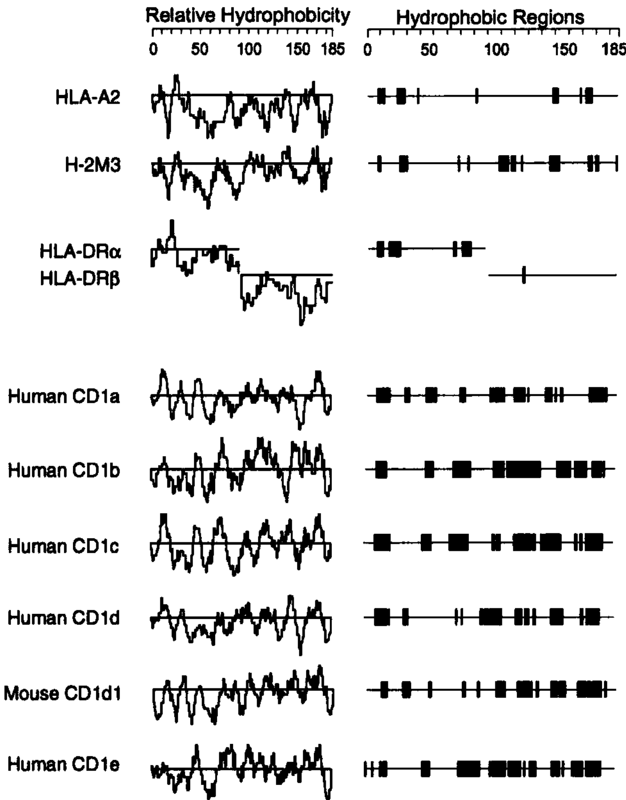

FIG. 10. Marked hydrophobicity of CD1 α1 and α2 domains. On the left are Kyte–Doolittle plots of relative hydropathy for the α1 and α2 domains of representative MHC class I and CD1 proteins and for the α1 domain of HLA-DRα and the β1 domain of HLA-DRβ (DRw 8.2). Hydropathy was averaged over a window length of nine amino acids. Deflections above the horizontal line indicate hydrophobic regions, and deflections below the line are hydrophilic regions. The lengths and positions of hydrophobic regions in each protein are shown on the right. In both cases, the scale at the top indicates position number in the amino acid sequence. References for sequences are given in the text.

fined CD1 antigens to include any proteins encoded by genes belonging to the CD1 family. This broadening of the cluster definition allows the recently defined product of the CD1D gene to be designated as CD1d, even though its pattern of tissue expression does not include pronounced expression on cortical thymocytes. In similar fashion, if and when a polypeptide product of the CD1E gene is discovered, it

is anticipated that it will be designated "CD1e" no matter what its protein structure or tissue distribution prove to be.

As a result of the collaborative efforts of the first workshop meeting, four monoclonal antibodies (NA1/34, OKT6, D47, and M241) were assigned to this cluster. Even at that time, some evidence suggested that there might be heterogeneity within CD1, since biochemical studies showed that the heavy-chain component of the antigen recognized by M241 was significantly smaller than that immunoprecipitated by NA1/34 and OKT6. This heterogeneity of human CD1 was confirmed as more monoclonal antibodies became available (see Table III). By the late 1980s, the existence of three distinct serologically defined proteins within CD1 was firmly establised (Boumsell and Knowles, 1987). These correspond to separate but related heavy chains, each present on the cell surface in noncovalent association with β2-microglobulin. These three proteins have since come to be known as CD1a, CD1b, and CD1c. Very recently a fourth human CD1 molecule, which is the product of the CD1D gene, has been defined serologically and designated CD1d (Bilsland *et al.*, 1991; Blumberg *et al.*, 1991). As can be seen from Table III, a substantial number of anti-CD1 mAbs now exist, especially for human CD1a. The reason for the abundance of antibodies to CD1a is not known, but most likely this is due to the relatively higher expression of CD1a on thymocytes (i.e., the immunogen used for most attempts at production of these mAbs) or else reflects greater immunogenicity in mice of CD1a relative to other CD1 proteins.

A. Properties of CD1-Specific Monoclonal Antibodies

All of the CD1-specific monoclonal antibodies defined to date recognize monomorphic determinants in their target antigens. Thus, they react with the appropriate CD1-expressing cells or tissues from all outbred individuals. This is not surprising, given that biochemical and molecular studies demonstrate that CD1 proteins from different individuals have little or no polymorphism, so that very few if any serologic epitopes on these molecules would be expected to correspond to allelic variants. Nearly all CD1-specific monoclonal antibodies that have been characterized so far appear to recognize the mature, fully assembled form of the protein that is expressed on the cell surface. There have been very few reports showing the reactivity of any anti-CD1 monoclonal antibodies in Western blotting or in other procedures that would indicate their reactivity with denatured or with β2-microglobulin-unassociated CD1 heavy chains. Thus, the majority of anti-CD1 monoclonal antibodies may have properties similar to

TABLE III
CD1-SPECIFIC MONOCLONAL ANTIBODIES

CD1 Antigen	Epitope Group	Antibody Name	Isotype[a]	Reference
Human CD1a[b]	A	NA1/34	Ig2a,κ	McMichael *et al.*, 1979
		L119	IgG1	Amiot *et al.*, 1986
		VIT6b	IgG1	ILWS[c]
		1OH3.9	IgG2a,κ	Olive *et al.*, 1984
		D47	IgG2,κ	Kanitakis *et al.*, 1983
		WM-33	IgG2a	Favaloro *et al.*, 1987
		WM-36	IgG2a	Favaloro *et al.*, 1987
		WM-39	IgG2a	Favaloro *et al.*, 1987
	B	VIT6	IgG2a	ILWS
	C	I19	IgG2b	Amiot *et al.*, 1986
		OKT6	IgG1	Reinherz *et al.*, 1980
		L544	IgG1	Amiot *et al.*, 1986
		L232	IgG1	ILWS
		VIT6d	IgG3	ILWS
		BL6	IgG1,κ	Yonish-Rouach *et al.*, 1982
		1OD12.2	IgG1,κ	Olive *et al.*, 1984
		WM-35	IgG2b	Favaloro *et al.*, 1987
	D	L404	IgG1	Amiot *et al.*, 1986
		Leu-6	IgG2a,κ	Wood *et al.*, 1983

Human CD1b	A	4A7.6	IgG2a,κ	Olive *et al.*, 1984
		WM-25	IgG1	Favaloro *et al.*, 1987
		7C4	IgG1	ILWS
	B	NU-T2	IgG1	ILWS
Human CD1c	A	M241	IgG1	Knowles and Bodmer, 1982
		L161	IgG1	Amiot *et al.*, 1986
		10C3	IgG1	ILWS
		7C6	IgM	ILWS
Human CD1d	—	NOR3.2	IgG1	Bilsland and Milstein, 1991
		1H1	IgM (rat)	Bleicher *et al.*, 1990
		3C11	IgM (rat)	Bleicher *et al.*, 1990
Mouse CD1 (CD1d1, ?CD1d2)	—	1H1	IgM (rat)	Bleicher *et al.*, 1990
		3C11	IgM (rat)	Bleicher *et al.*, 1990
Sheep CD1 (group 1 CD1)	—	SBU-T6	IgG1	Mackay *et al.*, 1985
		VPM5	?	Bujdoso *et al.*, 1989
Cow CD1 (group 1 CD1)	A	TH97A	IgG2a	MacHugh *et al.*, 1988
		CC13	IgG3	MacHugh *et al.*, 1988
		CC14	IgG1	MacHugh *et al.*, 1988
Rabbit CD1b	—	5E2	IgG1	Wang *et al.*, 1987

[a] All mAbs are of mouse origin unless otherwise noted.
[b] Other CD1a-specific mAbs not assigned to epitope groups are not included. For a listing of these, consult Boumsell (1989).
[c] ILWS: mAb described in proceedings of International Leukocyte Typing Workshops. Consult Boumsell (1989) and Boumsell and Knowles (1987).

mAbs specific for monomorphic determinants of MHC class I molecules, which with few exceptions also tend to recognize preferentially conformation-dependent epitopes of the mature heavy chain/β2-microglobulin complex (Stamm *et al.*, 1986). The only apparent exceptions to this rule involve mAbs that are reactive with human CD1d. Two such mAbs, 3C11 and 1H1 (raised against the mouse homologue of human CD1d and described below in greater detail), appear to immunoprecipitate a β2-microglobulin-unassociated and nonglycosylated form of the CD1d heavy chain from certain cell types (Balk *et al.*, 1994; see below). These mAbs have also been found to react with denatured CD1 heavy chains in Western blotting (Blumberg *et al.*, 1991). Another mAb against human CD1d has been produced by immunization of mice with a bacterial fusion protein corresponding to the human CD1d heavy chain. This mAb, NOR3.2, reacts exclusively with denaturated CD1d protein (Bilsland and Milstein, 1991).

Because of the relatively large number of mAbs that have been generated against CD1, it has been possible to map specific serologically defined epitopes on these proteins by cross-inhibition studies. This approach confirms that the CD1a, -b, and -c proteins as defined by mouse monoclonal antibodies are serologically very distinct. This is apparent from the fact that the binding of monoclonal antibodies reacting with any one of these proteins does not in any case cause marked inhibition of the binding mAbs to the other two (Olive *et al.*, 1984; Kahn-Perles *et al.*, 1985; Amiot *et al.*, 1986; Favaloro *et al.*, 1987a). Minor cross-reactivity between different forms or isotypes of CD1 by a few monoclonal antibodies has occassionally been suggested in such studies (Olive *et al.*, 1984; Favaloro *et al.*, 1987), but these have not been directly confirmed by other techniques. In general, CD1 proteins are also serologically distinct from MHC class I proteins in cross-inhibition studies, although one study reported that two mAbs against HLA class I proteins partially blocked the binding of three of four anti-CD1a mAbs tested (Kahn-Perles *et al.*, 1985). It is unclear whether this could be due to a true serologic cross-reactivity between these proteins or perhaps reflects a physical association between MHC class I and CD1a proteins on the cell surface (see Section V,E).

It has also been possible through the use of cross-inhibition studies to define separate serologic epitopes associated with individual CD1 heavy chains (Table III). In this way, at least four separate epitopes associated with the CD1a heavy chain have been identified, indicated as epitopic groups A–D (nonmenclature after Amiot *et al.*, 1987b). Epitopes A, C, and D appear to be entirely distinct, as binding of mAbs against these epitopes does not inhibit binding of mAbs against

the others (Olive *et al.*, 1984; Kahn-Perles *et al.*, 1985; Amiot *et al.*, 1986; Favaloro *et al.*, 1987). In contrast, the B epitope (defined by the mAb VIT6) appears to overlap the A and C epitopes, as antibodies to epitope B inhibit binding of antibodies to epitopes A and C, and vice versa (Amiot *et al.*, 1986; Favaloro *et al.*, 1987a). In similar fashion, two separate epitopes have been defined on the CD1b molecule, whereas the four anti-CD1c mAbs so far examined in this fashion appear to bind to the same or overlapping epitopes (Amiot *et al.* 1986, 1987b; Favaloro *et al.* 1987a). The relationship of these serologic epitopes to the protein structure of CD1 is currently unknown. Anti-CD1a mAbs were also subclustered in one study into those that react with a trypsin-cleaved 27-kDa form of the heavy chain (e.g., mAb L544) and those that only recognize the native 49-kDa form (Dezutter-Dambuyant *et al.*, 1989). This variable reactivity with trypsin-cleaved CD1a further underscores the heterogeneity of anti-CD1a mAbs and may be of practical significance when these antibodies are used to examine cells isolated from trypsin-treated tissues or cell monolayers.

B. Serologic Definition of CD1 in Rodents and Other Species

Serologic definition of CD1 proteins in experimental animals has been limited in comparison to what has been accomplished in humans (Table III). Two monoclonal antibodies against mouse CD1, 1H1, and 3C11 have been described (Bleicher *et al.*, 1990). Both were produced by immunization of Lewis rats with mouse L cells that had been transfected with a cDNA expression construct encoding the mouse CD1D1 gene product (i.e., one of the two mouse genes encoding the homologue of human CD1d). Although these mAbs are of IgM isotype, both have been shown to immunoprecipitate the mouse CD1 heavy chain plus variable amounts of β2-microglobulin. It has been noted that the β2-microglobulin coprecipitation is much more apparent with 1H1 than with 3C11, suggesting that the 3C11 mAb may preferentially recognize mCD1 heavy chains that are not β2-microglobulin associated. These mAbs are unusual in that they react with the native form of mouse CD1 on the cell surface as detected by flow cytometry, but also react with SDS-denatured mouse CD1 heavy chains in Western blotting. These antibodies have been used in immunohistology, immunoprecipitation, and flow cytometry studies and have been useful in defining the structure and tissue expression of mouse CD1. Both the 1H1 and the 3C11 mAbs have also been reported to cross-react with CD1 molecules expressed by epithelial cells of the intestine in Sprague–Dawley rats and by an epithelial cell line derived from Wistar

rats (Burke *et al.*, 1994). This is surprising in view of the fact that these mAbs were originally produced by rats and could possibly reflect the existence of allelic differences between the different rat strains examined. However, the 1H1 and 3C11 mAbs have also been reported to cross-react with the human CD1d molecule in both flow cytometry and immunoprecipitation studies (Blumberg *et al.*, 1991), suggesting that they must recognize a highly conserved CD1d epitope.

The serologic definition of CD1 antigens in other species has received relatively little attention. One monoclonal antibody, 5E2, raised against thymocytes from new Zealand White rabbits was extensively characterized and found to recognize a protein designated R-Ta with features analogous to human group 1 CD1 (Wang *et al*, 1987). Subsequent purification and N-terminal sequencing of the heavy-chain subunit of this protein confirmed that it is indeed a form of CD1 (Wang *et al*, 1988). Sixteen of 18 residues from this sequence are identical to the predicted amino acid sequence of the protein encoded by the CD1B gene isolated from a domestic rabbit genomic library (Calabi *et al.*, 1989a). It thus appears likely that the 5E2 mAb recognizes the rabbit homologue of human CD1b. Monoclonal antibodies that appear to recognize CD1 antigens of sheep (Mackay *et al.*, 1985; Bujdoso *et al.*, 1989), cow (MacHugh *et al.*, 1988), and possibly swine (Pescovitz *et al.*, 1984) have been reported, but have not been the focus of detailed molecular or biochemical studies. Thus, it is unclear which form(s) of CD1 are recognized by these mAbs, although it is likely that the sheep and bovine mAbs recognize group 1 CD1 proteins based on the patterns observed in tissue staining. No antibodies against CD1 molecules of birds or other nonmammalian species have yet been described.

V. Biochemical and Immunochemical Studies of CD1 Proteins

The availability of monoclonal antibodies against CD1 antigens has facilitated direct biochemical analyses of these proteins. Such studies were responsible for the initial recognition of the structural relationship between CD1 and MHC class I proteins, since they revealed the distinctive heavy-chain plus β2-microglobulin subunit structure of both types of proteins. Immunochemical studies also provide information on post–translational modification of CD1 proteins and their association with other proteins on the cell surface.

A. Labeling and Immunoprecipitation of CD1 Proteins

CD1 proteins are readily labeled by surface iodination of intact cells and can be specifically immunoprecipitated from detergent lysates prepared from such cells. Such features are obviously compatible with these molecules being transmembrane cell surface proteins, as they are predicted to be. Most of the initial studies defining the biochemical features of CD1 proteins used human thymocytes or thymic leukemia cell lines (e.g., MOLT-4, Jurkat, HPB-ALL) as the source of protein for analysis since these constitutively express substantial levels of all three human group 1 CD1 proteins. CD1a was the first protein in this family to be defined biochemically, revealing a 49-kDa heavy chain associated with a 12-kDa light chain (McMichael *et al.*, 1979; Terhorst *et al.*, 1981; Cotner *et al.*, 1981). Shortly thereafter, monoclonal antibodies against the other CD1 proteins expressed strongly by human thymocytes, CD1b and CD1c, were produced and used in analogous biochemical studies (Knowles and Bodmer, 1982; Olive *et al.*, 1984). These demonstrate similar immunoprecipitation patterns from ^{125}I-labeled thymocyte lysates as do anti-CD1a mAbs, except that the apparent heavy-chain sizes are 45 kDa for CD1b and 43 kDa for CD1c. The peptide backbone sizes of both CD1a and CD1c after removal of N-linked glycans by endoglycosidase-F (Endo-F) treatment are 33 kDa, which are considerably different than the 40-kDa size determined for similarly deglycosylated HLA-A and -B proteins (Lerch *et al.*, 1983). Comparison of CD1a and CD1c by tryptic peptide mapping reveals results characteristic for distinct but closely related proteins (i.e., approximately 60–70% of peptides identical) (Van de Rijn *et al.*, 1983).

A peculiar feature of CD1 proteins identified by initial biochemical studies is their failure to label efficiently with methods involving the incorporation of radioactive amino acids or oligosaccharide precursors (i.e., metabolic labeling). Thus, incubation of thymocytes or MOLT-4 cells in appropriate media containing [^{35}S]methionine, [^{3}H]leucine, or [^{3}H]glucosamine for periods varying from 4 to 42 hr did not lead to detectable labeling of immunoprecipitated CD1a (Lerch *et al.*, 1983). This probably signifies an unusually slow biosynthetic rate and turnover of CD1 proteins relative to most other cell surface proteins. Although metabolic labeling of CD1a can be achieved in high expressing variants or transfectant cell lines (Calabi *et al.*, 1991; M. Sugita, personal communication), this requires long (e.g., >12 hr) periods of incorporation of ^{35}S-labeled amino acids and still results in relatively weak labeling. A similar phenomenon is encountered in attempts to

metabolically label CDlb and CDlc proteins (M. Sugita, personal communication). Although an interesting feature of the CD1 system, this apparent slow metabolism of CD1 proteins has greatly hampered the application of biosynthetic labeling for dynamic pulse–chase analyses. Thus, there have been no studies reported to date on the kinetics of biosynthesis, turnover, and assembly of CD1 proteins. It is also interesting that one report demonstrated a protein identified immunochemically as rat CDld in lysates of a rat fetal intestinal cell line labeled for only 4 hr with [^{35}S]methionine/cysteine (Burke *et al.*, 1994). If confirmed, this could suggest substantially different kinetics of biosynthesis and assembly for group 2 versus group 1 CD1 proteins.

B. Absence of Significant Allelic Polymorphism of CD1 Proteins

Isoelectric focusing of immunoprecipitated CDla has been used to examine the heterogeneity of CDla proteins isolated from different individuals (Van Agthoven and Terhorst, 1982). CDla proteins from 19 individual thymuses were analyzed, and no differences in isoelectric focusing pattern could be found. Similar results were also reported for CDlc proteins from 6 different thymus prepartations (Van de Rijn *et al.*, 1983). In another study, CDla from 10 different thymuses and CDlb and -c from 5 different thymuses were analyzed by two-dimensional peptide mapping, and again little or no evidence for polymorphisms of CD1 proteins was found (Amiot *et al.*, 1988b). Thus, although subtle exceptions may yet be found, it seems likely that CD1 proteins are essentially monomorphic with minimal or no significant allelic diversity. As discussed earlier (see Section III,A), this conclusion is supported by comparison of DNA sequences encoding human and mouse CD1 obtained from genetically disparate sources.

This absence of polymorphism in CD1 proteins among different individuals of an outbred population is an important difference between these proteins and the classical MHC class I and II proteins and is a feature that they share with the nonclassical MHC class I molecules. In the case of these latter proteins, the meaning of the absence of polymorphism has been interpreted in two very different ways. The marked allelic polymorphism of classical MHC-encoded antigen-presenting molecules is believed to arise as a result of positive selection for a high level of variation among amino acid residues participating in the peptide binding sites of these proteins. Thus, it has been proposed that nonclassical MHC class I molecules lack polymorphism because they are no longer effective antigen-presenting molecules and, consequently, are not subject to continued positive selection for

allelic variation (Klein and O'hUigin, 1994). This hypothesis leads to the conclusion that nonpolymorphic antigen-presenting molecules are no longer important in immune responses, although they may be retained because they provide a genetic substrate for further evolution of the MHC family. An alternate view of this question proposes that nonpolymorphic antigen-presenting molecules are under evolutionary pressure *against* the development of polymorphisms because they have evolved specialized functions that require precise maintenance of their structure. One apparent example of this has been provided by the H-2M3 protein, a mouse nonclassical MHC class I molecule with limited polymorphism that binds N-formyl-modified peptides and presents them to $CD8^+$ cytotoxic T cells. This example appears to demonstrate the principle that nonpolymorphic antigen-presenting molecules can be important in cellular immunity if they have acquired the ability to capture and present antigens with more limited structural variability than is inherent in typical peptide antigens. As will be discussed in detail in a following section, there is now evidence that such a principle also operates in the case of CD1 (see Section VII, D).

C. Association of CD1 Heavy Chains with β2-Microglobulin

The association of CD1 heavy chains with β2-microglobulin is a distinctive structural feature of these proteins. The ability of cellular proteins to associate with β2-microglobulin is apparently limited to a relatively small group of proteins, all of which appear to have functions related to the immune system. Initially, β2-microglobulin binding was believed to be restricted only to MHC class I proteins. However, CD1 and two other proteins are now known to be exceptions to this rule. These other two proteins are the rodent intestinal immunoglobulin receptor FcRn (Simister and Mostov, 1989) and the cytomegalovirus-encoded glycoprotein H301 (Beck and Barrell, 1988). The importance of β2-microglobulin to the structure and function of MHC class I and other β2-microglobulin-associated proteins is still not entirely clear. However, in the case of MHC class I proteins, β2-microglobulin is required for the proper assembly of the mature protein and for its efficient expression on the cell surface (Ploegh *et al.*, 1981; Zijlstra *et al.*, 1990). A requirement for β2-microglobulin association for efficient surface expression is presumed to exist for CD1 heavy chains as well, although this has not yet been formally analyzed for most CD1 proteins. Surprisingly, for the one CD1 protein for which the β2-microglobulin binding requirement has been analyzed directly (i.e., the human CD1d protein, as described in detail below, β2-

microglobulin was found not to be essential for the transport of human CD1d heavy chains to the cell surface (Balk *et al.*, 1994). In addition, cocapping experiments appear to support the possibility that a fraction of CD1a and -b heavy chains on the cell surface are not associated with β2-microglobulin (Kahn-Perles *et al.*, 1985; Amiot *et al.*, 1986). However, this could reflect the dissociation of β2-microglobulin from CD1 after it has been transported to the cell surface, as is known to occur for a proportion of MHC class I heavy chains (Rock *et al.*, 1991).

The demonstration of β2-microglobulin association with CD1a heavy chains was initially complicated by conflicting results, although subsequent studies have entirely resolved the resulting controversy and confusion. Initial reports of immunoprecipitations with the prototype anti-CD1a mAb NA1/34 did not clearly visualize the 12-kDa component of the protein (McMichael *et al.*, 1979). In retrospect, this was presumably due to the weak association of β2-microglobulin with the CD1a heavy chain which allows dissociation during immunoprecipitation, especially at temperatures greater than 20°C or at alkaline pH (>8.2) (Kefford *et al.*, 1984). Subsequently, it was shown that CD1a isolated from cultured MOLT-4 cell lysates does associate with a 12-kDa subunit, although this subunit was reported to be electrophoretically distinct from human β2-microglobulin. This apparently unique "β2-microglobulin-like" small polypeptide was termed β_T (Ziegler and Milstein, 1979). In contrast, the CD1a heavy chains isolated from human thymocytes were shown by immunochemical and isoelectric focusing analyses to be identical to human β2-microglobulin (Terhorst *et al.*, 1981). This issue was finally resolved with the realization that bovine β2-microglobulin from serum in tissue culture media can freely exchange with the endogenous human β2-microglobulin on CD1a, and that β_T is in fact passively acquired bovine β2-microglobulin (Kefford *et al.*, 1984; Bernabeu *et al.*, 1984). Other studies along these lines have shown that the association of β2-microglobulin with CD1b heavy chains is also weak and allows exchange with exogenous bovine β2-microglobulin (Amiot *et al.*, 1986), whereas CD1c heavy chains bind tightly to endogenous β2-microglobulin and do not undergo significant exchange (Knowles, 1984). Accurate quantitation of heavy chain and β2-microglobulin in purified CD1a and CD1c proteins has shown that the molar ratio of these two subunits in the mature protein expressed by thymocytes is approximately 1 : 1 (Lerch *et al.*, 1986). The observation that the β2-microglobulin band is often only faintly visible in immunoprecipitates of ^{125}I-labeled CD1 proteins most likely reflects differential labeling of the two subunits or dissociation of β2-microglobulin at some stage during the immunoprecipitation.

The importance (if any) of β2-microglobulin dissociation and exchange remains unknown for CD1 molecules. However, it is interesting to note that β2-microglobulin binding by MHC class I molecules is stabilized by the concurrent binding of peptide antigens. In addition, the presence of excess exogenous β2-microglobulin facilitates the incorporation of antigenic peptides into peptide/MHC class I protein complexes. This has been suggested as a mechanism by which MHC class I molecules may acquire exogenous peptide antigens at the cell surface (reviewed in Yewdell and Bennink, 1992). Thus, in light of current evidence for the presentation of exogenous antigens by CD1 (see Section VII), it is possible that a similar mechanism could apply for CD1 proteins, i.e., binding of a foreign or self-molecule to a putative antigen binding site could strengthen the interaction of the CD1 heavy chain with β2-microglobulin and stabilize the resulting complex. Conversely, dissociation of β2-microglobulin may occur rapidly from "empty" CD1 molecules, perhaps marking them for clearance from the cell surface and degradation. Dissociation of β2-microglobulin from empty CD1 molecules on the cell surface might also allow the CD1 heavy chains to assume a conformation that enables the binding of antigens that are particularly abundant in the extracellular environment, and this binding might be stabilized by the acquisition of extracellular β2-microglobulin.

Another perplexing aspect of the β2-microglobulin association of CD1 proteins has recently been demonstrated by a report of β2-microglobulin-independent expression of the human CD1d protein by intestinal epithelial cells. Using mAbs originally raised against the murine CD1D1 gene product and cross-reactive with human CD1d (i.e., mAbs 1H1 and 3C11), this protein was shown to be strongly expressed by epithelial cells from the human large intestine (Blumberg *et al.*, 1991). Immunoprecipitations from lysates of ^{125}I-labeled normal colonic epithelial cells performed with these mAbs, or with a mAb raised against a bacterial fusion protein constructed from the human CD1d cDNA sequence (Bilsland and Milstein, 1991), revealed a 100-kDa band under nonreducing conditions and a single 37-kDa band after reduction. This 37-kDa protein was confirmed to be related to CD1d by peptide mapping and by reprecipitation with a specific antiserum and has been proposed to be a cell surface form of CD1d heavy chain that is devoid of N-linked glycans. Although MHC class I heavy chains were coimmunoprecipitated with β2-microglobulin from these cells, β2-microglobulin was not detected in the anti-CD1d immunoprecipitates, and a mAb specific for β2-microglobulin was unable to coprecipitate the 37-kDa protein. Thus, it has been proposed that human

CD1d heavy chains lacking N-linked glycans and not associated with β2-microglobulin are the predominant form of this protein expressed on the surface of intestinal epithelial cells. In support of this unexpected finding, surface staining with anti-CD1d mAb was demonstrated after transfection of a human CD1d cDNA into a β2-microglobulin-deficient melanoma cell line (FO-1) (Balk *et al.*, 1994). These findings appear to oppose the general rule that β2-microglobulin is an obligatory part of most CD1 proteins found on the cell surface and also contradict the expectation that association with β2-microglobulin is necessary to achieve significant levels of stable CD1 surface expression. This is particularly surprising given the fact that human CD1d and its rodent homologues have previously been shown to be associated with β2-microglobulin on the surface of several different cell types (Bleicher *et al.*, 1990; Bradbury *et al.*, 1990; Bilsland and Milstein, 1991; Blumberg *et al.*, 1991; Ichimiya *et al.*, 1994). Therefore, it appears that assembly and post-translational modification of CD1d may vary markedly in different cell types. Although no functional role for the β2-microglobulin-unassociated CD1d molecule is yet known, it does appear to be the predominant or only form of CD1d expressed by intestinal epithelia, raising the possibility of a specialized function relevant to this particular anatomical site.

D. Post-Translational Modifications of CD1 Proteins

The predicted amino acid sequences of CD1 proteins show a minimum of three and as many as five potential sites for the attachment of asparagine (N)-linked oligosaccharide chains (Fig. 4). Some or all of these potential sites are presumed to be glycosylated in most cases, although only a few examples have been directly studied. Human CD1a and CD1c immunoprecipitated from thymocytes have been examined after treatment with Endo-F, which removes both complex and high mannose N-linked carbohydrates. For both proteins, a fully deglycosylated backbone size of 33 kDa is found, and deglycosylation intermediates of 40, 38, and 36 kDa are also observed. These results are consistent with the presence of at least four N-linked oligosaccharide side chains on mature CD1a and CD1c proteins (Van de Rijn *et al.*, 1983), which is in good agreement with the four potential sites for N-linked glycosylation predicted by the deduced amino acid sequences of these proteins (see Figs. 3 and 4). Since both proteins show considerable charge heterogeneity in isoelectric focusing gels, these oligosaccharides are presumed to be of the complex (i.e., sialic acid containing) variety. This is supported by the finding that a portion of the charge heterogeneity of CD1a is eliminated by sialic acid removal

by neuraminidase treatment (Van Agthoven and Terhorst, 1982). Treatment of Endo-F deglycosylated CD1a and CD1c with trifluoromethanesulfonic acid does not lead to any further reduction in molecular mass, consistent with the absence of O-linked glycan chains on these proteins. However, analysis of CD1a purified from the MOLT-4 T leukemia cell line shows a molecular mass approximately 3 kDa greater than that of thymocyte CD1a, and this difference persists after neuraminidase treatment (Van Agthoven and Terhorst, 1982). This was interpreted to be evidence of an additional oligosaccharide group on CD1a expressed by this tumor cell line. Since only four sites for N-linked glycan attachment are predicted by the protein sequence of CD1a, this may indicate the attachment of an O-linked glycan in this case. Lectin-binding studies suggest the existence of qualitative differences between the carbohydrate composition of oligosaccharide chains in MHC class I molecules and CD1a, although the nature of these differences is not known (Lerch *et al.*, 1983). Studies carried out on the rabbit CD1b molecule are also consistent with the presence of at least three N-linked and no O-linked oligosaccharides (Wang *et al.*, 1988).

With a single exception, all other reports of CD1 proteins immunoprecipitated from the cell surface have shown results consistent with glycosylation of the heavy chain. This is inferred by the broad bands observed on sizing gels and also by the increase in the observed molecular mass relative to that calculated for the predicted backbone polypeptide when this value is available from DNA sequence information. The single exception has been noted for CD1d expressed by human colonic epithelial cells. As discussed previously, this protein appears to be expressed on the surface of these cells without being associated with β2-microglobulin, possibly as disulfide-bonded CD1d heavy-chain homotrimers. The mass of the epithelial cell CD1d proteins is not affected by N-glycanase treatment, supporting the absence of N-linked glycans (Balk *et al.*, 1994). The significance of these nonglycosylated CD1 heavy chains is currently not known, and the problem of how epithelial cells selectively prevent the glycosylation of particular transmembrane proteins while normally glycosylating others (e.g., MHC class I) has not been resolved. CD1d molecules expressed by other cell types have been reported to have molecular masses between 48 and 54 kDa, consistent with the addition of several oligosaccharide chains (Bleicher *et al.*, 1990; Bradbury *et al.*, 1990; Bilsland and Milstein, 1991; Mosser *et al.*, 1991; Blumberg *et al.*, 1991; Ichimiya *et al.*, 1994). In addition, Endo-F treatment of mouse CD1d isolated from BW5147 thymoma cells caused a reduction in molecular mass

from 49–55 kDa to 36 kDa, consistent with the presence of several N-linked glycans (Mosser *et al.*, 1991). However, it is interesting that in one published example in which proteins extracted from adult mouse intestine were analyzed by Western blotting with a polyclonal anti-mouse CD1 antiserum, a band of approximately 100 kDa (and not the 49- to 55-kDa bands seen in several other tissues) was identified (Mosser *et al.*, 1991). Although not specifically commented on by the authors of this study, these extracts were apparently separated in nonreducing gels, so it is possible that this 100-kDa band could represent the murine homologue of the disulfide-bonded multimer of 37-kDa nonglycosylated CD1d observed in nonreduced samples of human intestinal epithelial cells.

Very little is known about other potential post-translational modifications in CD1 proteins. Attempts to derive N-terminal sequence information from purified CD1a and CD1c proteins have failed because of resistance to Edman degradation (Lerch *et al.*, 1986). This indicates the existence of an N-terminal modification, the nature of which is currently not known. In contrast, successful N-terminal sequencing of purified rabbit CD1b protein has been reported (Wang *et al.*, 1988), indicating that N-terminal modification and blockade is not universal for CD1 proteins. Protein phosphorylation has been examined only for CD1a and is not detected under conditions that allow detectable phosphate labeling of MHC class I proteins (Blue *et al.*, 1989). This is not surprising given the predicted structure of the CD1a cytoplasmic tail which lacks threonine, serine, and tyrosine residues that would constitute potential sites for phosphorylation. Other known CD1 proteins all have at least one tyrosine and one serine or threonine residue in their cytoplasmic tails which could serve as potential phosphorylation sites (see Figs. 3 and 4). However, phosphorylation of these sites has not been investigated, and it is not currently known whether or not this occurs. No published studies have examined the possibility of acylation of CD1 proteins.

E. Intermolecular Complexes between CD1 and Other Proteins

CD1 heavy chains are known to associate with several other molecules in addition to β2-microglobulin on the surface of certain cell types. The first described and most well known of these intermolecular complexes is the association of CD1a heavy chains with the CD8α chain on human thymocytes. Whereas CD8α is found in disulfide-bonded multimeric forms of a 34-kDa glycoprotein and as a heterodimer associated with the CD8β subunit on peripheral T cells, on

thymocytes it is also disulfide linked to a 46-kDa transmembrane glycoprotein (Snow and Terhorst, 1983). This 46-kDa subunit has been identified as the CD1a heavy chain (Snow *et al.*, 1985; Ledbetter *et al.*, 1985). These complexes exist as multiple species with molecular masses greater than 120 kDa, probably consisting of one or more CD1a heavy chains disulfide linked to two or more CD8α chains. β2-Microglobulin is not found in these complexes, nor are CD1b or -c heavy chains seen in association with CD8α multimers. It is noteworthy that these complexes are immunoprecipitated by anti-CD8 mAbs, but only weakly or not at all by anti-CD1a mAbs, suggesting that the CD1a determinants are buried in the complex or that the conformation of the protein is altered. The basis for the interaction of the CD8α and CD1a polypeptides is not known, but it has been speculated that the Cys–Lys–Cys sequence in the cytoplasmic tail of CD8α may lie in close proximity to the Cys–Phe–Cys contained in the cytoplasmic tail of CD1a, with obvious potential for interchain disulfide bond formation. Although it is possible that artefactual disulfide bond formation during cell lysis could account for the existence of these complexes, the observed multimer formation is unaffected by addition of excess iodacetamide and is thus believed to be present prior to cell lysis. MHC class I molecules have also been found to be associated with CD8α molecules on the surface of normal peripheral T cells and thymocytes, although these complexes are noncovalently associated (Blue *et al.*, 1988; Bushkin *et al.*, 1988). Since CD8 is known to function as a coreceptor for MHC class I during T cell recognition of class I-presented antigens (Salter *et al.*, 1990), it is appealing to consider that the intermolecular complex formation between CD1a and CD8α could indicate a related role for CD8 on T cells during cell interactions with antigen-presenting cells expressing CD1a. At present there are no reports supporting or refuting this possibility.

CD1a is also found in association with HLA class I molecules (Amiot *et al.*, 1988a) and with CD1b and CD1c molecules (Amiot *et al.*, 1988b) on the surface of normal human thymocytes. Unlike the CD8α/CD1a complexes, these associations are noncovalent. The CD1a molecules present in these complexes are not recognized by anti-CD1a mAb, and the complexes can only be isolated by immunoprecipitation with mAbs against the other component (i.e., anti-HLA Class I, anti-CD1b, or anti-CD1c mAbs). It is unclear whether or not the CD1a heavy chains in these complexes are associated with β2-microglobulin, although the heavy chains of the other components of the complexes probably are β2-microglobulin associated. These complexes are observed only on normal thymocytes and not on CD1-expressing leuke-

mic cells, unlike the CD8α/CD1a intermolecular complexes that are observed on some but not all malignant cells that express these proteins (Amiot *et al.*, 1987a). No specific function has yet been proposed for these intermolecular complexes on normal thymocytes.

Other unidentified polypeptides have been occassionally observed to specifically coprecipitate with CD1 proteins, most notably a 16-kDa protein that is coprecipitated with CD1c from a variety of cell types including thymocytes, B cells (Knowles, 1987), and GM-CSF-activated monocytes (S. Porcelli, unpublished data). It is not known if this protein is related to the CD1c heavy chain (e.g., as a proteolytic degradation product) or if it is noncovalently associated or disulfide bonded to it. An unidentified polypeptide of approximately 200 kDa has been observed as a specifically coprecipitating protein with CD1b from ^{125}I surface-labeled MOLT-4 cells, and is thus believed to be specifically associated with CD1b (Knowles, 1987). It is not known whether or not this protein is also associated with CD1b on other cell types. One study has suggested that the Fc receptor for IgE on epidermal Langerhans cells may be physically associated with CD1a, although this has not been demonstrated by direct biochemical analysis (Bruynzeel-Koomen *et al.*, 1988).

VI. Tissue Distribution and Cellular Expression of CD1 Proteins

Studies of the expression of CD1 proteins in tissues by immunohistochemical techniques or on isolated cell populations by flow cytometry show that expression of these proteins is regulated in a tissue-specific and developmentally controlled manner. The classic or group 1 CD1 proteins (i.e., CD1a, -b, and -c) have been most thoroughly studied in this regard, revealing prominent expression in two hematopoietic cell lineages, lymphoid cells and myeloid/dendritic cells. Lymphoid cells expressing group 1 CD1 include the immature precursors of T cells in the thymic cortex and a subpopulation of B lymphocytes. Myeloid cells expressing these proteins include a variety of dendritic cells that are widely distributed throughout lymphoid and nonlymphoid tissues, as well as tissue macrophages in certain inflammatory lesions. Thus, in their overall pattern of cellular expression group 1 CD1 proteins are distinctly unlike the ubiquitously expressed classical MHC class I molecules and more closely parallel the restricted pattern of expression characteristic of MHC class II. The group 2 CD1 proteins (i.e., CD1d) have been less well studied, but initial observations suggest that their tissue distribution may be at least as widespread as that of the group 1 CD1 proteins, with one unique feature being their expres-

sion on epithelial cells in the gastrointestinal mucosa and elsewhere. The difference in patterns of cellular and tissue expression seen for group 1 versus group 2 CD1 provide an additional justification for this categorization into two distinct groups of CD1 proteins and is a further indication of the likelihood of important functional differences between members of the two groups. The pattern of cellular expression and tissue distribution for both groups of CD1 proteins is summarized in Table IV, and the potential functional implications of these data are discussed separately under Section VII.

A. Expression of Group 1 or Classic CD1 Proteins

1. Expression of Group 1 CD1 in the Thymus

Since CD1 was first discovered as a human thymocyte antigen, it is not surprising that initial studies on its cellular expression and tissue localization focused on the thymus. Studies using the first reported anti-CD1a mAb (NA1/34) showed that CD1a was strongly expressed on 85% of all thymocytes, whereas MHC class I molecules were detected on only 15% of thymocytes. When anti-CD1a and anti-MHC class I mAbs were used simultaneously, 99% of thymocytes were stained, indicating that MHC class I and CD1a are reciprocally expressed on human thymocytes (McMichael *et al.*, 1979). Although subsequent studies using more sensitive methods have shown that most $CD1^+$ thymocytes actually express low levels of MHC class I molecules, this reciprocal relationship between the level of CD1a and MHC class I expression has been confirmed (Blue *et al.*, 1989; Gambon *et al.*, 1988). Additional studies incorporating anti-CD1b and anti-CD1c mAbs demonstrate that most $CD1a^+$ cells also express CD1b and CD1c, with the relative levels of these proteins based on mean fluorescence intensity in flow cytometric studies being CD1a > CD1b > CD1c (Gambon *et al.*, 1988). Immunohistochemical staining of the thymus with anti-CD1a mAb shows that the protein is uniformly expressed in a cell surface pattern on most thymocytes of the thymic cortex, but only on rare cells (a proportion of which have dendritic morphology) in the thymic medulla (Bhan *et al.*, 1980). A similar pattern is also seen with mAbs specific for CD1b and CD1c (Favaloro *et al.*, 1989b). As for CD1a, CD1b and CD1c are never observed on resting or activated mature peripheral T cells either in the circulation or in lymphoid organs.

Further studies of CD1 expression in the thymus have attempted to correlate the expression of CD1 on cortical thymocytes with developmental stage. Thus, phenotypic analysis using various thymocyte differ-

TABLE IV
TISSUE DISTRIBUTION OF CD1 PROTEINS[a]

Cell Type or Tissue	Group 1			Group 2	
	Human CD1a	Human CD1b	Human CD1c	Human CD1d	Mouse CD1
Lymphoid cells					
Cortical thymocytes	+ + +	+ +	+ +	±	+
Medullary thymocytes	±	±	±	±	+
Peripheral T cells	−	−	−	−	−
Circulating B cells	−	−	±	±	?
Mantle zone B cells	−	−	+	−	−
Splenic B cells	−	−	±	±	?
Myelomonocytic cells					
Langerhans cells	+ + + +	±	+	−	−
LN interdigitating cells	+	+ +	+ +	−	−
Tissue dendritic cells	−	+	+ +	−	−
Thymic dendritic cells	+	?	+	−	−
Blood dendritic cells	−	−	−	?	?
Circulating monocytes	−	−	±	?	?
Tissue macrophages	±	±	±	?	?
Neutrophils	−	−	−	?	?
Epithelial cells					
Jejunal epithelium	−	−	−	+ +	+ +
Colonic epithelium	−	−	−	+ +	+ +
Renal tubules	−	−	−	+	−
Bile duct	−	−	−	+	−
Sweat glands	±	−	−	+	?
Other cells					
Hepatocytes	−	−	−	+	+
Pancreatic acinar cells	−	−	−	+	?
Vascular smooth muscle	−	−	−	+	?

[a] -, Not detected; ?, not examined or not reported; ±, present on a subpopulation; +, present on most cells at detectable levels; + +, highly expressed; + + +, very highly expressed; + + + +, most highly expressed.

entiation markers indicated that CD1 is expressed predominantly at the immature "common thymocyte" stage (Reinherz *et al.*, 1980), which is consistent with the localization of most $CD1^+$ cells to the thymic cortex. The vast majority of these cells coexpress CD4 and CD8 antigens, constituting the pool of "double-positive" thymocytes. Although most of these double-positive cells are known to be dying or destined for elimination, it is also widely believed that this population contains within it most or all of the precursors of $CD4^+8^-$ and $CD4^-8^+$ mature peripheral T cells (Von Boehmer, 1992). Three-color immunofluorescence analysis of thymocyte populations has led to further resolution of the phenotypic subsets of thymocytes and their expression of CD1 (Lanier *et al.*, 1986). These studies reveal that CD1 is absent from small populations of $CD3^-$ and highly $CD3^+$ thymocytes. Most likely, the former represent the earliest T cell precursors in the thymus, and the latter represent mature or nearly mature T cells that are close to exiting the thymus. The remainder of the thymocytes, most of which express low or moderate levels of CD3, are $CD1^+$ and also 85–100% positive for CD2 and CD5. These results have been integrated into a scheme for thymic development of T cells that is illustrated in Fig. 11. Thus, CD1 would appear to switch on early after the entry of thymocyte precursors into the thymic cortex, prior to the expression of CD3, CD4, and CD8 molecules that mediate the specific positive and negative selection events that subsequently occur. As CD3 levels rise (possibly as a result of positive selection), CD1 appears to decline and either CD4 or CD8 is shut off, thus giving rise to cells with the mature T cell phenotypes. Although the importance of the transient expression of CD1 on developing T cells is not known, it may be significant that CD1 proteins appear to be expressed during a phase in which CD3/T cell receptor complex (TCR)-mediated selection occurs. Thus, it is conceivable that interaction of the CD3/TCR complex with CD1 proteins expressed on thymocytes at this stage could mediate positive or negative selection events. This possibility seems plausible now that TCR/CD1 interactions have been demonstrated for mature circulating T cells, as discussed under Section VII. Alternatively, CD1 proteins may themselves be involved in mediating transmembrane signals by a pathway independent of TCR specificity. This possibility has been suggested by experiments showing that mAbs specific for CD1b or CD1c can induce a rapid increase in intracellular calcium in $CD1^+$ thymic leukemia cell lines (MOLT-4, Jurkat), consistent with their involvement in a TCR-independent signaling pathway (Theodorou *et al.*, 1990). Whether or not such signaling occurs in normal

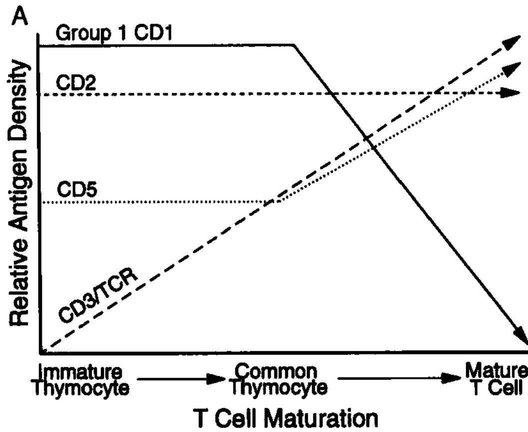

FIG. 11. CD1 expression in thymic development. (A) Expression of group 1 CD1 proteins (CD1a, -b, -c) is shown as a function of developmental stage and in relation to expression of other thymocyte differentiation antigens. (B) Hypothetical scheme for discrete stages of T cell development in the thymus, based on expression of group 1 CD1, CD3, CD4, and CD8 antigens. Stages that show CD1 expression are shaded. The bracket marked "thymic selection" indicates the stages during which TCR-mediated negative and positive selection are believed to occur. Both A and B are adapted from Lanier *et al.*, (1986) with permission.

thymocytes and what its relevance to the process of maturation might be are currently not known.

It is perhaps important to note that some investigators have suggested that since nearly all of the $CD1^+$ common thymocytes are destined to undergo cell death and will not give rise to functional T cells, this population may essentially represent a dead end of the thymic maturation process (i.e., a graveyard for the failures of thymic education). In this regard, it is of interest that a small population of $CD4^+CD8^+$ thymocytes that lacks expression of CD1 has been demonstrated (Blue *et al.*, 1987a). An alternative scheme for T cell development might thus propose that these $CD1^-$ double-positive cells are responsible for all of the productive thymopoiesis. In this scheme, CD1 would be viewed simply as a marker of cell death and may be expressed because of activation of the CD1 genes by factors related to cellular stress. Against this possibility is the observation that $CD1^+$ thymocytes can be activated to undergo DNA synthesis *in vitro* by stimulation through CD2 and the IL-2 receptor (Blue *et al.*, 1987b) and have been claimed to contain precursors capable of giving rise to mature T cells in limiting-dilution cultures (Lopez-Botet and

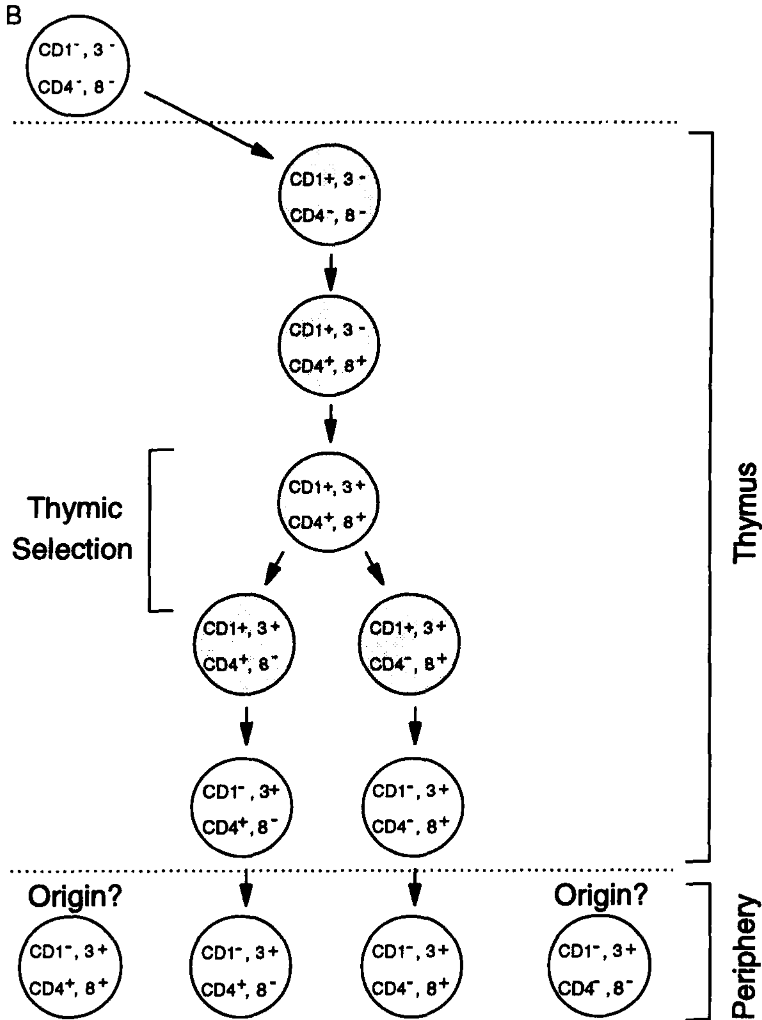

FIG. 11—*Continued*

Moretta, 1985). These experiments, although relying on artificial methods for thymocyte activation *in vivo*, appear to support the idea that at least some $CD1^+$ thymocytes are capable of continued growth and differentiation into more phenotypically mature cells. Ultimately, the resolution of this question will probably require a thorough investigation of the developmental potential of $CD1^+$ thymocytes either in *in vitro* thymic organ culture or *in vivo* in an animal model in which

group 1 CD1 proteins are expressed in the thymus. The relationship of CD1 expression to the development of mature $CD4^-CD8^-$ or "double-negative" T cells expressing either $\alpha\beta$ or $\gamma\delta$ TCRs is another area that has not been studied. This is currently of substantial interest, since most examples of functional recognition of CD1 molecules by human T cells reported to date have involved these double-negative subsets (see Section VII).

2. *Group 1 CD1 Expression by Dendritic Cells*

Dendritic cells (DCs) are a heterogeneous but probably related group of specialized antigen-presenting cells that play a key role in the priming of T cell responses to foreign antigens (Steinman, 1991). As their name implies, DCs are identified in tissues by their characteristic morphology, with multiple elongated and branching processes radiating from a central cell body. The precise pathway of differentiation that gives rise to these cells is still not entirely clear, although they are known to be of bone marrow origin and recent *in vitro* studies show that DCs and monocytes can both be derived from a common circulating $CD34^+$ precursor cell (Caux *et al.*, 1992). Other studies suggest that blood monocytes may also be able to develop into cells resembling DCs under certain conditions (Rossi, 1992). DCs typically express high levels of MHC class II molecules on their surface as well as other molecules involved in T cell activation (e.g., ICAM-1/CD54, LFA-3/CD58, and the costimulatory molecule B7/CD80) and are the most potent known accessory cells for activation of T lymphocyte reponses. A variety of distinct but probably related DC populations have been defined. These include the Langerhans cells of the epidermis, the so-called "veiled cells" found in afferent lymph, and the interdigitating cells (IDC) found in the T cell-rich areas of organized lymphoid tissues. In addition, DCs of unspecified type have been observed scattered throughout many tissues and also circulate in the blood in low numbers. Most of these cells have been found to display various patterns of group 1 CD1 proteins *in vivo* (summarized in Fig. 12). The complex interrelationships between these different DC populations and the evidence for their central role in the process of T cell activation have been the subject of several recent reviews (Steinman, 1991; King and Katz, 1990; Knight and Stagg, 1993) and will not be covered in detail here.

CD1 expression by DCs was first demonstrated for epidermal Langerhans cells (LCs). LCs represent an abundant DC population found in the skin and in other keratinizing epidermal sheets (e.g., oropharyngeal and vaginal mucosa). LCs are unlike most other DCs that have been

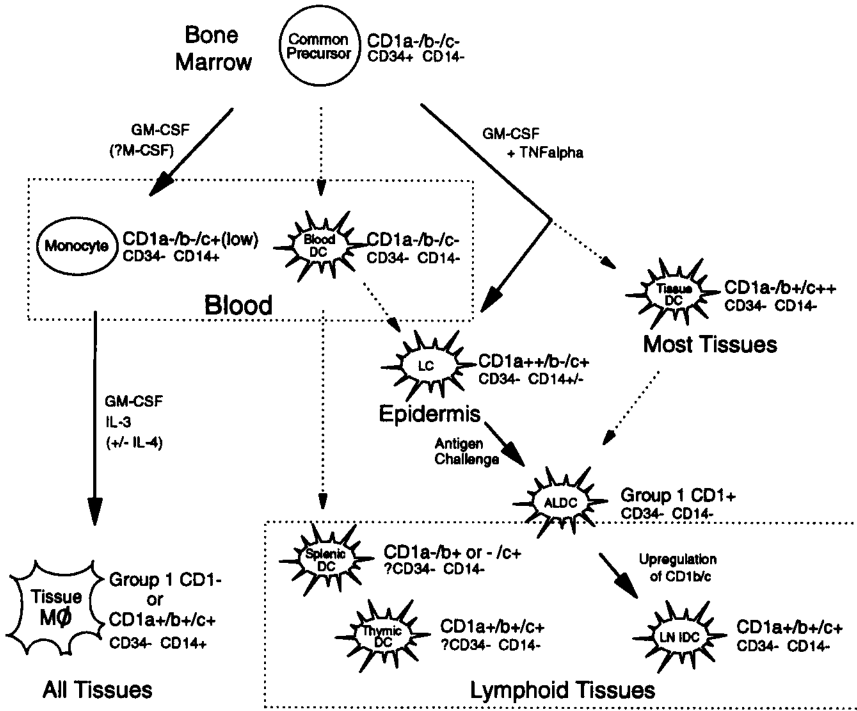

FIG. 12. Hypothetical scheme for the development and tissue distribution of group 1 CD1$^+$ macrophages and dendritic cells. Solid arrows indicate steps that are supported by *in vitro* or *in vivo* experimental results. Dotted arrows indicate steps or transitions that have been proposed but have not yet been confirmed or supported by experimental data. Expression of group 1 CD1, CD34 (a stem cell marker), and CD14 (a mature monocyte/macrophage marker) are indicated for each cell type. See text for details.

functionally examined in that they actively phagocytose antigens and also in their expression of Fc receptors (FcγRII and Fc7RI) and complement (C3) receptors that may facilitate their uptake of antigens that have been trapped as immune complexes. Evidence from a variety of *in vivo* studies suggests that the major role of LCs may be to take up antigens encountered in the epidermis and carry them via lymphatics to regional lymph nodes where they stimulate T cell responses (reviewed by King and Katz, 1990). Initial studies of LCs in normal skin revealed strong reactivity with anti-CD1a mAb (Fithian *et al.*, 1981). Immunoelectron microscopy demonstrates that the CD1a$^+$ epidermal cells have typical ultrastructural features of LCs, including the characteristic intracellular inclusions known as Birbeck granules (Chu *et al.*,

1982). The protein reactive with anti-CD1a mAb on LCs is indistinguishable in immunoprecipitation studies from CD1a expressed on thymocytes (Takezaki *et al.*, 1982). Although LCs often have been reported to be negative for CD1b and CD1c determinants in histologic sections of normal epidermis, analysis of suspensions of isolated LCs by flow cytometry reveals that low to moderate levels of CD1c and very low levels of CD1b may also be expressed by most of these cells (S. Porcelli, unpublished results). Furthermore, LCs in the epidermis of patients with contact dermatitis show induction of CD1b to readily detectable levels (Taylor *et al.*, 1991).

Langerhans cells are the only cell type for which studies of intracellular trafficking of CD1 molecules have been reported to date. Immunoelectron microscopy studies have shown that colocalization of CD1a and HLA-DR in coated pits is induced following incubation of LCs with mAbs specific for either of these proteins. Under these conditions, CD1a and HLA-DR molecules are also observed to cointernalize into coated vesicles, receptosomes, lysosomes, and Birbeck granules (Hanau *et al.*, 1987). This suggests that CD1 and MHC class II molecules may share common pathways of recycling and/or intracellular trafficking in LCs, and also raises the possibility that CD1a may be involved in the process of antigen uptake by LCs. In this regard, it is noteworthy that LCs, which express extremely high levels of CD1a, have generally been found to be more active at antigen uptake than other DCs, most of which seem to express much lower levels of this form of CD1. Another study has provided evidence that previously endocytosed CD1a molecules in LCs can recycle to the cell surface (Ray *et al.*, 1989).

Expression of group 1 CD1 proteins has also been demonstrated on many other types of DCs. In skin sections, anti-CD1c mAb was noted to stain a population of DCs located primarily adjacent to dermal capillaries (Van de Rijn *et al.*, 1984). These dermal DCs are distinct from LCs since they are located below the basement membrane and stain only weakly or not at all with anti-CD1a in tissue sections. CD1c$^+$ dermal DCs have recently been isolated and extensively characterized phenotypically and functionally, demonstrating that these cells also express CD1b and that they are potent stimulators of T cell responses (Nestle *et al.*, 1993; Meunier *et al.*, 1993). An extensive survey of CD1a, -b, and -c on DCs of fetal and adult tissues by immunostaining of frozen sections has also been reported in summary form (Cattoretti *et al.*, 1989a,b). In fetal samples, CD1c$^+$ DC were found in the soft tissues of the earliest samples examined (8 weeks gestational age) and were present in many organs (liver, spleen, mesentery, gut submucosa,

and dermis) of later (9–22 weeks gestational age) samples. $CD1b^+$ DC were also widely distributed, but 3–10 times less numerous, and $CD1a^+$ cells were found only in epidermis and lymph nodes. In adult samples, $CD1c^+$ DC were found dispersed in most tissues, and $CD1b^+c^+$ DCs were found in a perivascular distribution in liver, kidney, and lung. $CD1a^+$ DCs in the adult were found only in the epidermis, dermis, bronchial epithelium, and interfollicular regions of lymph nodes. These results indicate a widespread distribution of group 1 CD1 molecules (especially CD1c and -b) on tissue DCs. Immunohistochemical studies of bovine thymus using a mAb that appears to be specific for a subset of group 1 CD1 molecules also indicates that these proteins are expressed on dendritic cells in the thymic medulla (Bujdoso *et al.*, 1989). This appears to be the case for human thymus as well, in which medullary dendritic cells are reported to express CD1c and -a (Cattoretti *et al.*, 1989a).

A small population of circulating DCs in adult human blood can be isolated and generally has been found not to express detectable surface levels of group 1 CD1 proteins (Freudenthal and Steinman, 1990; Thomas *et al.*, 1993). However, this could reflect an artifact related to the complex and lengthy isolation procedure used to purify blood DCs since other studies show that a small but detectable fraction ($<1\%$) of circulating normal adult human peripheral blood mononuclear cells express CD1a (Dezutter-Dambuyant *et al.*, 1984). Furthermore, the proportion of such cells is generally increased to as high as 8% in adults with burn injuries (Gothelf, *et al.*, 1988) and to approximately 10–15% of mononuclear cells in the circulation of newborns (i.e., umbilical cord blood) (Gothelf *et al.*, 1986). These cells bear MHC class II and some myeloid differentiation antigens, and in the case of $CD1a^+$ umbilical cord blood cells electron microscopy reveals intracellular organelles similar to the Birbeck granules of LCs. An increase in $CD1a^+$ cells with dendritic cell features has also been observed in the blood of human immunodeficiency virus type 1-infected patients (Ree *et al.*, 1994). These results suggest that $CD1^+$ DCs or DC precursors, while not a prominent feature of healthy adult circulating leukocyte populations, may be more abundant in certain developmental stages or in states of physiologic stress and immunologic challenge.

The population of mobile DCs in afferent lymphatics known as veiled cells or afferent lymph dendritic cells (ALDCs) has also been studied with respect to CD1 expression. ALDCs are difficult to obtain in humans or in small animals, but are quite accessible to study in large animals, such as cow and sheep, in which afferent lymphatics can be cannulated for collection of draining lymph. ALDCs of sheep

and cow have been examined using mAbs that appear to be specific for group 1 CD1 antigens of these species [mAb TH97A in cow (MacHugh *et al.*, 1988) and mAbs SBU-T6 and VPM5 in sheep (Mackay *et al.*, 1985; Bujdoso *et al.*, 1989)]. These studies show that group 1 CD1 proteins are expressed at variable levels on 50% or more of these cells in both species (McKeever, *et al.*, 1991; Bujdoso *et al.*, 1989). An interesting point emerging from these studies is the finding that sheep ALDCs migrating from a site of intradermal antigen challenge carry the antigen with them and after isolation are strongly stimulatory *in vitro* for T cells specific for the challenge antigen (Bujdoso *et al.*, 1989). Intradermal antigen challenge has also been shown to cause increases *in vivo* of the levels of MHC class II and CD1 expressed by ALDCs (Hopkins *et al.*, 1989). These studies have strengthened the proposal that ALDCs are derived from Langerhans cells that exit the epidermis after encountering foreign antigens, migrating via the afferent lymphatics into the regional lymph nodes where they may populate the interfollicular T cell zones as "interdigitating cells." This migration appears to involve the upregulation of several group 1 CD1 proteins (e.g., probably CD1b and -c in humans), suggesting a potent pathway by which foreign antigens originating in the skin may enter a CD1-dependent pathway of T cell recognition. A hypothetical scheme of the relationship between different members of the DC family that incorporates these observations on their regulation of group 1 CD1 protein expression is presented in Fig. 12.

3. *Group 1 CD1 Expression by Monocytes and Tissue Macrophages*

Whereas the group 1 CD1 proteins are generally not expressed at significant levels on circulating monocytes, it is now known that these molecules can be induced to high levels on virtually all freshly isolated monocytes using cytokines *in vitro*. Monocytes isolated from human blood by adherence to plastic show strong induction of CD1a, -b, and -c molecules when cultured in media containing recombinant human GM-CSF (Porcelli *et al.*, 1992; Kasinrerk *et al.*, 1993) or Interleukin-3 (IL-3) (S. Porcelli, unpublished results). Weak induction has also been observed in some experiments by IL-1α and IL-1β (S. Porcelli, unpublished results). This induction is associated with the appearance of detectable levels of CD1-specific mRNA (Kasinrerk *et al.*, 1993) and is inhibited by cycloheximide (S. Porcelli, unpublished results), indicating that it most likely involves transcriptional activation of CD1 and new protein synthesis as opposed to mobilization of a preformed intracellular pool of CD1.

Other cytokines that are active at inducing MHC class I and class

II molecules (e.g., interferon-γ and TNFα) do not induce *de novo* expression of group 1 CD1 proteins on human monocytes (Karinrerk *et al.*, 1993), indicating important fundamental differences in the regulation of CD1 and MHC molecules on these cells (note that this is reminiscent of the reciprocal expression of CD1 and MHC class I on thymocytes). In fact, interferon-γ actually suppresses the induction of CD1 by GM-CSF (S. Porcelli, unpublished results). IL-10 also appears to antagonize the induction of group 1 CD1 proteins by GM-CSF (P. Sieling and R. Modlin, personal communication). Conversely, IL-4, which has no effect if used alone, shows a synergistic effect on the induction of CD1a (and in some experiments also CD1b and -c) when used in combination with GM-CSF (S. Porcelli, unpublished results). Overall, the cytokine regulation of CD1 expression by monocytes *in vitro* appears to be remarkably similar or identical in all aspects examined to that described for a protein previously reported as a novel monocyte activation marker called TOMS-1 (Maeda *et al.*, 1991). Limited biochemical studies of this protein suggest that it may in fact be CD1a (molecular mass of approximately 49 kDa, although no associated 12-kDa subunit was identified), although the possibility still remains that it is a separate monocyte differentiation antigen coordinately regulated with CD1. Induction of CD1 by GM-CSF is not seen on a variety of myelomonocytic cell lines (e.g., HL60, THP-1, KG1, U937, and others) (Kasinrerk *et al.*, 1993). In addition, this *in vitro* cytokine induction is inhibited in the presence of human serum or plasma, although it occurs readily in media supplemented with fetal bovine serum or in serum-free media (S. Porcelli, unpublished results). The presence of factors in adult human serum that inhibit this response on human monocytes seems paradoxical, but is consistent with the observation that CD1 expression is rarely seen on circulating myeloid or dendritic cells in normal adults, although it can be found on these cells in extravascular compartments (Fig. 12).

The surface phenotype of cells derived by culturing monocytes under conditions that induce CD1 expression has suggested to some investigators that these cells represent *in vitro* analogues of CD1-positive dendritic cells found in lymphoid and nonlymphoid tissues *in vivo*. Indeed, functional studies show that these cells are extremely efficient antigen-presenting cells for both MHC class II-restricted (Sallusto *et al.*, 1994) and for CD1-restricted T cell responses (Porcelli *et al.*, 1992). Nevertheless, the monocyte-derived cells do not appear obviously dendritic in morphology, and transmission electron microscopy reveals large phagolysosomes and an absence of Birbeck granules (S. Porcelli, unpublished results). This appearance is markedly differ-

ent from that reported for the more classic-appearing DC phenotype of cells derived *in vitro* from $CD34^+$ precursors stimulated with GM-CSF plus TNFα (Caux *et al.*, 1992). Overall, the phenotype and ultrastructure of the monocyte-derived cells suggests that they may represent the *in vitro* analogue of a type of tissue macrophage.

In support of this idea, there is evidence for the existence of macrophage-like cells expressing group 1 CD1 proteins in inflammatory or infectious lesions *in vivo*. CD1a was identified on large epithelioid cells (i.e., cells with typical macrophage morphology) in the outer layers of granulomas in the skin of patients with the tuberculoid form of leprosy (Modlin *et al.*, 1983), and CD1b mAb is reported to stain scattered epithelioid cells in chronic inflammatory mononuclear cell infiltrates (the precise disease state was not defined in the original report) (Cattoretti *et al.*, 1987). More recently, immunohistochemical studies of skin biopsy specimens from leprosy patients have shown that CD1a, -b, and -c are all expressed by large mononuclear cells distributed throughout the dermal granulomas of patients with the tuberculoid (i.e., immunologically responsive) form of leprosy. These cells are absent from the dermal lesions of patients with the lepromatous (i.e., immunologically nonresponsive) form of the disease, suggesting that their presence is positively correlated with the level of effective immunity to *Mycobacterium leprae* (P. Sieling and R. Modlin, personal communication). Although the precise origin and identity of these cells is not yet known, their size and morphology in immunoperoxidase-stained specimens and their location within granulomas are compatible with a macrophage subset. The existence of $CD1^+$ macrophages in chronic infectious and inflammatory lesions may be a consequence of *in situ* generation of cytokines that could induce CD1 during the process of monocyte migration and differentiation in tissues. In this regard, it is interesting that GM-CSF has been determined to be one of the cytokines that is abundantly produced in skin lesions of tuberculoid (but not lepromatous) leprosy patients (Yamamura *et al.*, 1991).

It should be pointed out that although there is evidence for expression of all three human group 1 CD1 proteins on a subset of tissue macrophages and on *in vitro* cultured monocytes, these molecules have by no means been found on all tissue macrophages *in situ*. For example, one study reported no detection of CD1a on a variety of different tissue macrophages in normal and in diseased (e.g., tuberculosis, sarcoidosis) tissues (Wood *et al.*, 1983). This could be due to technical problems relating to level of expression and sensitivity of detection, but may also reflect the complex regulation of CD1 on

monocyte-derived cells that could restrict expression to a limited subset of macrophages or restricted spectrum of pathologic states. It has also been reported that Kuppfer cells (a population of mononuclear phagocytes within the sinusoids of the liver) do not express surface CD1a, -b, or -c (Koller *et al.*, 1987), which seems to be consistent with the apparent exclusion of group 1 CD1 proteins from myeloid cells in the intravascular space in the absence of disease or infection. Thus, the extent and regulation of group 1 CD1 expression on extravascular tissue macrophages needs to be explored further. Nevertheless, it appears likely that the expression of group 1 CD1 by a subset of these cells greatly expands the potential role of these proteins in *in vivo* immune responses.

4. *Group 1 CD1 Expression by B Cells*

Other than cortical thymocytes, peripheral B cells are the only other known site of expression of group 1 CD1 proteins by lymphocytes. The expression of CD1c has been demonstrated on B cells purified from adult blood, spleen, and tonsil. Not all B cells in these sites are CD1c$^+$, and the percentage of CD1c$^+$ cells is greater for B cells purified from the spleen (44–49%) than from blood (28–37%) or tonsil (17–23%) (Small *et al.*, 1987a,b). Tissue staining of frozen sections of lymph node and tonsil is also consistent with CD1c being a marker of a B cell subset, since this shows fairly dense staining of the B cell rich "mantle zones" that surround the germinal centers in which growth and selection of antibody-producing cells occurs (Cattoretti *et al.*, 1987). Biochemical studies of CD1c expressed by peripheral B cells are consistent with it having the same protein structure as CD1c expressed by thymocytes or thymic leukemia cell lines, including the association with a coprecipitating 16-kDa unidentified polypeptide (Small *et al.*, 1987a).

The expression of CD1c by peripheral B cells also appears to be a developmentally regulated phenomenon. B cells isolated from umbilical cord blood taken at the time of birth are mostly CD1c$^+$ (range 83–93%, $N = 7$), and this percentage is observed to decline to adult levels during the first 2 or 3 years of life. CD1c was also observed on a high percentage (43–98%) of circulating B cells from infants and young children with severe combined immunodeficiency, although it is not entirely clear whether or not these levels are different from what would be expected for age-matched normal controls (Small *et al.*, 1989). These findings indicate the presence of developmental influences that direct the decline of the CD1c$^+$ B cell population during normal growth and aging. One isolated report has claimed that CD1c

can be upregulated on CD1c$^+$ B cells and induced *de novo* on a majority of CD1c$^-$ B cells isolated from either blood or spleen upon activation with either calcium ionophore plus phorbol ester or formalin-fixed *Staphylococcus aureus* Cowan strain 1 (Delia *et al.*, 1988). In general, the other group 1 CD1 proteins (CD1a and -b) have not been observed on B cells under any of these conditions. However, in one study of peripheral blood mononuclear cells from patients with acute hepatitis B infection, CD1a, -b, and -c were all observed on increased percentages of cells, ranging in most cases between 2 and 20%. At least in the case of the CD1a-expressing cells, a fraction (25%) was found to coexpress surface immunoglobulin, suggesting that they may be B cells. The relation of the CD1 expression to hepatitis B infection is unknown, but it appears to be at least partially specific to this infection since CD1$^+$ peripheral blood mononuclear cells were not observed in subjects with acute hepatitis A virus infection or autoimmune chronic liver inflammation (Roisman *et al.*, 1989).

5. *Other Sites of Group 1 CD1 Expression, Including Neoplastic Cells*

A small number of studies have claimed to find evidence of group 1 CD1 antigen expression on epithelial cells. CD1a has occassionally been observed on a fraction of keratinocytes in epidermal cell suspensions (Berti *et al.*, 1989), which may reflect passive acquisition of protein shed or secreted by adjacent Langerhans cells. It has also been reported that CD1a is induced on a few percent of cultured gingival epithelial cells by addition of interleukin-1 to the culture media (Walsh and Seymour, 1988). Perhaps the most convincing report of expression of a group 1 CD1 antigen by cells of epithelial origin involved the staining of eccrine sweat glands with anti-CD1a mAb in frozen sections of human skin. This staining is confined to epithelial cells lining the deep portion of the gland and appears to be mainly cytoplasmic (Kanitakis *et al.*, 1983). Overall, the available data indicate that group 1 CD1 proteins are at most only rarely expressed by normal epithelia. As discussed under Section VI,B, this may be a major difference in the pattern of cellular expression between group 1 and group 2 CD1 proteins. The possibility that group 1 CD1 proteins could be induced *de novo* on stressed, transformed, or infected epithelial cells has been suggested (R. Blumberg, personal communication), but no studies specifically examining this hypothesis have yet appeared. A single report has documented the expression of CD1c by endothelial cells in the dermis (Murphy *et al.*, 1985), but in general group 1 CD1 proteins have not been observed on endothelia in extensive surveys of a wide variety of tissues.

Group 1 CD1 proteins are well known to be expressed on a substantial fraction of several different hematologic malignancies, generally in a pattern reminiscent of their normal expression in cells of the T cell, B cell, and myeloid lineages. Thus, all three group 1 CD1 proteins have usually been found on the surface of blast cells of 20% to upwards of 50% of cases of T cell acute lymphocytic leukemias (Reinherz *et al.*, 1980; Salamone *et al.*, 1989; Amiot *et al.*, 1987a). Some of these tumors express only one or two of the three group 1 CD1 proteins, and the relative levels of CD1a, -b, and -c may vary considerably (Amiot *et al.*, 1987a). Group 1 CD1 proteins are also constitutively expressed on many T leukemia cell lines grown *in vitro*, including MOLT-4, HD-MAR, and HPB-ALL (Amiot *et al.*, 1987b). CD1a has been detected on a significant fraction of leukemic bone marrow cells in approximately 20% of cases of acute myeloid leukemias (Misery *et al.*, 1992). Malignant histiocytes infiltrating the skin in histiocytosis X, believed to be a malignant transformation of Langerhans cells, express high levels of CD1a as well as significant levels of CD1c and trace amounts of CD1b in tissue staining (Murphy *et al.*, 1983; Cattoretti *et al.*, 1987). In addition, CD1a is found on blasts from approximately one-fourth of cases of acute lymphocytic leukemia without markers of T cell origin (common ALL) (Hutchinson *et al.*, 1987). Several types of B cell neoplasms are known to express CD1c, including some cases of B cell chronic lymphocytic leukemia, hairy cell leukemia, and B cell non-Hodgkin's lymphomas, but not Burkitt's lymphomas. These tumors do not express CD1a, and CD1b has only been faintly detected in a few cases (Delia *et al.*, 1988; Cattoretti *et al.*, 1989a). CD1c is generally not found on B cell lines *in vitro*, including Epstein–Barr virus-transformed B lymphoblastoid cell lines and established Burkitt's lymphoma cell lines (Small *et al.*, 1987a). Group 1 CD1 antigens are not known to be expressed by malignant epithelia (carcinomas) or by other types of nonhematologic malignancies. Thus, in general the expression of group 1 CD1 proteins by malignant cells seems to reflect the lineage and state of differentiation of the tumor, and no specific role for these proteins in the process of malignant transformation has been proposed.

B. Tissue Distribution and Cellular Expression of Group 2 CD1 Proteins (CD1D)

Very recently, results from studies examining the tissue distribution of group 2 CD1 proteins in rodents and humans have become available. Although only a few studies have so far been reported, data pertaining to the tissue expression of group 2 CD1 proteins are now available at the mRNA and protein levels, and in a few cases from immunohisto-

chemical studies. The results available at this time suggest that the structurally distinct group 2 CD1 proteins do indeed have a pattern of cellular regulation and tissue expression that is significantly different than their group 1 counterparts. Overall, the emerging consensus at this point appears to be that group 2 CD1 proteins are not expressed prominently on the cells which strongly express group 1 CD1 (i.e., cortical thymocytes and dendritic cells), but are widely expressed in many tissues mainly by a variety of epithelial cell types.

1. *Expression of Group 2 CD1 Proteins in the Thymus*

The expression of the group 2 CD1 proteins was first examined at the RNA level in mouse thymus. RNase protection assays revealed prominent expression of mouse CD1D1 and CD1D2 gene transcripts in the thymus and in mouse cell lines of thymic origin (Bradbury *et al.*, 1988). Northern blot analysis also demonstrated that the majority of poly-A^+ CD1 mRNA in the mouse thymus is associated with membrane-bound polysomes, indicating that it is undergoing translation into protein (Bradbury *et al.*, 1988). A subsequent study revealed that mouse CD1D1 and CD1D2 transcripts are present in the thymus at approximately equal levels in most mouse strains, the one exception noted being strain SJL which shows several-fold higher levels of transcripts of CD1D1 compared to CD1D2. Much of the CD1 RNA in mouse thymus is incompletely spliced, although a small portion of transcripts from both genes do give rise to correctly spliced mRNA that is capable of encoding the mature CD1 polypeptides (Bradbury et al., 1990). Both mouse CD1 genes have also been shown by RNase protection assays to be expressed more strongly by immature cortical than mature medullary thymocytes, although this difference is not so marked as that observed for mouse TL genes (Bradbury *et al.*, 1990). No correlation has been found between the levels of TL (which appears to be regulated very much like the group 1 CD1 proteins are in the human thymus) and CD1-specific RNA in thymic RNA from different mouse strains, indicating that these genes are not coordinately regulated.

In contrast to these studies using the sensitive RNase protection method, Northern blotting of thymus RNA fails to demonstrate hybridization with a CD1D1 probe in the mouse (Balk *et al.*, 1991b) and shows relatively weak hybridization in rat (Ichimiya *et al.*, 1994). Northern blotting of human thymus RNA with a human CD1D-specific probe has shown expression of CD1D, although the signal is weak relative to that obtained with a probe hybridizing to group 1 CD1 gene transcripts (Balk *et al.*, 1989). These results are strongly sugges-

tive that, in contrast to the human group 1 CD1, expression of group 2 CD1 proteins in the thymus of both rodents and humans is relatively low, at least at the transcriptional level.

The availability of mAbs and antisera reactive with mouse CD1 proteins has recently enabled studies of group 2 CD1 expression at the protein level. Using two different anti-mouse CD1 monoclonal antibodies (3C11 and 1H1), low-level membrane staining of all lymphoid cells in the thymus has been detected and no distinction between the staining of cortical and medullary areas could be appreciated. This was confirmed by flow cytometry which showed dim staining of most thymocytes in suspension (Bleicher *et al.*, 1990). At present it is unclear if these mAbs (which were made by immunizing a rat with mouse CD1d1 protein) distinguish between CD1d1 and CD1d2, although it is suspected that they do not (S. Balk, personal communication). Immunoblotting (Mosser *et al.*, 1991) and immunoprecipitation (Bradbury *et al.*, 1990) studies have also confirmed the presence of CD1 protein in the mouse thymus. By immunoblotting, the thymus appears to be one of the more prominent sites of CD1 expression in adult mice. A developmental pattern of CD1 expression in the mouse thymus is also seen by immunoblotting of whole thymus extracts, with Day 14 prenatal thymus expressing relatively low levels consisting mainly of 49- and 51-kDa isoforms. This is followed by a gradual rise in total CD1 protein and a shift to two higher (53 and 55 kDa) molecular mass isoforms during later intrauterine and postnatal development (Mosser *et al.*, 1991). Immunoprecipitation of mouse CD1 from the surface of thymocytes revealed a 50- to 55-kDa heavy chain and a coprecipitating 12-kDa polypeptide, consistent with a β2-microglobulin-associated form of CD1 (Bradbury *et al.*, 1990).

In humans, detection of group 2 CD1 proteins on cells of thymic origin has been less consistent. Using the two anti-mouse CD1 mAbs [which have been found to cross-react with the human CD1d protein (Blumberg *et al.*, 1991)], flow cytometry of thymocyte suspensions failed to reveal significant surface expression of CD1d (Blumberg *et al.*, 1991). However, tissue staining of frozen sections of human thymus with the same mAbs was interpreted as showing faintly positive staining of scattered cortical and medullary thymocytes (Canchis *et al.*, 1993). This would be consistent with immunoprecipitation studies from the thymic leukemia cell line MOLT-4 using a mAb raised against a β-galactosidase–human CD1d bacterial fusion protein. This mAb shows weak precipitation of a 49-kDa β2-microglobulin-associated protein from MOLT-4 cells, with a crude estimate of expression level suggesting less than 4000 molecules per cell (Bilsland and Milstein,

1991). Similar immunoprecipitation studies from normal human thymocytes have not yet been reported. However, taken together these results indicate very low-level or absent expression of CDld by cells in the human thymus, again emphasizing the striking difference between expression of the group 1 and group 2 CDl proteins in this tissue.

2. *Other Sites of Group 2 CDl Protein Expression*

Whereas group 2 CDl proteins are apparently not as abundantly expressed in the thymus as the group 1 proteins, several studies now indicate that they do have a widespread distribution encompassing many extrathymic tissues. At the transcriptional level, RNase protection assays show expression of mouse CDlDl and CDlD2 RNA in a variety of mouse tissues, including spleen and liver, with lower levels in kidney and brain. In all of these extrathymic sites, CDlDl RNA levels are higher than those for CDlD2. Variable levels are also detected in a panel of cell lines encompassing the T, B, myeloid, and fibroblast lineages, again with CDlDl always exceeding CDlD2 (Bradbury *et al.*, 1988). Similarly, in rats, Northern blotting demonstrates CDlD RNA in spleen, liver, heart, kidney, and lung at comparable levels (and in all cases greater than that for thymus). A more sensitive reverse-transcriptase PCR (RT-PCR) technique also demonstrated expression of rat CDlD transcripts in intestinal epithelial cells (although this could not be detected by Northern blotting, presumably because the level of transcription is low). This RT-PCR technique also confirmed the absent or extremely low expression by peritoneal exudate cells (i.e., including macrophages and neutrophils) and cells in the brain, bone marrow, and testis (Ichimiya *et al.*, 1994).

Studies of extrathymic group 2 CDl expression at the protein level also indicate a widespread tissue distribution of these molecules in both rodents and humans, with one particularly interesting feature being their expression by intestinal epithelial cells in both species. Using the 1H1 and 3C11 anti-mouse CDl mAbs in a broad immunohistologic survey of mouse tissues, CDld protein was found to be most strongly expressed on epithelial cells of the stomach, small intestine, and colon. The observed staining of intestinal epithelia was predominantly on the apical membrane of the cells, with less intense staining also observed along the basolateral surface and on portions of epithelial cells that protrude into the lamina propria. Examination of longitudinal sections of intestinal villi showed that CDl expression is most intense on cells at the extrusion zone at the tips of the microvilli, and no staining is seen on the less mature cells in the crypts or on the M cells overlying Peyer's patches (Bleicher *et al.*, 1990). The other prominent

site of mouse CD1 expression detected in this study was the liver, in which prominent cytoplasmic staining of hepatocytes was observed. No staining was observed in brain, heart, skeletal muscle, kidney, lung, or esophagus, and minimal expression was observed on spleen cells. One study of CD1 expression in the rat using the same mAbs (i.e., 1H1 and 3C11, apparently cross-reactive with CD1 of Sprague–Dawley rats) showed a pattern of expression essentially identical to that of mouse (Burke *et al.*, 1994).

In contrast to the above-mentioned studies using immunohistochemical localization, biochemical studies have indicated a potentially wider range of tissue expression for mouse CD1 proteins. Thus, immunoblotting of tissue extracts with polyclonal antisera raised against a bacterial fusion protein corresponding to the MCD1D2 gene product showed expression of a group of polypeptides with molecular mass of 49–55 kDa (consistent with differentially glycosylated forms of the CD1d heavy chain) in thymus, spleen, liver, and lung, although not in brain, kidney, heart, or intestine of adult mice. Moreover, in fetal tissue samples, significant levels of CD1 protein were found at all of these anatomic sites. Interestingly, although CD1d protein was not noted in the intestinal sample by the authors of this study, the published reproduction of their Western blot does show a prominent band of approximately 100 kDa that is specific to this tissue (Mosser *et al.*, 1991). One intriguing possibility is that this could represent the nonglycosylated multimeric form of CD1d that has been identified on human intestinal epithelia (Balk *et al.*, 1994). To what extent any of these biochemically detected CD1 proteins are expressed on the surface of cells and the cell types responsible for their synthesis are at present largely unknown. However, flow cytometry shows surface staining of rat hepatocytes and transformed fetal intestinal cells with the 1H1 mAb (Burke *et al.*, 1994), indicating that these cells do express CD1d protein on the plasma membrane. In addition, proteins with molecular mass of 45–55 kDa have been specifically immunoprecipitated from ^{125}I surface-labeled rat splenocytes (Ichimiya *et al.*, 1994) and from mouse or rat fetal intestinal cell lines labeled for 4 hr with [^{35}S]methionine/cysteine (Burke *et al.*, 1994). Similar proteins were also identified in normal rat colonic epithelial cells and hepatocytes by Western blotting with the 1H1 mAb (Burke *et al.*, 1994).

The expression at the protein level of human group 2 CD1 (CD1d) has also been examined in a relatively small number of studies using the cross-reactive murine 1H1 and 3C11 mAbs in flow cytometry and immunohistochemistry (Blumberg *et al.*, 1991; Canchis *et al.*, 1993). These studies appear to confirm the intestinal epithelium as one of

the most prominent sites of expression of CD1d. Some subtle differences in the distribution of the staining were suggested compared to that reported for intestinal mouse CD1 (e.g., more staining in the crypts of microvilli in human than in mouse), but the basic pattern seems to be similar to that of the rodent intestine. Other prominent sites of tissue staining with the 1H1 mAb include kidney (including proximal and distal tubular epithelia), hepatocytes, and conjunctival epithelium. Overall, the tentative conclusions from these studies to date are that the CD1d protein is widely expressed in many tissues, but is preferentially found on certain cell types (i.e., epithelial cells, vascular smooth muscle of most tissues, and stromal and parenchymal cells of a few tissues) (Canchis *et al.*, 1993). Although most of these data are compatible with the reports on rodent CD1d, some caution should probably be exercised in interpreting the immunohistologic studies in human tissues. The mAbs used so far are both IgM antibodies that appear to be broadly cross-reactive with CD1d in several species, including the species in which the antibodies were produced (rat). Thus, the potential for additional cross-reactions with other tissue components may be substantial. However, limited biochemical analyses do suggest that, at least in some cases, the 3C11 and 1H1 mAbs recognize proteins on human cells that are biochemically and immunochemically similar or identical to human CD1d (Blumberg *et al.*, 1991; Balk *et al.*, 1994).

VII. The Immunological Function of CD1 Proteins

By reviewing the salient features of the structure and distribution of CD1 proteins and drawing attention to parallels with the MHC-encoded antigen-presenting molecules where appropriate, it is hoped that the foregoing sections have served to adequately set the stage for the following discussion of the function of CD1 proteins in cell-mediated immunity. Whereas the various aspects of CD1 structure and its regulated expression on specialized antigen-presenting cells certainly support the contention that these molecules mediate some form of antigen recognition by T lymphocytes, the recent functional studies that will now be reviewed provide the meat of the argument. As summarized in the section that follows, there is now clear evidence that the group 1 CD1 proteins are crucially involved in the specific recognition of self- and microbial antigens by a subset of T cells. Although the mechanism by which CD1 acts in this process is still not resolved at the molecular level, the available data point strongly to the conclusion that group 1 CD1 proteins do in fact present antigens

by a mechanism analogous to that which is used by MHC-encoded antigen-presenting molecules, although the chemical nature of the antigens presented may be surprisingly different than those handled by the MHC systems. Although less is known about the function of group 2 CD1 proteins at this time, some data have begun to emerge that also implicate them in a process of T cell-mediated specific recognition.

A. An Hypothesis: Nonclassical MHC Molecules and MHC-Related Molecules Present Antigen to Specialized T Cell Subsets

As our understanding of the phenotypic and functional properties of human and murine T cells has expanded over the past decade, it has become apparent that at least several distinct T cell subsets exist in addition to the relatively well understood classic $\alpha\beta$ TCR expressing $CD4^+$ (helper/inducer) and $CD8^+$ (cytotoxic/suppressor) subsets. Foremost among these at present are the cells expressing $\gamma\delta$ TCRs, many of which lack expression of CD4 and CD8 coreceptor molecules and are therefore referred to as double negative (DN) (reviewed in Porcelli *et al.*, 1991). In addition, a population of extrathymic, mature DN $\alpha\beta$ TCR bearing cells (DN$\alpha\beta$) has also been identified in both humans and mice (reviewed in Porcelli *et al.*, 1993; Takahama *et al.*, 1991). Finally, lymphocytes residing within epithelial sheets of various organs (so-called intraepithelial lymphocytes, or IEL) have also recently become the focus of intensive study as another distinctive T cell subset (Havran and Allison, 1991; Bonneville *et al.*, 1988; Jarry *et al.*, 1990). These novel T cell subsets generally represent minor populations in the circulation and specialized lymphoid organs, which probably explains why their existence was not appreciated in earlier studies of the cellular immune system. Nevertheless, they represent highly conserved components of vertebrate immune systems, and it is generally believed that they carry out important specialized functions that are largely unknown at this time.

Since all of the novel T cell subsets described above possess clonally variable TCRs, it is presumed that an important aspect of their function involves the specific recognition of self- or foreign antigens. Because of the distinctive structures of their TCRs which often show conserved or "canonical" features (Havran and Allison, 1991; Porcelli *et al.*, 1993; Dellabona *et al.*, 1993; Fowlkes *et al.*, 1987; Gross *et al.*, 1994; Regnault *et al.*, 1994), one reigning hypothesis has been that these cells may recognize a less structurally diverse group of antigens or

antigen-presenting molecules than $CD4^+$ or $CD8^+$ T cells (Janeway *et al.*, 1988; Strominger, 1989). The lack of CD4 and CD8 expression by many of these T cells (e.g., DN$\alpha\beta$ and DN$\gamma\delta$ T cells) also suggests that they may be less dependent on classical MHC molecules for antigen recognition, since these coreceptors are an important part of the MHC-restricted antigen-recognition process of conventional T cells. Thus, the nonpolymorphic nonclassical MHC class I molecules and non-MHC-encoded, MHC-related molecules (of which CD1 is the prototype) have come to center stage in the search for pathways of antigen recognition by these novel T cell subsets.

B. Specific Recognition of Group 1 CD1 Proteins by Human T Cells: A Novel Form of Autoreactivity

The first report linking specific T cell recognition to CD1 proteins demonstrated that both $\gamma\delta$ TCR^+ and DN $\alpha\beta$ TCR^+ T cell clones were capable of specific recognition of group 1 CD1 proteins (Porcelli *et al.*, 1989). In the course of examining the ability of randomly derived T cell clones from different donors to recognize and lyse a panel of tumor cell targets, two DN clones were identified that preferentially lysed only certain thymic leukemia cell lines. One of these clones, IDP2, is a DN $\gamma\delta$ TCR^+ cell isolated from the blood of an immunodeficiency patient (Brenner *et al.*, 1987). The other, BK6, is a DN $\alpha\beta$ TCR^+ T cell grown from the blood of a patient with systemic lupus erythematosus by mitogen (PHA) stimulation. On closer inspection, it became apparent that one feature of the preferred target cells that correlated with recognition by these T cell clones was their expression of group 1 CD proteins. Inhibition experiments with monoclonal antibodies specific for CD1a, -b, or -c showed that for one of the T cells (BK6) anti-CD1a mAbs were strongly inhibitory of target lysis, whereas for the other (IDP2), only anti-CD1c was inhibitory. The identity of the target structure as CD1a or CD1c was confirmed by expression of CD1a or CD1c in target cells that normally do not express these molecules (e.g., mouse L cells, mouse thymoma cells, or human rhabdomyosarcoma cells) by stable DNA-mediated transfection. Thus, for these T cell clones expression of the appropriate form of CD1 is both necessary and sufficient to confer specific target cell recognition by both T cell clones, proving that CD1a and -c are essential components of a pathway of T cell recognition used by certain DN T cell clones. This recognition is characterized by a high level of specificity since the related CD1a and CD1c proteins are completely discriminated by the two T cell clones, suggesting that the TCR is responsible for the recognition. This is further supported by the observation that the CD1-

mediated recognition is inhibited by mAbs specific for CD3 or for constant or variable region epitopes of the TCR (Porcelli *et al.*, 1989).

Subsequent studies have confirmed the existence of human T cells showing this direct recognition of specific group 1 CD1 proteins. In one study, 43 cloned and 11 polyclonal $\gamma\delta$ T cell lines grown from the blood of 19 different donors by nonspecific mitogen stimulation were examined for their ability to recognize and specifically lyse group 1 $CD1^+$ thymic leukemia cell lines. One T cell clone was found in this panel that showed CD1c-dependent target cell recognition, as indicated by specific blocking of the CTL response with anti-CD1c mAb (Faure *et al.*, 1990). Interestingly, this T cell clone expresses the same TCR Vδ1–Jδ1 rearrangement as does the CD1c-specific IDP2 $\gamma\delta$ T cell clone initially described, although the two clones differ in their TCRγ chain structure. T cells expressing Vδ1 are generally rare in the circulation of most normal humans, although they appear to be more prominent in lymphoid tissues. This sharing of TCRδ chain V and J segments suggests a correlation between TCR structure and recognition of CD1c, although this hypothesis has not yet been explored in a systematic fashion. Additional examples of DN $TCR\alpha\beta^+$ T cell clones specific for CD1c (Porcelli *et al.*, 1992) and for CD1a (Dellabona *et al.*, 1993) have also been reported, in both cases isolated by mitogen stimulation of circulating T cells without any deliberate selection for CD1 specificity. One example of a $CD8^+$ IEL T cell line showed apparently specific recognition of cells expressing one of several different CD1 proteins (CD1c > CD1b = CD1d > CD1a), and an IEL clone derived from this line showed significant recognition only of CD1c-transfected target cells (Balk *et al.*, 1991a).

The isolation of CD1-specific T cells in several different laboratories demonstrates the reproducibility of this phenomenon. It is also noteworthy that all of the T cell clones described in published reports to date were derived by nonspecific mitogen stimulation of isolated blood DN or intestinal lymphocytes without deliberate selection for CD1 recognition. This suggests that the frequency of T cells recognizing CD1 in these populations may be relatively high. In fact, preliminary analyses of precursor frequency confirm this suspicion, indicating a minimum precursor frequency of approximately 1 in 300 circulating DN$\alpha\beta$ T cells for recognition of CD1c (S. Porcelli, unpublished results). This frequency is actually comparable to that for alloreactive CTL precursors specific for individual alleles of MHC class I or II molecules (Kabelitz *et al.*, 1985). Although more extensive quantitative studies are needed to settle this point, the available data are supportive of the idea that group 1 CD1 proteins may be one major target structure

for specific recognition by circulating DN$\alpha\beta$ T cells. In addition, a role in specific recognition by some circulating $\gamma\delta$ T cells and possibly by intestinal IEL is also suggested by these initial reports.

The nature and significance of this initially observed form of specific recognition of CD1 by T cells is still unclear. Since CD1 appears to be recognized in these examples without any apparent structural variation or exogenous foreign antigen (E. Beckman and S. Porcelli, unpublished results), this would appear to be an instance of T cell activation by recognition of a normal self-protein. Thus, this form of recognition is perhaps most appropriately viewed as *autoreactivity* to CD1. As described previously, T cell clones showing autoreactivity to two of the group 1 CD1 proteins (i.e., CD1a and -c) have so far been identified. Ongoing studies in the author's laboratory indicate that autoreactivity to CD1c is a particularly prominent feature of circulating DN$\alpha\beta$ T cells, and that such T cells can be identified in virtually all normal blood donors. Such T cells may represent the DN counterpart of $CD4^+$ autoreactive T cells that recognize MHC class II proteins (Glimcher and Shevach, 1982), such as those that give rise to the autologous mixed lymphocyte response in humans. Presumably these CD1 autoreactive T cells, like their MHC class II-specific counterparts, are regulated *in vivo* in a way that prevents the expression of their effector functions under normal circumstances. However, once activated *in vitro* they are capable of mediating cytotoxicity against virtually any target cell expressing the appropriate form of CD1, and are also stimulated to produce cytokines by contact with their CD1 ligands (Porcelli *et al.*, 1989,1991; and additional unpublished data).

C. Restriction of T Cell Responses to Foreign Antigens by Group 1 CD1 Proteins

The discovery of T cell autoreactivity specific for CD1 provided the first direct functional parallel between CD1 proteins and MHC-encoded antigen-presenting molecules and set the stage for further studies to determine if CD1 could also restrict the responses of T cells to foreign antigens. This was undertaken directly by deriving DN T cell lines by repeated stimulation with microbial antigen preparations in the presence of monocytes treated with GM-CSF plus IL-4 to induce CD1 expression. Initial attempts using purified protein antigens (e.g., tetanus and diphtheria toxoids, and 65 or 70 kDa bacterial heat-shock proteins) did not result in outgrowth of antigen-specific T cells under these conditions (S. Porcelli, unpublished results). However, stimulation of isolated peripheral blood DN T cells with a crude sonicate of *Mycobacterium tuberculosis* and $CD1^+$ monocytes led to the deriva-

tion of a strongly antigen-responsive T cell line. This DN $\alpha\beta$ TCR$^+$ line (designated DN1), and T cell clones derived from it, proliferates upon addition of *M. tuberculosis* and sonicates only when CD1$^+$ monocytes are present in the culture. CD1$^+$ monocytes from any donor are able to stimulate the proliferative response to *M. tuberculosis*, indicating that the T cell/APC interaction is not restricted by polymorphic MHC antigen-presenting molecules. The proliferation is blocked completely by anti-CD1b mAbs, but not by mAbs to other CD1 or to MHC class I or II proteins. The apparent CD1b restriction of this response to *M. tuberculosis* antigen was confirmed using a series of transfectants of B lymphoblastoid cell lines expressing the different group 1 CD1 proteins. This showed that only transfectants expressing CD1b are lysed after exposure to the *M. tuberculosis* antigen (Porcelli *et al.*, 1992). Thus, CD1b is both necessary and sufficient to reconstitute antigen presentation by a CD1$^-$ target cell.

Further studies of the prototype CD1b-restricted bacterial antigen-specific DN1 T cell line have provided evidence for an antigen-processing step that precedes antigen recognition (Porcelli *et al.*, 1992). Antigen recognition is inhibited by prior aldehyde fixation of the antigen-presenting cells (APCs), indicating a possible requirement for internalization and surface reexpression of the antigen by the APC. Presentation of *M. tuberculosis* to DN1 is also inhibited by chloroquine and monensin (Porcelli *et al.*, 1992; and unpublished data), two agents known to disrupt the endosomal pathway of exogenous antigen processing generally associated with MHC class II-dependent antigen presentation (Margulies and Germain, 1993). To analyze the involvement of other components of the known pathways of antigen processing, CD1b transfectants of the antigen-processing mutant cell line T2 were studied. This cell line contains large homozygous deletions in both MHC loci, resulting in deletion of all functional MHC class II genes, the TAP-1 and TAP-2 peptide transporter genes required for endogenous peptide antigen presentation and MHC class I expression, and also the recently characterized HLA-DMA and -DMB genes that are now known to constitute part of the pathway of exogenous peptide antigen presentation (Riberdy and Cresswell, 1992; Cresswell, 1994; Morris *et al.*, 1994; Fling *et al.*, 1994). CD1b is well expressed after transfection of T2 cells, indicating that it is not critically dependent on TAP-1/2 function for its assembly and surface expression, as are MHC class I molecules. This observation is supported by the finding that group 1 CD1 proteins are normally expressed by GM-CSF-treated monocytes isolated from humans who carry homozygous mutations in their TAP-1 genes (de la Salle *et al.*, 1994). Most impor-

tantly, after exposure to *M. tuberculosis* antigen, CD1b-transfected T2 cells are efficiently recognized and lysed by DN1 cells. This indicates that the TAP-1/2 and DMA/DMB gene products, which are absent in these cells, are not required for processing and presentation of a CD1b-restricted antigen. Thus, the CD1 system appears to define a new pathway for presentation of exogenous antigens which shares some but not all of the features classically defined for the MHC class II pathway of exogenous peptide antigen presentation.

Additional attempts to derive CD1-restricted DN$\alpha\beta$ T cell lines using mycobacterial antigen preparations and CD1$^+$ monocytes as APCs have yielded an extended panel of CD1-restricted antigen-specific T cell lines (Table V; S. Porcelli, unpublished data). In some cases, these T cells actually have proven to be restricted by CD1c, although at least two additional examples of CD1b-restricted antigen-specific cell lines have been isolated. Interestingly, no examples of CD1a-restricted antigen recognition have yet been found. The reason for this is not known, but it is possible that CD1a presents antigens that are not contained in the bacterial extracts that have so far been tested, or that a different subset of T cells is involved. Alternatively, it may be that CD1a has evolved a distinctly different function than CD1b and -c, although their close structural relationships and coordinated tissue expression would seem to argue against this. All of the T cell lines listed in Table V respond by proliferating in the presence of the appropriate mycobacterial extracts and CD1$^+$ cytokine-activated monocytes and by cytolytic activity directed against CD1$^+$ target cells pulsed with the appropriate antigens. In each case, the restriction by CD1b or -c has been established both by specific anti-CD1 mAb blocking and by analysis of CTL activity against CD1b- and -c-transfected target cells (S. Porcelli, unpublished data). Interestingly, these T cells generally do not proliferate when antigen is presented to them by CD1$^+$-transfected B lymphoblastoid cell lines. This appears to be due to a novel costimulatory requirement for the induction of proliferation, which so far has been found only to be delivered by CD1$^+$ monocytes (S. Behar and S. Porcelli, manuscript in preparation). Another remarkable feature of these cell lines is the fine specificity of their antigen recognition. The majority of CD1-restricted T cell lines derived against mycobacterial antigens do not cross-react to any detectable extent between the closely related *M. tuberculosis* and *M. leprae* extracts, and none of the lines derived by stimulation with mycobacterial antigens respond to sonicates of Gram-negative (*Escherichia coli*) or Gram-positive (*S. aureus*) bacteria. Thus, these T cell responses are highly specific for the antigens against which they were

TABLE V

SPECIFICITY OF CD1-RESTRICTED T CELL LINES

T Cell Line	Source	Stimulating Antigen	Restriction	Antigen Recognition	
				M. tuberculosis	*M. leprae*
DN1	Normal blood	*M. tuberculosis*	CD1b	+ +	±
DN2	Normal blood	*M. tuberculosis*	CD1c	+ +	−
DN6	Normal blood	*M. tuberculosis*	CD1c	+ +	−
BDN2e	Normal blood	*M. leprae*	CD1c	−	+ +
BDN2w	Normal blood	*M. leprae*	CD1b	+	+ +
LDN1	Leprosy skin	*M. leprae*	CD1c	−	+
LDN4	Leprosy skin	*M. leprae*	CD1b	−	+ +

originally derived and restricted by discrete cell surface molecules on the APCs, which are the two hallmarks of specific T cell responses to antigen.

D. The Identification of CD1-Restricted Antigens

Given the demonstration of group 1 CD1 proteins as restriction elements for specific T cell responses to crude preparations of bacterial antigens, it has now become of crucial importance to identify the precise structures of the antigens that give rise to these responses. A first step in this direction has recently been accomplished with the identification of the general chemical structure of the *M. tuberculosis* antigen that is recognized by the prototype CD1b-restricted T cell line DN1. This antigen has now been shown to be a subset of mycolic acids, a class of complex and structurally variable mycobacterial lipids (Beckman *et al.*, 1994). This and other related findings now strongly suggest the remarkable conclusion that at least some CD1 proteins are antigen-presenting molecules that have evolved the function of presenting nonpeptide lipid antigens to T cells.

The identification of the antigen or antigens recognized by CD1b-restricted line DN1 as mycolic acid was initially accomplished by direct fractionation of mycobacteria and sequential purification of fractions that stimulated proliferation of the T cell line. After initial characterization revealed the antigen to be protease resistant, cell wall associated, and extremely hydrophobic, strategies were developed to specifically fractionate the lipids of *M. tuberculosis*. As shown in Fig. 13, the final step in this process involved separation of fatty acids and mycolic acids into homogeneous preparations by C18 reversed-phase high-performance liquid chromatography (RP-HPLC). In multiple such separations, all of the biologically active CD1b-restricted antigen was coeluted with the early portion of the complex absorbance peak that corresponds to purified mycolic acids. The identity of the antigen as mycolic acid was subsequently confirmed by demonstrating that homogeneous mycolic acid purified from a second source (i.e., previously purified mycobacterial cord factors, these being well-characterized mycobacteria-derived compounds that consist of the disaccharide trehalose with two covalently attached mycolic acids) could also stimulate the CD1b-restricted response of line DN1. In all cases, the responses observed to mycolic acids were restricted by CD1b molecules and were specific for DN1 since a variety of other T cell lines with different specificities did not respond to purified mycolic acids from either source (Beckman *et al.*, 1994). The identification of mycolic acid as a CD1b-restricted antigen may be the first demonstra-

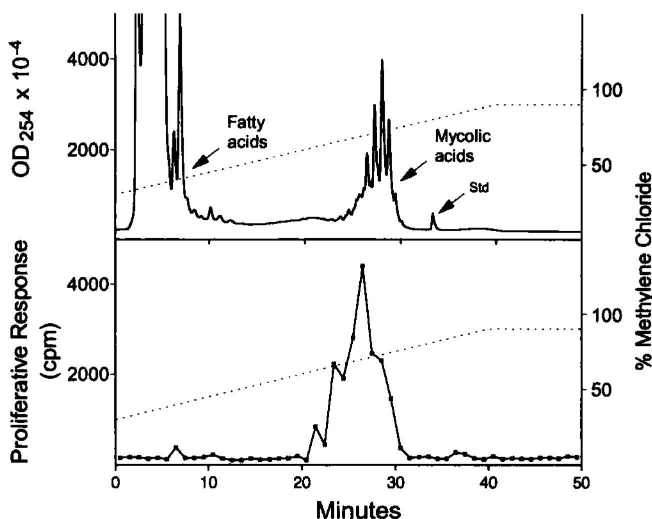

FIG. 13. Purification and identification of a CD1b-restricted mycobacterial antigen. (Top) C18 column reverse-phase HPLC that resolves a mycobacterial acyl chain preparation into the relatively short-chain fatty acids and the much longer-chain mycolic acids. (Bottom) the proliferative response of CD1b-restricted T cell line DN1 to each 1-min column fraction. Note that all of the antigen detected by the T cell assay elutes with the early portion of the complex mycolic acid peak. The dashed line represents the methylene chloride solvent gradient used to elute the column. Reproduced with permission from Beckman *et al.*, (1994).

tion of specific recognition of foreign lipid antigens by T cells and is another of a small but growing number of examples of naturally occurring nonpeptide ligands that appear to be specifically recognized by TCRs. Other recently identified examples of specific TCR-mediated recognition of nonpeptide antigens that have been well characterized include the recognition of small hydrophilic phosphorylated bacterial compounds by a major subset of human $\gamma\delta$ T cells (Constant *et al.*, 1994; Tanaka *et al.*, 1994). The manner in which these hydrophilic nonpeptide antigens are presented is not yet known, although it appears that the mechanism does not require group 1 CD1 molecules (C. Morita and S. Porcelli, unpublished data).

Although the mechanism of CD1b-restricted mycolic acid presentation and the nature of any antigen processing steps involved are still being worked out, the structure of mycolic acids indicates features that may relate to its antigenicity in the CD1-restricted system of cell-mediated immune recognition. The basic invariant structural features of these unusual lipids are shown schematically in Fig. 14. All mycolic

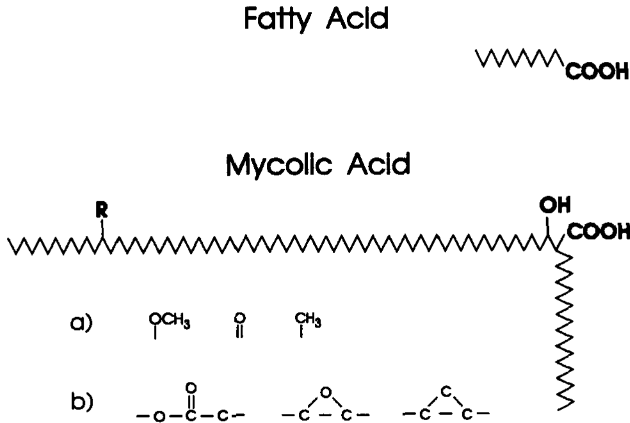

FIG. 14. Structure of a CD1b-restricted mycobacterial antigen. The basic structure of mycolic acids is shown and contrasted to the much simpler fatty acids. In a, three possible identities of the R group substitution are shown (i.e., keto, methoxy, and methyl substitutions). In b, three potential modifications of the bond structure of the long acyl chain are shown, including ester, epoxy, and cyclopropyl groups. In addition to these, variations in the number and position of double bonds, and in the length of the long acyl chain, are common.

acids have an α-branched, β-hydroxy structure at the free carboxyl group end, with an extremely long (generally C_{60} to C_{90}) main acyl chain. However, in addition to these invariant features, many potential and demonstrated possibilities for structural variation exist in these compounds. For example, most mycolic acids have at least one R group substitution on the long acyl chain, which can include keto, methoxy, epoxy, and methyl groups. In addition, variations in the main acyl chain length and number and position of modifications in bond structure (e.g., unsaturations and cyclopropyl groups) commonly occur (Goren and Brennan, 1982). These structural variations (especially the acyl chain length differences) account for the broad elution pattern of mycolic acids that is typically seen on RP-HPLC (Fig. 13). Although the mycolic acid antigen(s) recognized by the DN1 line have not yet been purified to homogeneity and structurally analyzed, it is already clear from preliminary results that not all mycolic acids of *M. tuberculosis* or other bacteria are recognized. Thus, it appears that the CD1-restricted system is able to discriminate fine structural variations in these lipids, which suggests a parallel with the ability of MHC class I- and class II-restricted T cells to distinguish patterns of protein variability as displayed by peptide antigens. Nevertheless, it should be pointed out that the level of structural variability of mycolic acids

is unlikely to be as great as the potential variability for octamer or nonamer peptides presented by MHC class I (up to 20^8 or 20^9 possible variations in theory) or the longer peptides presented by MHC class II proteins. Thus, mycolic acids would seem to satisfy the expectation that the nonpolymorphic CD1 proteins are involved in the presentation of a less structurally variable class of antigenic molecules than are presented by the polymorphic MHC molecules. As pointed out previously (Section III,E), the $\alpha 1$ and $\alpha 2$ domains of CD1 proteins are themselves unusually hydrophobic when compared to MHC proteins, which suggests that the extremely hydrophobic mycolic acid (or some portion of it) might be a suitable ligand for binding to a hydrophobic site formed by these domains.

Although the antigens recognized by other CD1-restricted T cells have not yet in most cases been precisely identified, the existence of other nonpeptide antigens distinct from mycolic acids is strongly suggested by preliminary studies. In fact, except for DN1, none of the DN$\alpha\beta$ T cell lines listed in Table V respond to purified acyl chain fractions (i.e., mycolic plus fatty acids), although most (and possibly all) appear to be specific for nonproteinaceous, hydrophobic or amphipathic bacterial antigens. Very recently, two of the T cell lines in this panel (LDN4 and BDN2w) have been shown to respond in a CD1b- or -c-restricted manner to purified lipoarabinomannan (LAM) of *M. leprae*, a well-characterized glycolipid structure of the mycobacterial cell wall. In this case, the lipid portion of the antigen appears to be necessary but not sufficient for CD1-restricted T cell recognition (P. Sieling, S. Porcelli, and R. Modlin, manuscript in preparation). Thus, it is not possible at this time to estimate how many different CD1-restricted antigens exist in mycobacteria, although it is clear that multiple structurally distinct antigens are present that can be recognized in the context of CD1b or CD1c. Mycolic acids and LAM are found mainly in the cell walls of mycobacteria and a few other related organisms and are consequently not widely distributed among microbial pathogens. Thus, the possibility that additional related or completely distinct CD1-restricted antigens are present in other microorganisms or even in eukaryotic cells remains entirely open to speculation.

An important lingering question left open by the findings described above is that of whether or not CD1 proteins under any conditions bind and present or in other ways restrict the recognition of peptide antigens. Our own studies have so far revealed no evidence for this. It appears that in the experimental system that we have developed and used, nonpeptide antigens are preferentially or exclusively recog-

nized. This is true despite the fact that the APCs and T cells are provided with a broad range of potential antigens during their stimulation, including the myriad of potential peptides that are present in crude mycobacterial sonicates. We therefore favor the hypothesis that, at least for T cell responses restricted by group 1 CD1 proteins, nonpeptide hydrophobic antigens (including the defined antigens mycolic acid and LAM) are preferentially or exclusively presented. In further support of this, attempts to isolate endogenous cellular peptides from large quantities (e.g., up to 3×10^{10} cell equivalents) of immunopurified CD1a protein from high-expressing transfected B lymphoblastoid cells have so far yielded no evidence of specifically bound peptides (M. DiBrino, J. Coligan, and S. Porcelli, unpublished data). Nevertheless, the possibility that peptides, especially hydrophobic peptides, might also be presented to CD1-restricted T cells remains a formal possibility that should not be discounted at this point. In fact, some recent studies of mouse CD1 proteins (i.e., group 2 CD1) have shown that they can bind certain peptides isolated from peptide display phage libraries. These peptides are typically hydrophobic and contain a sequence motif that includes two tryptophan residues (P. Peterson, personal communication). However, caution must be exercised in extrapolating these data to normal physiologic interactions, since certain peptides isolated by the screening of display libraries are known to bind to proteins that normally are not peptide-binding structures (e.g., lectins or avidin) (Devlin *et al.*, 1990).

E. The Mechanism of CD1 Restriction of T Cell Responses

The results summarized above demonstrate that one function for CD1 molecules is to restrict the responses of certain T cells to microbial antigens (Porcelli *et al.*, 1992). Clearly, the data are very consistent with CD1 actually presenting the identified nonpeptide antigens to T cells in a manner analogous to presentation by MHC class I and II. However, other models that could potentially account for the observed data are illustrated in Fig. 15. CD1 restriction as a result of a "coligand" interaction between the TCR and CD1 proteins (Fig. 15, left) is a formal possibility that could account both for the specificity of the response and for the restriction by a particular isotype of CD1. This model requires the existence of molecules on the APC (coligands) that interact specifically with certain TCR variable elements in a manner analogous to the binding of superantigens to the TCRβ chain (Janeway, 1992). However, endogenous TCR coligands of this sort have not been demonstrated to actually exist in other systems, and their existence

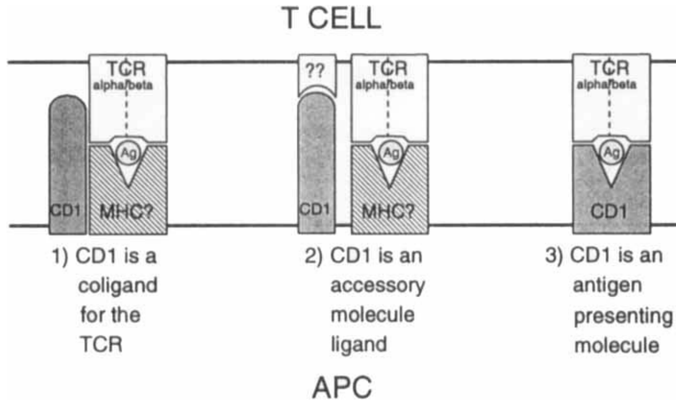

FIG. 15. Models for the mechanism of CD1-restricted antigen presentation. Three possible mechanisms to account for the observed CD1 restriction of responses to specific bacterial antigens are illustrated. See text for detailed explanation.

remains hypothetical at this time. In addition, this model would require that presentation be mediated by another unidentified molecule, and studies to date have essentially ruled out the participation of other known antigen-presenting molecules (Porcelli *et al.,* 1992). A mechanism by which CD1 functions as a ligand for putative counterreceptor or adhesion molecule on the T cell could also be considered (Fig. 15, center). However, such a model would have to propose the existence of a family of at least three such putative counterreceptors on DN$\alpha\beta$ T cells in order to account for the observed specific recognition of or restriction by CD1a, CD1b, or CD1c. This model would also require the involvement of another unidentified antigen-presenting molecule. Thus, the model that we currently favor is the simplest and does not require the involvement of any additional hypothetical elements (Fig. 15, right). This model is entirely analogous to the mechanism by which MHC-encoded antigen-presenting molecules function and proposes that CD1 proteins specifically bind foreign nonpeptide antigens to form a complex that is recognized by the TCRs of certain clonally distributed T cells. One obvious prediction of this model is that a direct binding interaction should be demonstrable between nonpeptide CD1-restricted antigens such as mycolic acid or LAM or fragments derived from these by antigen processing. This has not yet been shown, but methods to demonstrate such nonpeptide antigen-binding properties of CD1 proteins are now being developed.

F. The Function of Group 2 CD1 Proteins

Very little direct experimentation to discern the function of group 2 CD1 proteins (CD1d) has been reported, even in the mouse or rat where *in vivo* and *in vitro* manipulations should allow many questions to be readily assessed. Most attention has so far been directed on the gut lymphoid system because of the reported prominent expression of both mouse and human CD1d on intestinal epithelia. A widely circulating hypothesis has thus been that CD1d performs some role in the selection, activation, or immune recognition functions of intestinal intraepithelial lymphocytes (iIEL). However, to this date only very limited data have actually appeared that directly assess this idea. One $CD8^+$ iIEL polyclonal T cell line has been described that appears to weakly recognize target cells transfected with a human CD1d expression construct (up to about 18% lysis of $CD1d^+$ targets, as opposed to less than 8% for mock transfected targets). However, this line also lyses targets expressing CD1a, -b, or -c to an approximately equal extent, and the mechanism underlying the recognition has not been studied in detail (Balk *et al.*, 1991). It has also been observed that an anti-CD1d mAb (3C11) inhibits the proliferation of peripheral blood T cells that have been stimulated by freshly isolated allogeneic intestinal epithelial cells. This may suggest the involvement of CD1d in some form of activation pathway involving the recognition of molecules expressed on the surface of epithelial cells, but the mechanism of this effect has not been further investigated (Panja *et al.*, 1993).

As discussed earlier (see Sections V,C and V,D), CD1d appears to be expressed in two markedly different forms (i.e., with or without N-linked glycans and β2-microglobulin) (Balk *et al.*, 1994). The possibility that the two forms may carry out different functions in immune recognition has been suggested, but has not yet been assessed experimentally. Because of the location of group 2 CD1 proteins on epithelial cells, other roles for these molecules, such as the binding and transport of immunoglobulins or other molecules, have also been suggested (S. Balk and R. Blumberg, personal communications). A precedent for this usurpation of MHC-like structure for other functions separate from antigen presentation has already been provided by the FcRn protein (Burmeister *et al.*, 1994) and could prove true for these proteins as well. However, the demonstration that the closely related group 1 CD1 proteins are involved in specific T cell recognition and antigen presentation would seem to argue against this possibility. In addition, as alluded to above, some evidence has recently been obtained that mouse group 2 CD1 proteins may specifically bind certain peptides

of a defined sequence. It will obviously be of great interest to see if this peptide binding can be shown to correlate with CD1-restricted peptide recognition by mouse T cells *in vivo* and *in vitro*.

G. The Potential Immunologic Role of CD1-Restricted T Cells

There are as yet no *in vivo* data to address the question of what the importance of CD1-restricted T cells might be in terms of responses to specific immunological challenges or the overall maintenance of immunity. Based on the conservation of CD1 genes and proteins in all mammals so far examined, it appears that CD1 probably serves a function that is important to survival and maintenance of the breeding potential of these species. However, some questions are raised by the lack of group 1 CD1 proteins in rodents. This may suggest that CD1b- or CD1c-restricted responses to bacterial antigens are not crucial to mounting effective immune responses to bacterial pathogens, having been largely superseded by other mechanisms such as MHC-restricted antigen recognition. Alternatively, it may be that rodents are unique in having evolved or retained other mechanisms that make the group 1 CD1-restricted responses superfluous. It will be interesting to see if future studies reveal another system of antigen-presenting molecules in rodents that parallel the group 1 CD1 proteins in other mammals in terms of tissue expression and function. Although such a role was previously suggested for the mouse TL antigens (Bradbury *et al.*, 1990), this now seems unlikely given the marked divergence of CD1 both in structure and in function from the peptide-binding classical and nonclassical MHC class I proteins.

So far, the available data suggest that CD1 is required for responses to nonprotein, lipid-containing antigens of mycobacteria. This at least suggests the possibility that these responses play a role in immune resistance and containment of mycobacterial infections. For some mammals (especially humans), the challenge posed to the immune system by mycobacterial species is indeed formidable, as infection with these organisms (e.g., *M. tuberculosis* and *M. leprae*) accounts for extraordinary amounts of morbidity and motality. One intriguing hypothesis relating the presentation of lipid-containing antigens by CD1 to mycobacterial infections pertains to the manner in which these organisms are able to sequester themselves within macrophages in modified endosomes. In these compartments, mycobacteria may in some cases successfully avoid immune detection by sequestering their antigens away from the classical pathways of exogenous (class II-restricted) and en-

dogenous (class I-restricted) antigen presentation. However, recently it has been shown that the membrane defining such modified endosomes undergoes exchange with other membranes within the cell as well as with the plasma membrane, and that this membrane exchange is accompanied by the escape of lipid-containing mycobacterial antigens (e.g., LAM) into other compartments within the endosomal network (Xu *et al.*, 1994). This suggests that lipid-containing antigens, such as LAM or mycolic acid, may be efficiently carried out of these vacuoles containing sequestered viable mycobacteria and subsequently delivered to other compartments in which antigen processing and association with CD1 proteins may occur. This would result in antigen recognition by CD1-restricted T cells, leading to lysis of the infected cell, granuloma formation, or other beneficial cellular responses. Such a mechanism would appear to fit well with the recently demonstrated inducibility of group 1 CD1 proteins on monocytes and macrophages, since macrophages are the favored cell for the growth and persistence of many intracellular bacterial pathogens including mycobacteria. It should also be pointed out that antigens from pathogens other than mycobacteria have not yet been specifically examined for presentation by the CD1 system. Thus, it remains very possible that CD1 may have a role in immunity to a much broader range of specific pathogens than has so far been suggested by direct observations.

Finally, it is important to note that the distribution of CD1-restricted T cells within different phenotypic subsets of T cells has not yet been carefully defined. Whereas our studies have focused until now on circulating DN$\alpha\beta$ T cells, it is possible that T cells belonging to other phenotypic subsets or in other anatomic sites will also prove to show significant levels of CD1 restriction. In fact, preliminary studies now show that CD1b restriction of circulating DN$\gamma\delta$ and CD8$^+\alpha\beta$ T cell responses to mycobacterial lipids can also be demonstrated (J.P. Rosat and M. Brenner, pesonal communication). Other T cell subsets, including the intraepithelial lymphocytes of the gut and skin and the expanded circulating DN$\alpha\beta$ T cells seen in some patients with autoimmune diseases, should also be investigated with regard to the role of CD1 molecules in their immunologic function.

VIII. Concluding Remarks

Largely as a result of the abundance of structural information on CD1, it has recently been possible to demonstrate a function for at least some of the molecules in this family. Thus, it now appears very

likely that group 1 CD1 proteins are antigen-presenting molecules in the same sense as the classical MHC class I and II molecules. Although it seems clear that MHC-restricted T cells are predominant in most immune responses, it still remains possible that CD1-restricted responses may take on a substantial importance in certain anatomic sites or in the setting of specific diseases or infections. This possibility is emphasized by the recognition that CD1 differs from MHC-encoded antigen-presenting molecules in a number of ways. These include their patterns of cellular and tissue expression, the predicted structures of their membrane distal domains, and perhaps most importantly the chemical nature of the antigens presented. Therefore, an appreciation of how the CD1 system contributes to immunity may well enhance our ability to manipulate the immune system in predictable and useful ways. In addition, the demonstration of a third lineage of antigen-presenting molecules separate from those encoded by the MHC should have significant implications for our understanding of how the immune system has evolved to its present awesome state of complexity and extraordinary efficiency.

Acknowledgments

I gratefully acknowledge many valuable discussions and collaborations with Drs. Evan Beckman, Michael Brenner, Craig Morita, Sam Behar, Steve Furlong, Masahiko Sugita, Augustin Melian, Jean Pierre Rosat, Peter Sieling, Robert Modlin, Steven Balk, and Richard Blumberg. I especially thank Dr. E. Beckman for contributing many new ideas on the proposed structure–function relationships of CD1 proteins in the binding of lipid antigens, and for helping to prepare some of the illustrations that appear in this chapter. I also thank Catherine Morlino for assistance in preparing the tables for this chapter. This work was supported by Clinical Investigator Award AR01854 from the NIAMS/NIH.

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This article was accepted for publication on 20 January 1995.

ADVANCES IN IMMUNOLOGY, VOL. 59

Positive Selection of Thymocytes

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I. Introduction

There are many things wrong with the hypothesis to explain the somatic generation of the antigen receptor repertoire in lymphocytes that was put forward by Niels Jerne in 1969 and published in 1971 (Jerne, 1971). But in that document is a revolutionary idea: Jerne proposed that germline genes for lymphocyte receptors encode structures that recognize the set of highly polymorphic major histocompatibility antigens of the species. According to the Jerne hypothesis, the subset of newly formed thymocytes which score as anti-self-major histocompatability complex (MHC) would be driven to proliferate, accumulating somatic mutations in the receptor genes. In some cases this would result in the loss of self-reactivity of the selected thymocytes, which would then be allowed to populate the peripheral lymphoid organs and respond to conventional foreign antigen using the mutated anti-self-receptor. In an MHC^A animal, a different subset of clonally expressed germline-encoded receptors would be selected compared to those selected in an MHC^B individual. To this day there is little evidence that T cell receptor genes undergo mutation after expression on the cell surface. Yet the idea that the self-MHC antigens

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expressed in the thymus select the T cell receptor (TCR) repertoire is correct and is what we now call "positive selection."

Jerne did not originally distinguish B and T lymphocyte compartments or receptor genes; in those days the T cell receptor for antigen was unknown, and the phenomenon of MHC restriction of T cell recognition had not yet been discovered. There is no evidence that immature B cells expressing surface immunoglobulin are subject to any requirement for self-recognition to promote their differentiation. The rationale for the requirement in T cells comes from the way in which T cells recognize foreignness. The discovery in the early 1970s of the phenomenon of the MHC restriction of T cell recognition provided great insight into T cell physiology. Twenty years later, we know that T cells recognize short peptide fragments of foreign antigens tightly bound in a groove on the MHC-encoded class I and class II molecules. More than a dozen crystal structures of MHC plus peptide have been solved, showing that some peptide side chains interact largely with the MHC molecule and serve as anchors, while other peptide side chains are accessible to solvent and presumably to the TCR. These recent crystal structures, such as the one presented in Fig. 1, give us a molecular picture of what MHC-restricted recognition means. At present there are no crystal structures of the $\alpha\beta$ TCR but there is good reason to picture it as an immunoglobulin-like structure which overlays its complex ligand, making contacts with MHC and peptide components. MHC polymorphism, expressed as substitutions in the groove resulting in differences in peptide binding specificity, was probably selected because it ensured that a portion of any pathogen would be presented to T cells. While an unselected, germline TCR repertoire could manifest reactivity to MHC/peptide complexes in any individual, it obviously became advantageous to preselect this repertoire for some reactivity to self-MHC (plus self-peptides) in the absence of foreign antigen. This self-reactivity is the driving force behind the positive selection of thymocytes. As we shall see, the repertoire positively selected on MHC^A plus self-peptides has a much higher frequency of reactivity to foreign antigen plus MHC^A than to foreign antigen presented by MHC^B. Unlike Jerne's suggestion, positively selected thymocytes are not driven to proliferate, and their receptors do not mutate away from self-recognition. Instead, cells are rescued from programmed cell death on the basis of self-recognition and go on to serve in the periphery as self-tolerant T cells. This different response in immature versus mature T cells to recognition of antigen through an unmutated TCR may be controlled by poorly understood changes in the triggerability of the cells.

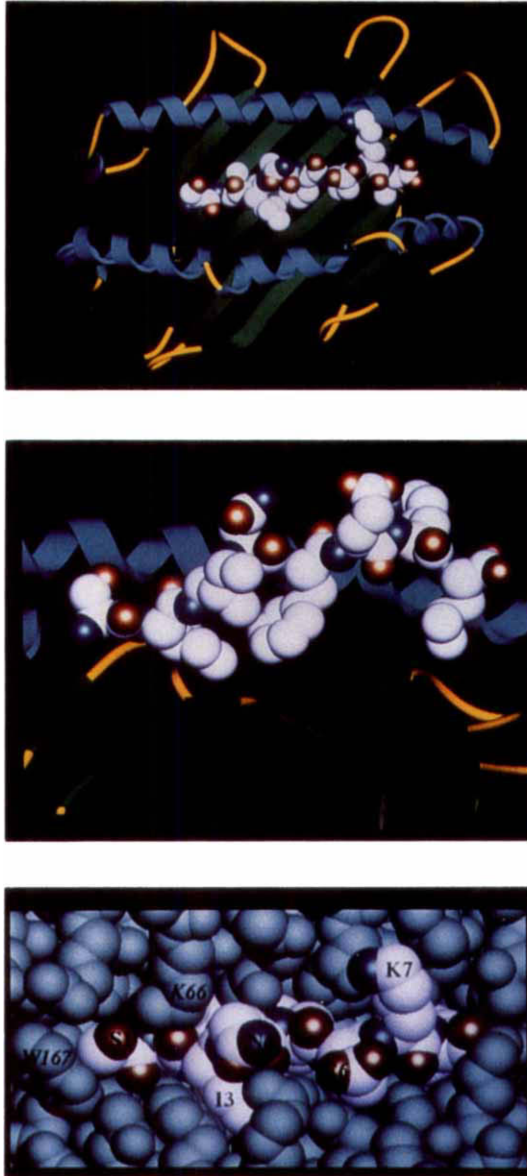


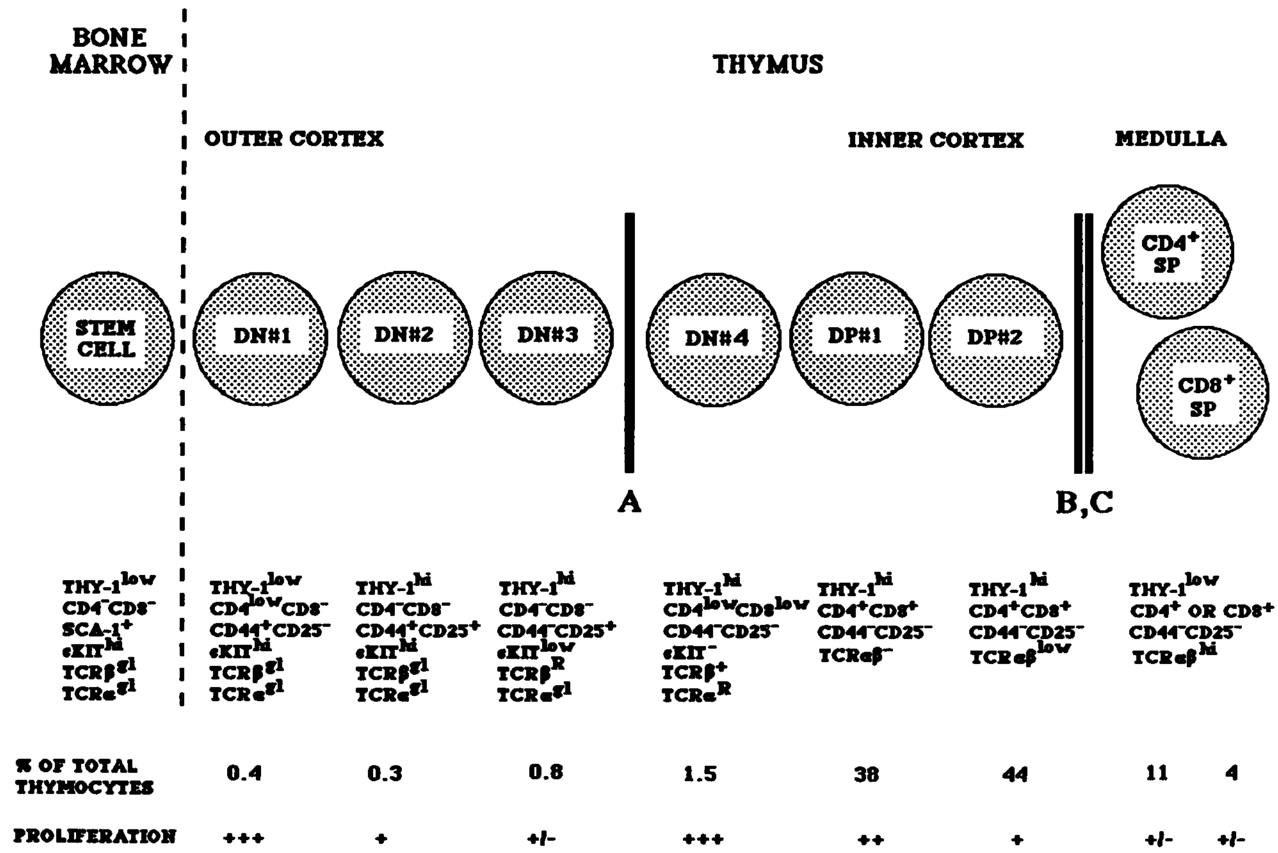
FIG. 1 The ligand for MHC-restricted T cell recognition. Three views of the interaction of the murine MHC class I molecule, H-$2K^b$, with an antigenic peptide, SIINFEKL, derived from chicken ovalbumin. (Top) The octameric ovalbumin peptide with the amino-terminus at the left lies in an extended configuration in the peptide-binding groove of H-$2K^b$; A top view with the class I $\alpha1$ helix at the top and the $\alpha2$ helix at the bottom. (Middle) A side view with the $\alpha2$ helix stripped away. (Bottom) A space-filling model viewed from the top. The side chains of peptide residues S1, N4, E6, and K7 point up, out of the MHC groove. Residues F5 and L8 fit in constrained pockets of the groove (for further details see Fremont *et al.,* 1995).

In this chapter on the nature of the self-recognition event that promotes maturation of the $\alpha\beta$ TCR^+ lineage of thymocytes, we provide a brief overview of T cell development, describe the experiments that demonstrate positive selection, and discuss recent work on the nature of the self-ligand that is recognized in the thymus.

II. An Overview of $\alpha\beta$ TCR^+ Thymocyte Development

The 100 to 1000 bone marrow-derived stem cells that daily enter the thymus of a young adult mouse begin the approximately 3 week long process of differentiation that results in functional, self-MHC-restricted, self-tolerant T cells (Fowlkes and Pardoll, 1989; Shortman *et al.*, 1990). As illustrated in Fig. 2, the various stages of intrathymic development can be monitored by the status of the TCRα and -β chain genes and the cell surface expression of molecules such as CD44 (Pgp-1), CD25 (the α-chain of the IL-2 receptor), Thy-1, and the TCR and its CD4 and CD8 coreceptors (Nikolíc-Zugíc, 1991; Godfrey and Zlotnik, 1993). Thymocytes can be divided into three main categories based on their expression of CD4 and CD8 molecules. Double-negative or "DN" thymocytes are $CD4^-CD8^-$ (or low), double-positive or "DP" thymocytes are $CD4^+CD8^+$, and single-positive or "SP" thymocytes are either $CD4^+CD8^-$ or $CD4^-CD8^+$. The bulk (approximately 80%) of thymocytes are DP, while DN cells comprise 3–5% and SP comprise 15% of the total lymphoid compartment in a young adult thymus.

The lineage relationships between the myriad of intrathymic subpopulations have been established by analyses of the progeny of highly purified cells injected intravenously or directly intrathymically into genetically marked recipients. These experiments have revealed that as maturation progresses, thymocytes generally proceed in an orderly fashion from DN to DP to SP compartments (Fowlkes *et al.*, 1985; Shimonkevitz *et al.*, 1987; Scollay *et al.*, 1988; Guidos *et al.*, 1989). The differentiation of the principal $\alpha\beta$ TCR^+ lineage of thymocytes follows a generally centripedal pattern, beginning with the least mature DN cells in the outer rim of the thymic cortex and ending with functionally and phenotypically mature SP cells in the medulla (Sprent and Webb, 1987). Although the most recent thymic immigrants express low levels of CD4, they are operationally defined as DN because they are resistant to complement-mediated lysis in the presence of anti-CD4 and anti-CD8 antibodies. They also express CD44, and the hyaluronic acid-binding function of that glycoprotein may facilitate the homing of the lymphoid stem cell to the thymus (Lesley *et al.*, 1985). These stem cells progress to the stage we have designated DN 2 when they


BONE MARROW
THYMUS
OUTER CORTEX
INNER CORTEX
MEDULLA
STEM CELL
DN#1
DN#2
DN#3
DN#4
DP#1
DP#2
CD4^{+} SP
CD8^{+} SP
A
B,C
THY-1^{low} CD4^{-}CD8^{-} SCA-1^{+} cKIT^{hi} TCRβ^{gl} TCRα^{gl}
THY-1^{low} CD4^{low}CD8^{-} CD44^{+}CD25^{-} cKIT^{hi} TCRβ^{gl} TCRα^{gl}
THY-1^{hi} CD4^{-}CD8^{-} CD44^{+}CD25^{+} cKIT^{hi} TCRβ^{gl} TCRα^{gl}
THY-1^{hi} CD4^{-}CD8^{-} CD44^{-}CD25^{+} cKIT^{low} TCRβ^{R} TCRα^{gl}
THY-1^{hi} CD4^{low}CD8^{low} CD44^{-}CD25^{-} cKIT^{-} TCRβ^{+} TCRα^{R}
THY-1^{hi} CD4^{+}CD8^{+} CD44^{-}CD25^{-} TCRαβ^{-}
THY-1^{hi} CD4^{+}CD8^{+} CD44^{-}CD25^{-} TCRαβ^{low}
THY-1^{low} CD4^{+} OR CD8^{+} CD44^{-}CD25^{-} TCRαβ^{hi}
% OF TOTAL THYMOCYTES
0.4
0.3
0.8
1.5
38
44
11
4
PROLIFERATION
+++
+
+/-
+++
++
+
+/-
+/-

become committed to the T cell lineage, lose CD4 expression, and become $CD25^+$ (Fig. 2). Rearrangement of the TCRβ chain gene begins at the DN 3 stage, also characterized as $CD44^-CD25^+$, while α-chain gene rearrangement follows in DN 4, the most mature (operationally defined) DN compartment (reviewed in Godfrey and Zlotnik, 1993). Surface expression of CD4 and CD8 molecules is initated in the DN 4 compartment (and is in fact triggered by productively rearranged TCRβ chain genes, see below) and increases until the cells are readily defined as DP. The populous DP compartment of thymocytes can be subdivided into two roughly equal categories, those that do and those that do not express cell surface $\alpha\beta$ TCR molecules. The former subpopulation of DPs has a 3 or 4 day life span in the thymus, either dying within this time frame or giving rise to SP thymocytes that express levels of $\alpha\beta$ TCR molecules comparable to those expressed by mature peripheral T cells (Shortman *et al.*, 1990).

The progression from DN to DP to SP compartments is accompanied by at least two episodes of discontinuous clonal expansion and is, upon cursory inspection, an extremely wasteful process (Shortman *et al.*, 1990). Although most of the SP thymocytes are exported to the peripheral lymphoid organs at a rate of approximately 2×10^6/day, greater than 90% of the total cells generated in the thymus die there by apoptosis and are rapidly engulfed by macrophages (Surh and Sprent, 1994). This cycle of lymphopoiesis and cell death is "wasteful" in appearance only, for as most readers are aware, the selective forces that initiate this massive cell death are those that help mold the TCR repertoire into one that is maximally useful for the animal (reviewed in Nikolíc-Zugíc, 1991; Godfrey and Zlotnik, 1993; von Boehmer, 1994; Janeway, 1994; Robey and Fowlkes, 1994, Jameson *et al.*, 1995).

We emphasize three main control points in thymocyte maturation.

FIG. 2. Schematic diagram of the intrathymic maturation of $\alpha\beta$ TCR^+ T cells in young adult mice. The cells are organized from the least mature bone marrow-derived stem cell on the left of the figure to the most mature single positive thymocytes on the right. Only the principal cell types are illustrated; transitional populations, such as the TCR^{hi} $CD4^{low}CD8^+$ and $CD4^+CD8^{low}$ cells, are excluded for simplicity. The solid black lines represent the control points discussed in the text. A refers to the control of TCRβ chain gene rearrangement, thymocyte proliferation, and the initiation of CD4 and CD8 expression that is associated with productive TCRβ chain gene rearrangement. B and C refer to positive and negative selection of $\alpha\beta$ TCR^+ thymocytes that serve to mold the self-MHC-restricted, self-tolerant TCR repertoire. The germline configuration of the TCR genes is denoted gl, and genes undergoing somatic rearrangement are marked with the superscript R.

TCR-β chain expression plays a critical role in the first major control point (barrier A in Fig. 2) at a developmental stage that precedes TCRα chain gene rearrangement and expression. It is at this juncture that cells terminate TCRβ chain gene rearrangement, transit from the DN to the DP compartment, and begin clonal expansion. Several lines of experimentation have demonstrated that productive TCRβ chain gene rearrangement is required to traverse this barrier. The thymuses of mice genetically lacking TCRβ chain genes are very small and contain thymocytes arrested at the DN 3 stage (Mombaerts *et al.*, 1992). The introduction of a productively rearranged TCRβ chain gene into these mice results in thymuses of nearly normal cellularity and permits the initiation of both CD4 and CD8 expression (Kishi *et al.*, 1991; Shinkai *et al.*, 1993). Furthermore, the fact that there is selection for cells carrying productive rearrangements of TCRβ chain genes *prior* to the rearrangement of TCRα chain genes strongly suggests that β-chain protein is a key factor in early T cell differentiation (Dudley *et al.*, 1994). The biochemical basis for this selection is currently being unraveled. Recent evidence demonstrates the surface expression on immature thymocytes of TCRβ chains disulfide-bonded to a 33-kDa glycoprotein designated pre-TCRα or pTα (Groettrup and von Boehmer, 1993; Saint-Ruf *et al.*, 1994). Although pTα genes are not somatically rearranged, their expression is under tight developmental control. In analogy to the expression of λ5 and $V_{pre}B$ that serve as substitute immunoglobulin light chains during early B cell development (reviewed in Melchers *et al.*,1993), it is likely that pTα serves as a surrogate TCRα chain, enabling the surface expression of TCRβ chains prior to the appearance of their TCRα chain partners. Crosslinking of TCRβ chains or CD3ε molecules (to which the pre-TCR is tightly associated) on immature thymocytes induces mobilization of intracellular stores of Ca^{2+}, indicating that the pre-TCR can transduce signals (Groettrup *et al.*,1992; Groettrup and von Boehmer, 1993). The subunit responsible for this signal transduction is likely to be the pTα chain, whose cytoplasmic tail contains two potential phosphorylation sites and a Src homology sequence (Saint-Ruf *et al.*, 1994). The signaling seems to also require $p56^{lck}$, since thymocyte maturation to the DP stage is blocked in mice deficient in $p56^{lck}$ and overexpression of $p56^{lck}$ kinase activity drives the DN to DP transition in thymocytes that lack a productive TCRβ chain gene rearrangement (Molina *et al.*, 1992; Levin *et al.*, 1993; Mombaerts *et al.*, 1994). What is the nature of the interaction involving the pre-TCR that results in the transduction of a maturational signal? Maturation has been shown to proceed past this first control point even in the absence of a TCR Vβ domain, leading

to speculation that *cell surface* expression of the pre-TCR complex may not be required for the DN to DP transition (Krimpenfort *et al.*, 1989; von Boehmer, 1992; Palmer *et al.*, 1993; Jacobs *et al.*, 1994).

The second two principal selective events in the thymus, labeled B and C in Fig. 2, are positive and negative selection. These two control points require the surface expression of both TCRα and -β chains and are initiated by the interaction between the $\alpha\beta$ TCR and an intrathymic ligand. This interaction during positive selection leads to the termination of TCRα chain gene rearrangement brought about by the extinction of RAG-1 and RAG-2 gene expression (Turka *et al.*, 1991; Brandle *et al.*, 1994). Positive selection also results in the rescue of thymocytes from programmed cell death and initiates the transition of thymocytes from the DP to SP compartments (reviewed in Fowlkes and Pardoll, 1989; Nikolíc-Zugíc, 1991; Godfrey and Zlotnik, 1993; von Boehmer, 1994; Janeway, 1994; Robey and Fowlkes, 1994; Jameson *et al.*, 1995). Thymuses from mice deficient in TCRα chain gene expression exhibit nearly normal cellularity and are filled with DP cells (Mombaerts *et al.*, 1992; Philpott *et al.*, 1992). Negative selection is responsible for the induction of self-tolerance through the clonal elimination of thymocytes whose TCRs recognize self-MHC (filled with self-peptides) with an inappropriately high affinity or avidity (reviewed in Sprent and Webb, 1987; Blackman *et al.*, 1990; von Boehmer, 1992; Janeway, 1994; Robey and Fowlkes, 1994). Negative selection can occur at any time in the life history of an immature thymocyte once it expresses an $\alpha\beta$ TCR at the cell surface. Thus, if the deleting self-antigen is expressed in the thymic cortex, clonal elimination can remove cells at an early developmental stage, whereas if thymic expression of the deleting antigen is limited to the medulla, clonal elimination spares the DP stage and operates at the DP to SP transition (Blackman *et al.*, 1990; von Boehmer, 1992; Janeway, 1994; Robey and Fowlkes, 1994). While some thymocytes may never succeed in expressing a TCR on the cell surface, the vast majority of cells that die do so after surface expression of a TCR and upon failure to traverse the barriers imposed by positive and negative selection (Surh and Sprent, 1994).

III. Experimental Systems for Analyzing Positive Selection

We begin our detailed examination of the evidence for intrathymic positive selection with a brief summary of some of the experimental systems employed over the years to expand our understanding of T cell development.

A. Radiation Bone Marrow Chimeras

The initial discovery of positive selection resulted from the experimental manipulation of the MHC restriction specificity of T cells of a given genotype (Bevan, 1977). This separation of the thymocyte's genotype and functional phenotype resulted from allowing hematopoietic stem cells of one MHC genotype to mature within the thymus of a second MHC genotype. This artificial situation was engineered by reconstitution of the hematopoietic system of a lethally irradiated host animal with donor-derived bone marrow cells depleted of mature T cells. High doses of irradiation destroy both the stem cell and mature cell compartments of the host's lymphoid system, enabling efficient engraftment by injected donor cells and ensuring the establishment of lymphoid chimerism (van Bekkum and de Vries, 1967). Such radiation chimeras can be further manipulated by thymectomy and surgical implantation of lymphocyte-free thymic tissue of any desired genotype. In such thymic chimeras, stem cells of one genotype can be forced to differentiate into mature T cells in the thymus of another genotype (Fink and Bevan, 1978; Zinkernagel *et al.*, 1978a,b). The advantage of such a procedure is its technical simplicity and the rapidity with which "mix and match" experiments using various MHC-different recipients, thymus donors, and bone marrow donors can be conducted. The disadvantages include the possible contamination of the donated thymic tissue or bone marrow preparation with mature lymphocytes, potential differences in the relative radiation sensitivity of lymphocytes, antigen-presenting cells, and thymic epithelial tissue, nonimmune resistance to allogeneic stem cell engraftment in some donor–host combinations, and the widespread radiation damage apparent even in the fully reconstituted host animal.

B. Acute Negative Selection

In contrast to the construction of radiation bone marrow chimeras, in which the developmental environment of a thymocyte is experimentally altered, procedures using acute negative selection center on the removal of alloreactive T cells from the mature, peripheral pool of lymphocytes, thereby allowing inspection of MHC restriction specificity in the absence of an otherwise overwhelming response to allogeneic MHC molecules. Acute negative selection can be achieved through both the *in vivo* and the *in vitro* means described below.

1. Biological Filtration

In this system, a recipient animal of one genotype is used as an "affinity column" for mature lymphocytes from a genetically different

donor animal. The recipient animal is irradiated, marked donor cells are injected, and the recirculating fraction of the donor cells is collected by cannulation of the recipient's thoracic duct (Ford and Atkins, 1971). If the donor cells are collected within 4 days after injection, the recirculating population will be specifically deleted for those donor lymphocytes that can recognize recipient alloantigens. The cause of this negative selection is the retention at the site of antigen recognition of antigen-reactive donor cells, a process that temporarily pulls them from the pool of recirculating lymphocytes (Sprent and Miller, 1976). The technical difficulty of thoracic duct cannulation in mice is the main drawback to this procedure.

2. *BrdU and Light-Induced Suicide*

In this *in vitro* system for inducing acute negative selection, proliferating cells in a mixed lymphocyte culture are first labeled with the thymidine analog bromodeoxyuridine. The cells are then exposed to light, the BrdU-containing DNA is fragmented, and the cells are killed (Zoschke and Bach, 1970). In this way, cultures can be specifically depleted of cells that respond to the antigens of choice. This technique requires a delicate balancing act between efficiency (removal of all antigen-reactive cells) and specificity (removal exclusively of antigen-reactive cells).

C. TCR Transgenic Mice

The study of T cell maturation has been revolutionized by the direct genetic alteration of strains of mice through the random chromosomal incorporation of DNA injected into embryos prior to their first cell division. The embryos are transplanted into the oviducts of pseudopregnant females who may carry these manipulated embryos to term (reviewed in Jaenisch, 1988). If DNA encoding productively rearranged TCR genes under the control of endogenous promoters and enhancers is successfully incorporated into the germline of recipient animals, the processes of allelic exclusion of TCRα and -β chain genes ensures that the T cells maturing in this new mouse strain will express the TCR transgenes to the near exclusion of endogenous TCR genes. This ability to create mice with nearly monoclonal T cells has greatly simplified the study of positive selection, shifting it from an analysis of MHC restriction specificity among properly tolerized T cells to a straightforward analysis of surface antigen phenotype. Complex TCR repertoire analyses can be replaced by using anti-idiotypic antibodies to follow cells expressing a single well-characterized TCR of known antigen and MHC specificity. There are also risks inherent in applying what we learn from analyzing thymic selection of cells expressing a

single type of TCR to the unmanipulated thymus in which heterogenous populations of lymphocytes mature. We know that the levels and timing of the intrathymic expression of TCR transgenes can differ greatly from those in nontransgenic mice, and these differences may alter some of the details of intrathymic maturation. The avidity of the particular TCR for its complex ligand and the anatomic location and level of expression of the positively and negatively selecting peptides are all characteristics predicted to influence either the timing or the efficiency of positive selection.

D. Gene Inactivation by Homologous Recombination

Our current understanding of the role played in intrathymic maturation by the interaction between TCR, coreceptor, and the MHC/peptide complex is indebted to technology that permits the targeted inactivation of genes encoding these various components. The gene encoding the protein of interest is first disrupted *in vitro*, usually by the deletion of particular nucleotide sequences, and then inserted into a targeting vector that permits selection for homologous recombination events. The inactivated gene is next introduced into a line of pluripotent murine embryonic stem cells, and cells are selected in which the disrupted gene has integrated into the DNA by virtue of homologous recombination, thereby replacing the endogenous gene on one of the two homologous chromosomes. The stem cells are injected into the blastocysts of embryos that are subsequently implanted and (hopefully) carried to term. The injected stem cells will contribute to some but not all tissues in any given embryo, so the mice that develop from these manipulated embryos will be mosaics and heterozygous for the disruption. Successful germline transmission and matings between heterozygotes enable the establishment of novel lines of genetically deficient "knockout" mice (reviewed in Frohman and Martin, 1989). These mice, although technically very challenging to engineer, have unequivocally established the role in positive selection for each of the four categories of interacting molecules—MHC, TCR, peptides, and coreceptors.

E. *In Vitro* Systems

1. *Fetal Thymus Organ Culture (FTOC)*

Fetal thymic rudiments maintained in tissue culture were the first *in vitro* system capable of supporting thymocyte maturation. In this system, fetal thymuses are explanted at Days 14–16 of gestation and maintained in culture at an air/liquid interphase on polycarbonate filters floating on a gelatin foam sponge (Jenkinson and Ander-

son, 1994). Thymocyte maturation proceeds with roughly the same kinetics as that observed in intact embryos. The lymphocytes and dendritic cells contained within explanted thymic rudiments can be easily eliminated by an initial period of culture in the presence of 2-deoxyguanosine. The thymic microenvironment can be reconstructed by seeding the remaining epithelial shell with genetically distinct stem cells or thymocyte subpopulations to create chimeric thymuses (Jenkinson and Anderson, 1994). Although relatively small numbers of maturing thymocytes can be obtained from this *in vitro* technique, the small volume of these cultures and the accessibility of the developing thymocytes to experimental manipulations, such as antibody or peptide addition, have been a boon to immunologists in recent years.

2. *Reaggregate Cultures*

In this technique, deoxyguanosine-treated thymic lobes are disaggregated by trypsinization, and the resulting stromal cells (either with or without further cell selection) are mixed with defined thymocyte subpopulations. After pelleting and placement into organ culture, these mixed cells rapidly reaggregate to form intact lobe-like structures (Jenkinson and Anderson, 1994). These aggregates can contain larger numbers of lymphocytes than are readily achieved by reseeding undispersed thymic rudiments and are not limited to those thymocytes with the potential for thymus recolonization. Thus, DPs can be used, and they can differentiate to SPs in a few days.

IV. The Discovery of Positive Selection

Interest in T cell biology surged with the discovery of the MHC-restricted interaction between helper T cells and B cells (Katz *et al.*, 1973), macrophages and T cells (Rosenthal and Shevach, 1973), and CTL and their target cells (Zinkernagel and Doherty, 1974). Much early attention focused on whether MHC restriction results from a required "like–like" interaction between the MHC molecules of the T cell and its partner, or whether profitable interactions can occur between H-2-incompatible T and B cells for example. Efficient collaboration between MHC-allogeneic T and B cells was clearly demonstrated in tetraparental radiation bone marrow chimeras. These animals are constructed by the reconstitution of irradiated MHC F_1 (A × B) hosts with a mixture of bone marrow derived from parent A and parent B animals. In such $[(A + B) \rightarrow F_1(A \times B)]$ chimeras, T cells of both MHC A and B origin are tolerant of MHC A and B antigens and can collaborate equally well with B cells of either haplotype (von

Boehmer *et al.*, 1975). These experiments helped foster the idea that T cells can interact with MHC-incompatible partner cells if they have differentiated in the presence of cells of that haplotype and have thereby undergone a process of "adaptation."

The demonstration that this adaptation involves something other than antigen presentation and tolerance induction was provided by analyses of [$F_1 \rightarrow$ parent] radiation chimeras (Bevan, 1977). In these experiments, the same pool of T cell-depleted $F_1(A \times B)$ bone marrow cells was used to reconstitute both irradiated parent A and parent B recipients. Despite the fact that both sets of animals were tolerant of MHC A and B antigens and were reconstituted with antigen-presenting cells of F_1 origin, the CTL from [$F_1 \rightarrow A$] chimeras showed a marked preference for lysis of targets expressing antigen in the context of MHC A and vice versa for CTL derived from the [$F_1 \rightarrow B$] chimeras.

Shortly after the 1977 publication of this work, two groups used thymic chimeras to demonstrate that the MHC antigens expressed by the thymic microenvironment provided the specificity of this learned bias in MHC restriction specificity (Zinkernagel *et al.*, 1978a,b; Fink and Bevan, 1978). Thus, skewing toward recognition of thymic MHC antigens was apparent in $F_1(A \times B)$ mice after thymectomy, irradiation, reconstitution with T cell-depleted $F_1(A \times B)$ bone marrow cells, and grafting with thymic tissue derived from either parent A or parent B. Prior irradiation of the thymus graft did not alter the extent of the bias in MHC restriction specificity, leading to the interpretation that radioresistant thymic epithelial cells are responsible for imprinting MHC restriction specificity.

However, here enter the first discords of controversy into the field of positive selection. If restriction to self-MHC antigens is as stringent as many experiments with radiation chimeras suggested, it should have proven very difficult to demonstrate the presence of mature T cells in normal animals capable of interacting in an antigen-specific manner with allogeneic MHC molecules. These cells did not seem to be as rare as expected. Acute negative selection to allogeneic MHC antigens by biological filtration (for example, Wilson *et al.*, 1977) or by BrdU-induced *in vitro* suicide (for example, Ishii *et al.*, 1981) frequently revealed T cells that were apparently restricted to that allogeneic MHC molecule. Thus, T cell populations from normal H-2^b mice in which K^k- and I-A^k-specific alloreactivity has been eliminated can generate CTL specific for a viral antigen plus K^k, while no K^k-restricted activity could be generated in [$F_1(H\text{-}2^k \times H\text{-}2^b) \rightarrow H\text{-}2^b$] radiation chimeras (Doherty and Bennink, 1979).

Reconciliation between the reported stringency of self-MHC restriction in radiation chimeras, on the one hand, with the apparent laxity in self-preference in unmanipulated animals on the other was initially attempted by invoking a type of suppression specifically induced by the chimeric environment. However, despite some initial suggestion that MHC haplotype-specific suppressor cells were responsible for the relative absence of MHC B-restricted T cells in [$F_1 \rightarrow$ A] chimeras (Smith and Miller, 1980), these experiments did not hold up under further scrutiny. Cell mixing experiments ruled out the active suppression of MHC B-restricted CTL responses in [$F_1 \rightarrow$ A] chimeras (Fink and Bevan, 1978; Zinkernagel and Althage, 1979) and additional work failed to uncover evidence for either haplotype-specific suppression or help operating on maturing thymocytes (Fink and Bevan, 1981a,b). These experiments were interpreted as evidence of selection *for* thymocytes bearing receptors restricted to thymic MHC antigens, rather than selection *against* cells bearing receptors restricted to nonthymic MHC molecules.

How then were the experiments with radiation chimeras and those with unmanipulated animals reconciled? By still more controversy, this time revolving around the differentiation of T cells in wholly allogeneic [A $\rightarrow$ B] chimeras. Although such animals were initially reported to be immunoincompetent (Zinkernagel *et al.*, 1978a,b), other investigators disagreed (Matzinger, 1978; Onoe *et al.*, 1980; Wagner *et al.*, 1980). It seemed that the MHC restriction specificity in such animals, at least for virus-specific CTL, could be mainly to thymic MHC, to donor MHC, or to neither, depending both on the type of virus and on the MHC combinations used (Zinkernagel *et al.*, 1984). Thus, rather than focusing on why radiation chimeras differed in the stringency of self-MHC restriction from normal animals, immunologists were driven to explain why this imprinting of restriction specificity is not absolute. Is this due to the simultaneous recognition by a T cell population of antigen in the context of both thymic and nonthymic MHC molecules? Experiments that revealed the bias toward A-restricted recognition in [(F_1(A $\times$ B) $\rightarrow$ A] chimeras demonstrated that T cells restricted to the nonthymic MHC did not recognize the *same* antigens in the context of MHC B molecules as were recognized by A-restricted T cells (Bevan and Fink, 1978). However, by the early 1980s, it was demonstrated that T cells capable of recognizing antigen 1 in the context of MHC A could also recognize antigen 2 in the context of MHC B (Hunig and Bevan, 1981). Cross-reaction or degeneracy at this level ("self + X mimicry," Sprent and Webb, 1987) could certainly account for some or all of the apparent laxity in thymic imprinting.

It could also indicate that while self-MHC restriction specificity of developing thymocytes is largely selected on the basis of "self" as defined by thymic epithelium, some positive selection can be inefficiently driven by the MHC molecules expressed by bone marrow-derived cells. There is some evidence for this latter possibility (Bix and Raulet, 1992). The differences in the stringency of self-MHC restriction specificity highlighted above could be a function of the nature of the antigen and the MHC molecules involved, as well as the health and immunization history of the experimental animals.

One final set of explanations was offered for why positive selection does not appear to be absolute, centering on the hypothesis that positive selection is not an obligate requirement for intrathymic T cell differentiation. Over time, this hypothesis was refined to exclude class II-restricted T cells. Thus, it was proposed that while class I-restricted CTL undergo a somewhat lax selection for thymic MHC restriction specificity, class II-restricted T cells are absolutely restricted to recognition of antigen in the context of thymic class II molecules (Singer *et al.*, 1981; Bradley *et al.*, 1982). These data led to the interpretation that class I-restricted T cells can mature either intrathymically, in which case they are selected for recognition of antigen in the context of thymic MHC molecules, or extrathymically, in the absence of positive selection (Kruisbeek *et al.*, 1981,1983). Although extrathymic sites for T cell maturation almost certainly exist (reviewed in Rocha *et al.*, 1992; Abo, 1993), studies on TCR transgenics and various knockout mice (see below) do not support this pathway of T cell maturation as an explanation for a preference rather than an absolute requirement for recognition of antigen in the context of thymic MHC molecules. These most recent studies also do not support earlier hypotheses that class I-restricted cells show less strict self-preference than do class II-restricted T cells. The explanation for these earlier findings is still unclear.

Thus, the decade following the first articulation of the notion that thymocytes are selected for recognition of the MHC molecules expressed by the thymic epithelium was one of controversy. Was this selection a requirement of T cell differentiation? Can self + X mimicry alone explain why self-MHC preference is not absolute? Do class I- and class II-restricted T cells differ fundamentally in their bias for self-MHC restriction specificity? Are T cells that have differentiated in the presence of allogeneic MHC molecules *not* expressed by thymic epithelial cells more stringently restricted to thymic MHC antigens than are T cells from unmanipulated animals? Is the bias for self-MHC restriction specificity influenced by the introduction of antigen that is

essential for a functional readout of MHC restriction specificity? The following experiments utilizing key technological advances have helped answer some of these questions.

V. Positive Selection in Genetically Manipulated Animals

A. T Cell Receptor Transgenics

In 1988 two groups independently published the characterization of T cell development in mice which carried transgenes encoding the TCRα and -β chains derived from mature T cells. In these mice, the antigen/MHC specificity of one mature T cell clone is expressed by all immature T cells as they develop in the thymus. Such TCR transgenic systems have provided elegant ways of analyzing both tolerance induction (negative selection) and positive selection in T cell development.

TABLE I
$\alpha\beta$ TCR Transgenic Mice: Source of the Rearranged Genes

TCR Specificity	H-2 of Origin	Positive Selection[a]	Reference
		CD8$^+$ T cells	
H-Y plus D^b	H-2^b	D^b	Kisielow *et al.*, 1988
Allo-H-$2L^d$	H-2^b	K^b	Sha *et al.*, 1988a,b
LCMV plus D^b	H-2^b	D^b	Pircher *et al.*, 1989
Flu NP plus D^b	H-2^b	(D^b)	Mamalaki *et al.*, 1993
Allo-H-$2K^b$	H-2^k	K^k	Schönrich *et al.*, 1991
SV40 T plus K^b	H-2^k	(K^k)	Geiger *et al.*, 1993
Ovalbumin plus K^b	H-2^b	K^b	Hogquist *et al.*, 1994
Allo-I-A^k	H-2^{bq4}	D^q	Suzuki *et al.*, 1994
		CD4$^+$ T cells	
Cytochrome c plus I-E^k	H-2^a	I-E^k	Berg *et al.*, 1989
Cytochrome c plus I-E^k	H-2^a	I-E^k	Kaye *et al.*, 1989
Ovalbumin plus I-A^d	H-2^d	(I-A^d)	Murphy *et al.*, 1990
λL-chain plus I-E^d	H-2^d	(I-E^d)	Bogen *et al.*, 1992
Islets plus I-A^{nod}	H-2^{g7}	(I-A^{nod})	Katz *et al.*, 1993
MBP plus I-A^u	H-2^u	(I-A^u)	Goverman *et al.*, 1993
MBP plus I-A^u	H-2^u	(I-A^u)	Lafaille *et al.*, 1994
Flu HA plus I-E^d	H-2^d	I-E^d	Kirberg *et al.*, 1994
Flu HA plus I-A^d	H-2^d	(I-A^d)	Scott *et al.*, 1994
C5 plus I-E^k	H-2^a	(I-E^k)	Zal *et al.*, 1994

[a] Parentheses imply that the specificity of positive selection has not formally been mapped to the restricting MHC allele.

Since the original description of TCR transgenic mice, more than a dozen other TCR specificities have been transferred to the germline of mice. A list of these is presented in Table I.

The original reports of TCR transgenic mice described rearranged α- and β-chain genes from $CD8^+$ cytotoxic T cell clones. In one case the CTL clone was derived from an $H\text{-}2^b$ mouse and recognized an undefined "male-specific" H-Y antigen in the context of the class I molecule $H\text{-}2D^b$ (Teh *et al.*, 1988; Kisielow *et al.*, 1988). In the other case, the donor T cell clone was an alloreactive $H\text{-}2^b$ anti-$H\text{-}2L^d$ $CD8^+$ CTL (Sha *et al.*, 1988a,b). Expression of these receptors by developing thymocytes in $H\text{-}2^b$ mice resulted in the preferential selection of $CD8^+$ over $CD4^+$ single-positive mature T cells. These data indicated the obligatory relationship between CD4/CD8 coreceptor expression and TCR specificity and introduced the analysis of surface antigen phenotype as a simple and direct means of detecting positive selection. While most of the $CD8^+$ cells in these animals expressed high levels of both transgenic TCRα and -β chains, the few $CD4^+$ cells that developed expressed low levels of the TCRα chain and high levels of the β-chain transgene. This phenotype suggested the association of the transgenic β-chain with the product of an endogenously rearranged TCRα chain gene, endowing the $CD4^+$ cells with a new specificity. This hypothesis was clearly proven by the absence of functional $CD4^+$ T cells in anti-H-Y transgenic mice bred onto a SCID background which obviates any endogenous TCR gene rearrangement (Scott *et al.*, 1989).

As would be expected from the cytolytic specificity for H-Y plus D^b of the $CD8^+$ clone that donated the TCR genes, thymocytes bearing this receptor required $H\text{-}2D^b$ expression on thymic stroma in order to progress to mature $CD8^+$ T cells. For example, while HTG mice (K^d IA^d IE^d D^b) and $H\text{-}2^b$ mice (bb-b) did select a large fraction of $CD8^+TCR^{hi}$ thymocytes, R107 mice (bb-d) did not. In the case of the transgenic mice expressing a receptor from an alloreactive CTL clone derived from $H\text{-}2^b$ mice that specifically lysed targets expressing $H\text{-}2L^d$, there was no obvious prediction as to which MHC molecule in $H\text{-}2^b$ mice might be responsible for positive selection of thymocytes expressing this receptor. The selecting molecules turned out to be $H\text{-}2K^b$ (Sha *et al.*, 1990).

The reciprocal experiment, in which rearranged TCRα and -β chain genes were taken from a $CD4^+$ MHC class II-restricted T cell clone, was reported by two groups a year later. Both groups took receptor genes from T cells that were specific for a peptide fragment of pigeon cytochrome c plus $I\text{-}E^k$ (Berg *et al.*, 1989; Kaye *et al.*, 1989). These

TCR transgenic mice were found to have an overabundance of TCR^{hi} thymocytes maturing to the CD4 subset, an observation that was dependent on the coexpression in the thymus of I-E^{k} (or a closely related I-E molecule). In one case, it was shown that targeted expression of an I-Eα transgene to the thymic cortex was sufficient to restore positive selection in H-2^{b} mice that are genetically deficient in I-Eα but express functional I-Eβ. In this setup, the I-Eα chain restores expression of an Eβ^{b}Eα molecule on the cell surface. Targeted expression of I-Eβ^{b}Eα to the thymic medulla and the lymphoid periphery by a different form of the promoter did not restore positive selection (Benoist and Mathis, 1989; Berg *et al.*, 1989). This result emphasized the importance of the location of MHC-expressing cells within the thymic architecture in rescuing immature DP thymocytes from programmed cell death.

Since 1989, a large number of lines of other TCR transgenic mice have been made and are available for study (Table I). In many cases, mapping the MHC molecule that serves as the positively selecting restriction element with the use of MHC recombinants or MHC transgenes has not been performed. The skewing of T cell development to CD8 or CD4 subsets depending on the origin of the TCR has been demonstrated in most systems in which it has been analyzed. What follows is a description of two recent studies that serve as exceptions to the rule of the correlation between coreceptor expression and MHC restriction specificity.

Kirberg and co-workers (1994) have described mice transgenic for a TCR cloned from a CD4^{+} T cell specific for an influenza hemagglutinin peptide plus I-E^{d} which apparently break the correlation of TCR specificity and commitment to the CD4^{+} or CD8^{+} subset. In mice that express no endogenously rearranged TCR genes, the surprising result is that transgenic TCR^{hi} cells are found in the CD8^{+} subset as well as in the (expected) CD4^{+} subset. These transgene-positive CD8^{+} cells have cytolytic activity directed against cells presenting the HA peptide plus I-E^{d}. This could be a case of a TCR which cross-reacts at the positive-selection step with self-I-E^{d} and self-class I. However, maturation of the CD8^{+} cells depends not only on expression of I-E^{d} but also on the simultaneous expression of class I. The authors prefer the interpretation that this may be a case in which positive selection is triggered by a high-affinity interaction of the TCR with self-class II in combination with a noncognate CD8-class I interaction. Thus, their notion is that when DP thymocytes are signaled by binding to self-I-E, they randomly downregulate either CD8 or CD4. In the latter case of mismatched TCR specificity and coreceptor molecule, most T cell specificities would be lost. But this particular receptor may have a

high enough affinity for self-class II MHC to allow survival of the cells which downregulate CD4.

In another recent publication, Suzuki and colleagues (1994) set out to study the positive selection of a TCR expressed on $CD8^+$ CTL specific for allogeneic class II MHC molecules. This discordance between CD8 or CD4 surface phenotype and specificity for class I or class II MHC is not uncommon among CTL specific for allogeneic class II MHC, as many are $CD8^+$. In the resulting line of mice, the transgenic TCR appears to be selected in a conventional way on self-H-$2D^q$ and not on self-class II molecules. This observation led to the suggestion that the ability of the cells to react to allogeneic class II represents a chance cross-reaction.

B. Gene Knockouts

Our understanding of the involvement in positive selection of TCR, MHC, coreceptors, and peptide has been greatly expanded by the use of targeted gene disruption. A partial list of such knockout mice, together with their associated phenotypes, is given in Table II. Disrupting both copies of the β2m gene in mice has the immediate consequence of extinguishing MHC class I expression at the cell surface. The trimeric complex of class I heavy chain, β2m light chain, and peptide is unstable in the absence of any one component. The secondary consequence of the lack of β2m is that the mice are almost devoid of $CD8^+TCR^{hi}$ cells in the thymus or periphery. Thus, $CD8^+$ cells need MHC class I expression to mature in the thymus (Koller *et al.*, 1990; Zjilstra *et al.*, 1990).

Mice of the H-2^b haplotype express only one classical class II molecule at the cell surface, namely I-A^b. Disruption of the I-Aβ chain results in "class II negative" mice which have a profound deficit in mature $CD4^+$ T cells (Cosgrove *et al.*, 1991; Grusby *et al.*, 1991). MHC class II expression in the thymus is required for the generation of mature, $CD4^+$ SP T cells.

Shutting off the main supply route of peptides that load onto class I molecules by disrupting one component of the heterodimeric transporter associated with antigen presentation (TAP) results in low cell surface expression of class I and a decreased appearance of $CD8^+$ T cells (van Kaer *et al.*, 1992). Disruption of the class II-associated invariant chain, which protects the class II groove in the endoplasmic reticulum and directs the newly synthesized class II to endosomes, results in a similar decrease in both class II surface expression and $CD4^+$ T cell differentiation (Viville *et al.*, 1993).

Totally obliterating the expression of the coreceptor molecules at

TABLE II
GENE KNOCKOUTS: CONSEQUENCES FOR POSITIVE SELECTION

Target Gene	Surface Phenotype	Effect on Thymus	References
β2m	No class I MHC	No CD8$^+$ cells	Koller *et al.*, 1990; Zjilstra *et al.*, 1990
Class II I-Aβ	No class II MHC	Decreased CD4$^+$ cells	Cosgrove *et al.*, 1991; Grusby *et al.*, 1991
TAP-1	Low class I MHC	Decreased CD8$^+$ cells	van Kaer *et al.*, 1992
CD8α	No CD8	No class I-restricted T cells	Fung-Leung *et al.*, 1991
CD4	No CD4	Decreased class II-restricted T cells	Rahemtulla *et al.*, 1991; Killeen *et al.*, 1993
CD8β	CD8$\alpha\alpha$ only	5× Decrease in CD8$^+$ cells	Crooks and Littman, 1994; Fung-Leung *et al.*, 1994
Invariant chain	Reduced class II	Decreased CD4$^+$ cells	Viville *et al.*, 1993
Tdt	Lack of N regions	More efficient selection of SPs	Gilfillan *et al.*, 1994

the thymocyte cell surface by disrupting the CD4 or CD8α genes obviously prevents the phenotypic appearance of the SP subset. In each case, however, the absence of one coreceptor seems to have no effect on the generation of large numbers of immature "DPs" nor on the development of the other SP subset (Fung-Leung *et al.*, 1991; Rahemtulla *et al.*, 1991; Killeen *et al.*, 1993). The CD8α and CD4 knockout mice are not exact mirror images; while CD8α knockout mice seem to be devoid of any MHC class I-restricted T cells, the CD4-deficient mice appear to have some peripheral T cells which are MHC class II-restricted and functional.

Knocking out only the CD8β gene allows T cells to express CD8$\alpha\alpha$ homodimers at the cell surface at a level only slightly below the expression level of CD8 $\alpha\beta$ dimers in a wild-type mouse. In CD8β-deficient mice, an approximate five-fold diminution in the level of production of CD8$^+$ SP T cells has been reported (Crooks and Littman, 1994; Fung-Leung *et al.*, 1994). It thus seems likely that the β-chain in the CD8 heterodimer has a role in the recognition or signaling event in positive selection.

During the process of TCRα and -β chain gene rearrangement, the enzyme terminal deoxynucleotidyl transferase (Tdt) adds bases at the V_β-D-J and the V_α-J junctions. These noncoded bases, designated N regions, have the effect of increasing sequence diversity in the CDR3 region of the TCR, that region thought to overlay the peptide bound in the groove of the MHC (Davis and Bjorkman, 1988). In the absence of Tdt, Gilfillan and co-workers (1994) have reported that there is a more efficient selection of DP thymocytes into the SP compartment. This is expressed as both an increased fraction of SPs in the thymus and a faster transit time through the thymus. Thinking back to Jerne's suggestion (1971) this may mean that the germline TCR repertoire, encompassing all of the combinatorial and junctional diversity of the genes, is more likely to react with self-MHC in the thymus and trigger positive selection than are TCRs with inserted N-region diversity. Remarkably then, we conclude that the insertion of N regions *spoils* positive selection to some degree, since fewer immature T cells are suitable for positive selection in wild-type mice than in Tdt knockouts. Perhaps wild-type receptors have a more stringent requirement for recognition of the self-peptides during the selection event.

VI. Commitment to the CD4$^+$ or CD8$^+$ Lineage: Is It Instructive or Stochastic?

This great debate has raged for the past 4 years since the alternatives were first put forward (Robey *et al.*, 1991; Borgulya *et al.*, 1991). The

instructive theory of lineage commitment proposes that during the positively selecting interaction of the TCR and its MHC ligand, the DP thymocyte receives directions: an interaction of the TCR with MHC class I signals the cell to downregulate CD4, while a TCR class II interaction signals CD8 downregulation. The stochastic theory, on the other hand, proposes that the TCR/MHC ligand interaction for positive selection sends no directional signals to the cell. According to this hypothesis, thymocytes randomly downregulate either CD4 or CD8, regardless of the nature of their expressed TCRs. Selection for cells whose TCRs and coreceptors match in specificity accounts for the observed correlation between MHC restriction specificity and coreceptor expression.

The initial tests of the two theories questioned whether forced, constitutive expression of transgenic CD8 could rescue $CD4^+$ T cells with a class I-restricted transgenic TCR. The appearance of such $CD4^+CD8tg^+TCR^+$ cells would imply that endogenous CD8 had been downregulated, despite the expression of a class I-restricted TCR, and would be taken as evidence for the stochastic model. The fact that no such rescue was seen favored the instructive notion that positive selection on class I signaled CD4 downregulation (Robey *et al.*, 1991; Borgulya *et al.*, 1991). However, since that time, opinion has swayed back to the idea that following positive selection, DPs stochastically move to the CD8 (killer) lineage or CD4 (helper) lineage preceding a selective step involving the CD8 or CD4 coreceptor plus the TCR. At this time only cells bearing the correctly matched coreceptor survive, while cells that made the "wrong" choice of coreceptor die (Chan *et al.*, 1993). Evidence supporting the stochastic/selective theory has come from the analysis in MHC knockout mice of coreceptor–TCR mismatched thymocytes with a transitional $CD4^+CD8^{lo}$ or $CD8^+CD4^{lo}$ phenotype (Chan *et al.*, 1993; Davis *et al.*, 1993; van Meerwijk and Germain, 1993). Also, in some cases, transgenic expression of one coreceptor *can* rescue cells which apparently made the wrong coreceptor choice (reviewed in Robey and Fowlkes, 1994). As a modification of the two-step stochastic/selective theory, Pircher and co-workers (1994) have suggested that the TCR-MHC interaction might result in continuous, coreceptor-dependent signaling. How far down the wrong pathway a cell may go will depend on the strength or the coreceptor independence of the TCR–MHC interaction. Cells whose receptors mediate low-affinity (or avidity) TCR–MHC interactions will not travel far down the wrong lineage pathway. Eventually, as the matched coreceptor level decreases in such cells, signaling will cease and the thymocyte will die or possibly reverse its coreceptor decision.

VII. The Cell Type Mediating Positive Selection

The self-recognition event that rescues $CD4^+CD8^+$ DP thymocytes from programmed cell death occurs in the thymic cortex. Thymocytes themselves express a low level of MHC class I molecules and no endogenously synthesized class II molecules. There are rather small numbers of other marrow-derived cells in the cortex. The epithelial cells provide an extensive meshwork throughout the thymic cortex expressing a high level of both classes of MHC molecules. The original radiation chimera and thymus grafting model system that demonstrated positive selection implied strongly that the cell type which provided the MHC ligand was (a) radiation-resistant and (b) resident in the thymus, i.e., probably the cortical epithelial cells. An early suggestion that the cell responsible for selecting the class II-restricted T cells is fundamentally different from that selecting class I-restricted T cells (Longo and Schwartz, 1980; Longo and Davis, 1983) was eventually put to rest (Lo and Sprent, 1986). Thymic sections revealed the clustering of TCR at the site of interaction between thymocytes and cortical epithelial cells (Farr *et al.*, 1985). Shortly thereafter, it was shown that positive selection requires the targeted expression of the relevant MHC molecules to cortical and not medullary epithelial cells in transgenic mice (Benoist and Mathis, 1989; Berg *et al.*, 1989). These and other experiments have demonstrated that cortical epithelial cells are responsible for the positive selection of most class I- and class II-restricted T cells. What is not clear is whether this is simply because the cortical epithelial cells happen to be in the right place with the right ligand, or whether they also have a specialized propensity for positive selection.

Thymus epithelial cell (TEC) lines injected directly into the thymus can apparently promote the positive selection of $CD8^+$ and $CD4^+$ T cells with antigen reactivity restricted by the TEC MHC type (Vukmanović *et al.*, 1992; Hugo *et al.*, 1992). The TEC lines which do this express conventional MHC class I, in the sense that the endogenous peptides they present in association with class I are largely overlapping with those presented by lymphoid cells (Vukmanović *et al.*, 1993). Thus, there is no suggestion that positive selection requires an "altered ligand" compared to negative selection.

Further studies with intrathymic injections have suggested that even fibroblasts can promote positive selection (Hugo *et al.*, 1993; Pawlowski *et al.*, 1993). In addition, in radiation chimeras whose thymic stromal cells lack MHC class I expression, MHC class I expressed only on marrow-derived cells (T cells, macrophages, dendritic cells) can

substitute for selection of a relatively small number of mature $CD8^+$ cells (Bix and Raulet, 1992). The converse experiment with MHC class II knockout mice (wild-type marrow populating a class II knockout thymus) did not show an enhancement of $CD4^+$ T cell maturation over the background (Markowitz *et al.*, 1993). This difference between $CD8^+$ and $CD4^+$ positive selection may be due to the fact that very few bone marrow-derived cells in the thymus cortex express MHC class II.

Much of these data seem to imply that thymic epithelial cells provide the ligand for positive selection simply because they happen to be in the right place, and that no other special property is required. However, several lines of evidence have recently emerged to suggest that thymus cortical epithelium may indeed be unique in its ability to positively select. One report, in which H-$2K^b$ expression was driven by the CD2 promoter, implied that class I expressed on thymic lymphocytes could not drive positive selection of transgenic thymocytes expressing an alloreactive TCR that is normally positively selected by K^b (Schönrich *et al.*, 1993). Thus, it may be that the weak selection observed by Bix and Raulet (1992) in mice with a normal TCR repertoire is receptor specific, or perhaps mediated not by lymphocytes, but by macrophages or dendritic cells. In an extensive series of studies, Anderson and colleagues have used a 3-day thymus reaggregate culture system to analyze the stromal cell types required to promote the differentiation of TCR^-DP thymocytes to TCR^+SP cells (Jenkinson *et al.*, 1992; Anderson *et al.*, 1993,1994). In this system, purified thymus epithelium promotes selection, while salivary and gut epithelium do not. Remarkably, chemically fixed thymic epithelium also works in this system, implying that a specialized surface property is the key. In other studies, it has been shown that thymus epithelium is uniquely endowed with promoting the differentiation of a class II-restricted, TCR^+ thymoma from the DP to the $CD4^+SP$ phenotype (Poirier *et al.*, 1994).

These latest experiments suggest that thymus epithelium *is* specialized to mediate positive selection. It should be remembered that the *in vivo* experiments showing that fibroblasts or hematopoietic cells can provide the MHC ligand for positive selection were all performed in the thymus, i.e., in the presence of thymic epithelial cells. In these systems the thymocytes may have recognized the MHC ligand on fibroblasts, while getting some other essential signal from the TEC *in trans*. Determining the nature of this unknown specialized property of TEC would greatly facilitate the *in vitro* analysis of positive selection.

VIII. The Ligand for Positive Selection

A. Peptides Are Involved in Positive Selection

The recognition requirement for positive selection is remarkably specific for the particular MHC allele. Thus, as analyzed in TCR transgenic systems, a receptor specific for the class I molecule H-$2D^b$ plus H-Y that is positively selected in a thymus expressing H-$2D^b$ (without H-Y) is selected neither by any MHC class II molecules nor by other unrelated MHC class I molecules such as H-$2D^k$, D^d, K^b, K^d, or K^k. Similarly, the transgenic TCR specific for allo-H-$2L^d$ that is positively selected by K^b is not positively selected by a dozen other MHC molecules (reviewed under Section V,A). This strict MHC allele specificity breaks down when one examines closely related variants of the selecting MHC molecule. Spontaneously occurring H-$2D^b$ or K^b mutants have various abilities to select the TCRs mentioned above. Similarly, in the case of MHC class II-restricted TCRs specific for I-E^k plus foreign peptide, there can be some degree of positive selection in a thymus expressing I-E^b. In this case, the I-$E\beta^k$ chains differ in only four residues and the I-$E\alpha$ chain is invariant. There can also be unexpected cross-reactivities in positive selection: Kaye and coworkers (1992) showed that a TCR specific for I-E^k plus cytochrome c peptide was positively selected on I-A^b as well as on I-E^k.

Some of the comparisons of selection on variant MHC molecules can also shed light on the requirement for the recognition of the self-peptides bound by the selecting self-MHC molecule. In studies with nontransgenic mice, small changes in the positively selecting MHC molecule can alter the pattern of responsive T cells in the periphery. For example, $CD8^+$ T cells expressing a large variety of TCRs able to respond to a chicken ovalbumin peptide (SIINFEKL) plus H-$2K^b$ are selected in H-2^b mice or in (H-2^b × H-2^{bm8} → H-2^b) [F_1 → parent] radiation chimeras. However, (H-2^b × H-2^{bm8} → H-2^{bm8}) chimeras make no $CD8^+$ T cell response to SIINFEKL plus K^b (Nikolić-Zulić and Bevan, 1990). The wild-type K^b and K^{bm8} variant molecules differ by four amino acid residues and are serologically indistinguishable. Three of these residues appear to be silent changes for T cell contact or peptide binding, while the fourth can affect peptide binding (Pullen *et al.*, 1991). Further, allelic differences in I-E molecules that are predicted to affect peptide binding also influence the selection of the T cell repertoire as measured by immunization and fine specificity analysis (Bhayani *et al.*, 1991). Similarly, changes in selecting MHC class I (Jacobs *et al.*, 1990; Sha *et al.*, 1990) or class II (Berg *et al.*, 1990) molecules that are predicted to alter the range of self-peptides

bound have been shown to affect the selection of thymocytes expressing a homogenous transgenic TCR.

These results make it likely that self-peptides do play a role in TCR repertoire selection, but they leave open the question of how precisely peptides are recognized by the TCR. There are two extreme viewpoints on this issue: (a) the peptide role is not very specific; they simply must not obstruct the TCR from contacting residues on the MHC α-helices during positive selection; and alternatively (b) the recognition of self-peptide during positive selection is highly specific, such that perhaps only one self-peptide/MHC ligand of the 10^4 or so expressed can select a certain TCR. According to the former view, if empty MHC molecules were stable and could be expressed at the cell surface (under physiological conditions, both empty class I and class II molecules are unstable), most TCRs that are normally selected would still be selected (Schumacher and Ploegh, 1994). For a slightly higher degree of peptide recognition, one could propose that only a few residues of the peptide contact the TCR, adding some energy and specificity to positive selection, while the other side chains must simply keep out of the way. Also included in the first hypothesis is the notion that certain categories of peptides impose certain defined conformations on the MHC helices and each conformation selects a subset of the T cell repertoire.

Only very recently have materials and methods become available to allow the study of the single peptides on the process of positive selection.

B. Synthetic Peptides Restore Positive Selection

In order to perform "peptide add-back" experiments, the first requirement is a genetic system resulting in a deficiency in peptide loading onto MHC molecules. The second requirement is an *in vitro* system which supports the process of positive selection. In the case of MHC class I, two lines of knockout mice have provided the genetic system, and FTOC has provided the *in vitro* system to study positive selection. Mice in which the β2m gene has been disrupted by homologous recombination cannot make stable class I trimers of heavy chain, β2m, and peptide, and thus do not express class I antigen on the cell surface. As a result of this lack of class I expression on thymic epithelial cells, β2m-deficient mice show a profound depletion of $CD8^+$ T cell development and carry about 2% of the wild-type numbers of $CD8^+$ peripheral T cells (Koller *et al.*, 1990; Zjilstra *et al.*, 1990). The key to using this genetic system was the finding that class I trimers can be reformed at the cell surface by the exogenous addition of an appro-

priate class I-binding peptide *plus* β2m. Evidently, free heavy chains traffic to the cell surface in these β2m knockout cells. Only very low levels of class I reconstitution at the surface are achieved with this system: $CD8^+$ T cells can appreciate it, but the reconstitution is below the levels of detectability by flow cytometry. Probably fewer than 1000 copies/cell of class I are refolded in β2m mutant lymphoid cells by this procedure.

The other genetic system that has been exploited for analyzing the peptide requirement for positive selection is TAP-1 knockout mice. In this case, disrupting the function of the transporter in the endoplasmic reticulum membrane that shunts peptides from the cytosol to the ER lumen for class I association again results in an impaired expression of class I and a concurrent decrease in $CD8^+$ T cell selection in the thymus. Adding appropriate class I-binding peptides in this system can restore surface MHC class I expression to near wild-type levels. Thus, tens of thousands of homogeneous peptide/class I ligands can be reformed on the surface of TAP-1 knockout cells (van Kaer *et al.*, 1992).

1. *Studies with Ova/K^b TCR Transgenic Mice*

The antigenic Ova peptide (SIINFEKL) complexed to the H-$2K^b$ molecule has been used extensively in fine-specificity analyses (Jameson and Bevan, 1992; Jameson *et al.*, 1993) and the structure of the complex has been solved by X-ray crystallography (Fremont *et al.*, 1995). Residues S1, N4, E6, and K7 point out of the K^b groove and can be varied quite extensively without reducing the peptide affinity for K^b. Many "up" residue variants become weak targeting peptides for CTL clones specific for Ova (Jameson *et al.*, 1993). These are referred to as weak agonists and it seems likely from their targeting activity, their frequency, and now from Biacore measurements of peptide/K^b binding to soluble TCR that when bound to K^b, they provide lower-affinity ligands for the TCR. Many other up variants of Ova, when added to target cells, do not induce CTL-mediated target lysis at any concentration, but instead serve as antagonists for the TCR. That is, they bind K^b on a presenting cell and interact with the TCR specifically, without triggering lysis, in a way that can decrease agonist-directed lysis. Peptides with mixed (weak agonist and antagonist) properties also exist. Again, Biacore measurements and the ease of identifying antagonist peptides suggest that many of these peptides provide low-affinity ligands for the TCR.

In FTOC studies of the Ova/K^b TCR transgenic mice crossed to the β2m knockout background, it was reported that the antigenic Ova

peptide caused deletion of DP thymocytes and showed no enhancement of positive selection at any dose (Hogquist *et al.*, 1994). However, variant Ova peptides, in particular those that scored as antagonist peptides in CTL assays, provided quality ligands for positive selection. For example, the R4 variant peptide is a TCR antagonist for mature T cells bearing this receptor and induces positive selection in FTOC at saturating concentrations (Fig. 3). The related variant, K4, serves neither as an antagonist nor as an agonist and also fails to induce positive selection in this β2m knockout system. E1 is a weak agonist/antagonist which also induces positive selection. The double-variant peptide, E1R4, is null in CTL assays (i.e., is neither an agonist nor an antagonist) and is also inactive in FTOC (Jameson *et al.*, 1994). Also

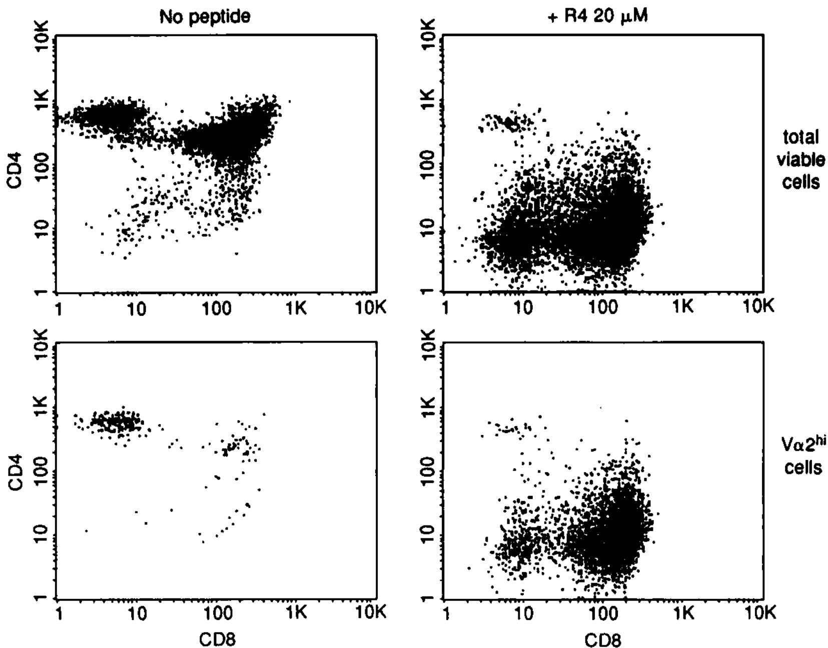


FIG. 3. An example of peptide-induced positive selection obtained in 7 day fetal thymus organ cultures of TCR transgenic, β2-microglobulin knockout animals. The $V_{\alpha}2V_{\beta}5$ transgenic TCR is specific for the ovalbumin peptide SIINFEKL plus H-2K^b. The R4 variant peptide (SIIRFEKL) is a pure antagonist peptide (Hogquist *et al.*, 1994; Jameson *et al.*, 1994). The left panels show that with no added peptide during organ culture, there are few CD8$^+$ cells produced (top) and very few cells express the transgenic TCR at high levels (bottom). The addition of the R4 peptide results in a dramatic selection of CD8$^+$, TCR^{hi} cells (right panels).

scored as null in both assays are SSYSYSSL, an unobstructive peptide in which the MHC anchors are preserved and all of the up side chains are small, as well as two H-2K^b-bound self-peptides that are naturally processed and presented by mouse cells (Hogquist *et al.*, 1994).

The weak agonist, E1, as well as a much stronger agonist variant peptide, A2, both phenotypically induce the differentiation of CD8$^+$CD4$^-$, TCR^{hi} thymocytes in β2m knockout FTOC. However, these CD8$^+$ cells frequently have a CD8^{low} phenotype and, most importantly, do not have a normal functional response to antigen (Jameson *et al.*, 1994). The A2-selected cells, for example, do not respond to the original Ova antigen.

To summarize the experiments performed using the β2m knockout system, where only low levels of class I can be reformed at the cell surface: the peptides that work best in positive selection are pure antagonist peptides. These peptides have no ability to delete immature thymocytes. Weak agonists also work, but the selected cells do not display wild-type reactivity to antigen. Any peptide which is null in both agonist and antagonist assays using mature T cells is also null in the β2m knockout system for studying positive selection. Thus, with this transgenic TCR on the β2m-deficient background, positive selection is highly peptide specific.

2. *Studies with LCMV/D^b TCR Transgenic Mice*

The same TCR transgenic mouse (Pircher *et al.*, 1989) has been used by two independent groups to study peptide-induced positive selection in FTOC in either the background of the TAP-1 knockout (Ashton-Rickardt *et al.*, 1994) or the β2m knockout (Sebzda *et al.*, 1994). The antigenic nonamer peptide from the LCMV glycoprotein (KAVYNFATC) was used in a modified form to study positive selection, such that the C-terminal cysteine was changed to methionine, an alternative anchor residue introduced to prevent peptide dimerization. The conclusion of both papers is that the same strongly antigenic peptide (KAVYNFATM) can induce positive selection of cells expressing this TCR in FTOC and, at a higher peptide concentration, can also cause delection. The results are therefore similar to those with the weak agonist peptide E1 reported by Hogquist and co-workers (1994). It is also notable that the CD8$^+$ TCR^{hi} cells that are positively selected by the strong agonist peptide used by Ashton-Rickardt and co-workers (1994) and Sebzda and co-workers (1994) actually express a lower surface density of the CD8 coreceptor. Neither group has reported whether these CD8^{low} cells respond to antigen. It is likely, based on the results with a strong agonist in the Ova/K^b system, that these cells may also be functionally inert.

In the TAP-1 knockout background, an unobstructive polyserine peptide which binds D^b did not induce positive selection, in agreement with the results of Hogquist and co-workers (1994). Variants of the original antigenic peptide were able to stimulate positive selection in this system as well (Ashton-Rickardt *et al.*, 1994).

These two papers allow a comparison of the efficacy of the same strongly antigenic peptide to induce the appearance of $CD8^+$ cells bearing the same TCR in TAP-1 knockout versus β2m knockout mice. Surprisingly, the optimal peptide concentration for positive selection in the TAP-1 knockout FTOC was reported to be 3×10^{-5} *M* (Ashton-Rickardt *et al.*, 1994), while in the β2m knockout experiments, 10^{-12} *M* peptide induced positive selection and 10^{-6} *M* induced negative selection (Sebzda *et al.*, 1994). This discrepancy is difficult to understand since peptide loads on surface class I to a higher level in TAP-1 knockouts than in β2m knockouts. Some technical difference in peptide addition or degradation may turn out to be the key.

C. So What Is the Natural Ligand for Positive Selection?

The ligand on thymus epithelial cells that is recognized by the TCR on immature thymocytes and permits their survival is, without doubt, an MHC molecule occupied by self-peptide. However, the question that opened this section still remains: how specific is the recognition of the self-peptide in this process?

Peptides with short side chains protruding out of the groove of class I (the polyserine peptides) did not provide ligands for positive selection of the two TCR transgenics tested (Hogquist *et al.*, 1994; Ashton-Rickardt *et al.*, 1994). In the case of the β2m-deficient FTOC, low levels of K^b reconstitution were probably achieved, though in the case of the TAP-1-deficient FTOC, higher levels of D^b stabilization could be expected. Together, these results dispute the simplest notion that the only role of peptide is to stabilize MHC and allow the TCR to contact the α helices. However, the negative results apply only to these two TCRs and at the ligand density achieved in the FTOC.

In the case of the β2m-deficient FTOC, only peptides that scored (at least) as antagonists for mature CTL expressing the transgenic TCR were able to promote positive selection. Weak agonist peptides also apparently worked, but resulted in $CD8^+$ single-positive cells with an altered reactivity to foreign peptide. Stronger agonists gave phenotypic selection, but the selected cells were unable to respond to the original antigen. These studies demonstrate the dangers of using flow cytometric analysis alone to define positive selection, and argue against the notion that strong agonist peptide ligands will be involved in positive selection. Indeed, when natural peptides purified by immunoprecipita-

tion of MHC molecules and HPLC fractionation are loaded onto TAP-1 knockout target cells, agonist activity is undetectable. Using the same peptide extraction technology to search for the natural foreign agonist is often successful, and the active peptide fraction can frequently be diluted many-fold and still score in the assay (Rotzschke *et al.*, 1990,1991; Falk *et al.*, 1990). By this criterion then, natural self-peptides do not contain agonists for effector T cells even when their level of cell surface expression is experimentally upregulated in this way.

If low-affinity ligands are the naturals for positive selection, the question remains: how low can you go? Again, in the β2m knockout FTOC/Ova-specific TCR system, the E1 and R4 variants of SIINFEKL promote positive selection, but the K4 and combined E1R4 variants do not. At the level of K^b stabilization achieved in this system, this drop in affinity for the TCR correlates with a lack of positive selection in FTOC and a lack of antagonism of mature CTL. But if these null peptides (or the polyserine peptide) could be loaded to a higher ligand density onto the presenting cell, it is conceivable that positive selection would be observed on these ligands. Since self-MHC is normally stabilized by a variety of self peptides, each at rather low occupancy, this could mean that different self-peptides cooperate in the selection of one TCR. This hypothesis has been labeled peptide gemisch selection (Bevan *et al.*, 1994). If a thymic epithelial cell expresses 10^5 MHC molecules, and presents a self-peptide complexity of 10^4, the average peptide occupancy would be 10 copies/cell. Actual analyses of MHC-associated self-peptides give great ranges of occupancy, up to 5% of the MHC molecules may be occupied by one peptide in some cases, representing 2000 copies/cell (Falk *et al.*, 1991). According to this, some abundant self-peptides may work alone in selecting a TCR with very low affinity for the MHC/peptide complex.

At this point we do not know the identity of any self-peptide which is involved in the positive selection of cells bearing any TCR. It seems likely that some TCRs will be selected by one peptide/MHC complex, while others may use cooperating peptide/MHC ligands. In any of these schemes, however, it is apparent that the diversity of the self-peptide/MHC repertoire is exploited in selecting the peripheral T cell receptor repertoire for foreign antigen.

IX. Concluding Remarks

The requirement for immature thymocytes to demonstrate that their surface $\alpha\beta$ TCRs have some reactivity to self-MHC class I or class II molecules is the barrier enforced by positive selection. Most $\alpha\beta$ TCR

thymocytes simply don't pass this test. Cells that initially fail positive selection may briefly continue to rearrange TCRα chain genes and finally succeed in expressing a selectable TCR. However, most DP thymocytes undergo a programmed cell death, ensuring that the selected repertoire is reactive with foreign peptides presented by self-MHC molecules.

The nature of the signal triggered by the TCR–self-MHC interaction that rescues thymocytes from death remains an enigma. The interaction between the TCR and its MHC ligand that stimulates positive selection in FTOC is so weak that it does not stimulate any measurable signaling in mature T cells. For this reason, mature T cells cannot serve as model systems for the signal transduction pathway operating in thymocyte selection. This information is likely to come instead from a couple of sources. The first is the ever-expanding use of genetically manipulated animal model systems to elucidate the role of various gene products in thymocyte selection. The second is the use of *in vitro* reaggregate culture systems that allow the study of positive selection from separated stromal and stem cell components, in which it may soon be possible to introduce probes directly into the DP stem cells for positive selection.

Acknowledgments

Pamela J. Fink is supported by NIH Grant AG-13078. Michael J. Bevan is supported by NIAID Grants AI-19335 and AI-29802 and the Howard Hughes Medical Institute. We thank S. R. Dillon for suggestions on the manuscript and C. Cherry for secretarial assistance.

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This article was accepted for publication on 26 January 1995.

ADVANCES IN IMMUNOLOGY, VOL. 59

Molecular and Cellular Aspects of X-Linked Agammaglobulinemia

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I. Introduction

X-linked agammaglobulinemia (XLA) was the first immunodeficiency described more than 40 years ago by Dr. (Colonel) Ogden C. Bruton (Bruton, 1952). The disease is characterized by an increased susceptibility to mainly bacterial infections although enteroviral infections have become increasingly important due to their resistance to existing therapies (Lederman and Winkelstein, 1985; McKinney *et al.*, 1987).

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The study of XLA has had a major impact not only on the field of immune deficiency research, which was established as a discipline after the description of XLA, but also on the understanding of fundamental principles in basic immunology. Thus, the study of XLA was of crucial importance for evaluating the protective role of humoral immunity, for defining lymphocyte cell lineages, and dissecting the B cell developmental process. The recent cloning of a gene encoding a cell signaling molecule that is mutated in XLA patients (Vetrie *et al.*, 1993b; Tsukada *et al.*, 1993) has made XLA a prototype model system for elucidating the role of signaling molecules in cell differentiation. These developments have raised the hope for future gene therapy treatment that will eventually replace the immunoglobulin substitution instituted already by Bruton (1952).

In this review we summarize the current knowledge in the field of XLA and discuss it in the context of the recent developments. A large number of excellent reviews on XLA have already been written, several of which cover clinical aspects, (Good and Zak, 1956; Firkin and Blackburn, 1957; Good *et al.*, 1962; Rosen and Janeway, 1966; Burgio and Ugazio, 1982; Lederman and Winkelstein, 1985; Conley and Puck, 1988b; Hendriks and Schuurman, 1991; Timmers *et al.*, 1991; Conley, 1992; Hermaszewski and Webster, 1993; Kinnon *et al.*, 1993; Rawlings and Witte, 1994; Smith *et al.*, 1994b) and in particular we refer to these surveys for clinical manifestations as these will only be briefly described.

As the role of the XLA product, the protein tyrosine kinase (PTK) designated *Btk* (*B*ruton's agammaglobulinemia *t*yrosine *k*inase), has just begun to be unraveled it is obvious that this review can only summarize the limited knowledge that exists today. However, given the fact that it was only 2 years since the cloning of the gene was reported, substantial information on many aspects of Btk has accumulated. Thus, the genomic organization has been characterized, more than 100 mutations have been identified, the 3-dimensional structure of parts of the molecule has been predicted, its potential role in some signaling pathways has been described, a mouse with a genetic defect in the *Btk* gene has been recognized, and additional mouse mutants are being created by homologous recombination.

II. The Original Report—Bruton (1952) and Its Implications

In 1952 Bruton reported an 8-year-old boy that had suffered since the age of 4½ years from recurrent bacterial infections, including 19 episodes of clinical sepsis often caused by pneumococci. The patient

received antibiotics and there were unsuccessful attempts to boost his humoral immunity against pneumococci (Bruton, 1952). Initiating from the observation that the boy had undetectable levels of serum immunoglobulins as determined by electrophoresis, Bruton went on to show that the child was suffering from a more generalized inability to synthesize antibodies of any kind. After recognizing the putative cause of the problems, Bruton demonstrated the cause–effect relationship between the absence of immunoglobulins and the clinical profile of the child by supplying him subcutaneously with preparations of normal human gamma globulin that dramatically improved his condition. In his own words: "With the demonstration of the absence of gamma globulins, and the apparent control of infection by furnishing gamma globulins, there seems little doubt of the existence of a direct relationship in this case" (Bruton, 1952). While patients with reduced levels of gamma globulins in the serum had been described before (Longsworth and MacInnes, 1940; Schick and Greenbaum, 1945; Krebs, 1946) this was the first time that severe hypogammaglobulinemia was presented as a distinct disease entity, thus, admittedly marking the beginning of the field of immunodeficiency.

It has been noticed by several investigators that the boy described by Bruton in 1952 may not have had XLA (Conley and Puck, 1988b; Burgio *et al.*, 1993). Thus, the patient was healthy until the age of 4½ years, considerably longer than most patients with confirmed XLA (Lederman and Winkelstein, 1985). He was tonsilectomized and there was no mentioning of abnormal architecture of the removed tissue. There was no family history of disease, although this is not to be expected in one-third of patients with XLA (Morton and Chung, 1959; Chase and Murphy, 1973). It is interesting to note that in his original article (1952) Bruton himself writes: "However, two possibilities seem apparent; (1) a congenital dysfunction in the mechanism of gamma globulin production and (2) an acquired dysfunction in this mechanism. The fact that the patient survived 4½ years and without severe infection appears to make the first unlikely."

Already the same year Bruton together with Apt, Gitlin, and Janeway described two male siblings with agammaglobulinemia (Bruton *et al.*, 1952) and stated: "It is postulated that these children have a congenital defect of the plasma proteins analogous to hemophilia or afibrinogenemia." In the discussion following the presentation of this paper to the Society for Pediatric Research, Dr. Lytt Gardner, Baltimore suggests: "we might be dealing with a simple Mendelian recessive trait" (Bruton *et al.*, 1952). Through the work mainly of Janeway and his associates resulting in the identification of nine boys with deficiency

of serum gamma globulins the concept that agammaglobulinemia may manifest X-linked inheritance emerged (Janeway *et al.*, 1953). The originality of Bruton's findings and conclusions is clearly stated in a remark made by Janeway himself (Janeway, 1954):

> To begin with, we should have been, but were not, bright enough to discover the disease, because the first patient with symptom complex, the basic cause of which was the inability to produce gamma globulins was seen by us as long ago as 1942. . . . It was one of those situations in which all of us said 'There must be something wrong with this boy,' but we did not know what it was. Then I received a letter in 1950 by Colonel Bruton, Chief of Pediatrics at the Walter Reed Army Hospital. He wrote me a long letter, describing a case which since has been published in Pediatrics.

Patients with mild forms of XLA resembling common variable immunodeficiency (CVID) have been described (Buckley and Sidbury, Jr. 1968; Goldblum *et al.*, 1974; Wedgwood and Ochs, 1980; Leickley and Buckley, 1986; Conley and Puck, 1988a) and recent mutation analysis confirms that certain individuals indeed have a mild form of the disease (Vorechovsky *et al.*, 1993c; Bradley *et al.*, 1994; Ohta *et al.*, 1994; Saffran *et al.*, 1994; Vihinen *et al.*, 1994c). In fact, in a recent survey 21% of XLA patients presented between the ages of 3 and 5 years (Hermaszewski and Webster, (1993). Thus, it cannot with certainty be excluded that the patient described by Bruton indeed had XLA.

However, much more importantly, the description of this patient heralded a new era in immunology, the study of immunodeficiencies. Within 10 years after Bruton's original report approximately 300 patients with immunoglobulin deficiency were described in 214 references as reviewed by Good *et al.*, (1962). In their 1984 review on primary immunodeficiencies, Rosen, Cooper, and Wedgewood (Rosen *et al.*, (1984) stated: "Some 30 years since its initial description, this syndrome remains the prototype for discussion of primary humoral immunodeficiencies. Known by many names—including "Bruton's. . . ."

Today the study of immune deficiencies is a major tool in basic research as novel forms are constantly being created by current state-of-the-art technologies, such as gene targeting, to investigate the putative role of genes thought to be active in the immune system.

III. Congenital and Acquired Agammaglobulinemia in the Early 1950s

Apart from the studies of Bruton (1952) and Janeway and his associates (Janeway *et al.*, 1953; Janeway, 1954), several reports appeared

in 1953 and 1954 describing patients with agammaglobulinemia (Jean, 1953; Keidan *et al.*, 1953; Olhagen, 1953; Hayles *et al.*, 1954; Jacobsen, 1954; Lang *et al.*, 1954; Sanford *et al.*, 1954; Moncke, 1954; Prasad and Koza, 1954; Saslaw and Wall, 1954). The obvious difficulty in these early days was the correct classification of patients. Gamma globulins were amenable for analysis, whereas defects in cellular immunity were not clearly recognized as the concept of different forms of lymphocytes, B and T cells, was developed later (Miller *et al.*, 1962; Mitchell and Miller, 1968; Good, 1973). In the early 1950s the debate on the cellular origin of gamma globulins as being lymphocytes or plasma cells was still ongoing (Good, 1973), although previous studies had indicated that plasma cells were the main source (Kolouch, 1938; Fagraeus, 1948). In 1956 the Bursa of Fabricius was recognized as a site for antibody-producing cells (Glick *et al.*, 1956) and it was not until the next decade that the X-linked hyper-IgM syndrome was first described as a separate disease entity (Israel-Asselain *et al.*, 1960; Burtin, 1961; Rosen *et al.*, 1961).

Patients were frequently classified as having agammaglobulinemia with or without lymphopenia (Gitlin and Craig, 1963). As an example the patient described by Keidan *et al.*, (1953) who was frequently refered to as having "agammaglobulinemia" (Martin, 1954; Elphinstone *et al.*, 1956; Wallenborn, 1960) most likely had an autosomal recessive form of severe combined deficiency (SCID) as she was the child of first cousins, developed fatal vaccinia, and was found to have both agammaglobulinemia and lymphocytopenia. As discussed in a clinical staff conference (Martin *et al.*, 1957), based on the findings of Good and Zak (1956) agammaglobulinemia was considered to be caused by a general disease of the reticulum having different features as lymphocytopenia, plasma cell defect, and recurrent neutropenia. Also in surveys published much later (Hill, 1971) difficulties in the classification are apparent (Hermaszewski and Webster, 1993). Another example of the understanding of the immune system in the early 1950s is given in the discussion of the report by Jean (1953). The paper ends with the sentence mentioning the possibility that the "nonspecific" gamma globulins, given as substitution therapy, may be transformed into specific antibodies.

Furthermore, the observation in the early 1950s of congenital agammaglobulinemia in females (Pearce and Perinpanayagam, 1957) as well as adult onset (Olhagen, 1953; Saslaw and Wall, 1954; Jacobsen, 1954; Lang *et al.*, 1954; Prasad and Koza, 1954) clearly demonstrated that there were other forms of agammaglobulinemia than XLA.

IV. Early Studies on the Origin of Agammaglobulinemia

In the 1950s experiments were carried out in individuals with agammaglobulinemia demonstrating that the half-life of gamma globulins was normal as first described by Bruton *et al.*, (1952) and Janeway *et al.*, (1953) and shortly after confirmed by other groups (Lang *et al.*, 1954; Young *et al.*, 1955; Good and Zak, 1956). Studies of patients with agammaglobulinemia revealing the absence of plasma cells further supported the notion that plasma cells were the main source of antibodies, and indicated that the underlying defect was in the synthesis rather than in the catabolism of gamma globulin (Olhagen 1953; Craig *et al.*, 1954; Good, 1954). Good mentions as a personal communication (Good and Zak, 1956) that N. Neuhauser called attention to the almost complete absence of adenoid tissue in patients with agammaglobulinemia. It was also demonstrated that immediate-type hypersensitivity could be transfered from an allergic child to a child with agammaglobulinemia using the Prausnitz–Kustner test (Good and Zak, 1956).

Kulneff *et al.* (1955) reported that BCG vaccination produced hypersensitivity in two out of three patients. Porter (1955) reported delayed-type hypersensitivity but the absence of tubercle polysaccharide antibodies following BCG vaccination in a child with XLA and that skin sensitivity to 2,4-dinitrofluorobenzine (DNFB) was normal. Good and Zak (1956) also found that skin sensitivity to DNFB was readily produced in agammglobulinemia, whereas the development of bacterial hypersensitivity was impaired. However, intravenous or intramuscular injection of 20 ml of blood cells from unrelated donors to patients with agammaglobulinemia was found not to result in the febrile reactions and changes in platelet and leukocyte levels seen in normal individuals (Good and Zak, 1956). Furthermore, Good and Varco (1955) reported that a skin graft from a 7-year-old boy with agammaglobulinemia, lacking plasma cells but showing normal blood lymphocyte levels, was rejected in a 2½-year-old girl after the expected time, whereas a skin graft from a healthy adult female was not rejected during an observation period of 11 months in the agammaglobulinemic boy. Thus, most of the early clinical studies were compatible with normal cellular immunity in XLA. However, exceptions, such as the lack of graft rejection, possibly related to disease classification, were found.

To investigate the possibility of a circulating substance that could inhibit antibody formation, 75 ml of blood plasma was transferred from a boy with agammaglobulinemia to a child with irreversible hydrocephalus. Subsequent vaccination induced a normal humoral immune

response without affecting the total gamma globulin level. These investigators also demonstrated that transfer of leukocytes from an agammaglobulinemic child to an adult healthy volunteer was uneventful (Good and Zak, 1956). To rule out an influence of the adrenal–pituitary system on the development of agammaglobulinemia the adrenal function was studied in five patients and found to be normal (Good and Kelley, 1955).

V. The Name Issue and the Locus Designation

The term agammaglobulinemia, originally coined by Bruton (1952), was based on the use of serum electrophoresis. When more sensitive techniques were employed it was soon realized that these patients had very low, but detectable levels of gamma globulin. On the name issue, Janeway (1954) stated "This disease should probably have been called "hypogammaglobulinemia"; we have used the term "agammaglobulinemia", since this had already been used and, for all practical purposes, the congenital cases do not have any gamma globulin." Good *et al.*, (1962) make the following analogy: "A substantial precedent exists in the use of the homologous term *anemia* although a deficiency of red cells and hemoglobin, rather than complete lack of these elements, is indicated by this expression". Several other names have been advocated, such as panhypogammaglobulinemia, as well as using agammaglobulinemia in combination with "Bruton's," "congenital," "infantile," and "sex-linked" (Pearce and Perinpanayagam, 1957; Seligmann *et al.*, 1968). Over the years XLA has become the generally accepted name (Cooper *et al.*, 1973; Rosen *et al.*, 1984; WHO Scientific group, 1992), and Bruton's disease/agammaglobulinemia is used synonymously. In the Mendelian Inheritance in Man classification the disease is called "Agammaglobulinemia [Bruton type]" with a MIM number "300300" (Online Mendelian Inheritance in Man, OMIM) (Pearson *et al.*, 1994) or Agammaglobulinemia [Bruton type, AGMX1] MIM number 300300 (The Johns Hopkins University, Baltimore, MD). The locus, formerly designated IMD1, is now called AGMX1, as assigned by the Human Gene Mapping Workshop (Mandel *et al.*, 1989).

There is at present no indication of an additional XLA locus. As will be reviewed in a following section, based on the observations in a single kindred it was erroneously anticipated that there was a second XLA locus (Hendriks *et al.*, 1989). However, the defective gene was inherited from a male demonstrating X chromosome mosaicism, and it is now agreed that there is no evidence for more than one X-chromosomal locus for XLA (Davies *et al.*, 1990). Of note is that the

incorrect locus, which was assigned to Xp22 and designated AGMX2, MIM300310, has not yet been removed from the databases.

VI. Incidence and Prognosis

Several investigators have estimated the frequency of XLA in the population. In most studies the incidence is around $5/10^6$ (Table I). It was discussed early that familial agammaglobulinemia might be a disease of the "Northern European stock" (Elphinstone *et al.*, 1956). However, despite that 89/96 (93%) of the XLA cases were Caucasian in a large American study (Lederman and Winkelstein, 1985), it seems likely that the frequency of XLA, being an X-linked, previously lethal trait, does not vary among ethnic groups. Prior to antibiotics and immunoglobulin replacement all XLA patients died at a young age (Jamieson and Kerr, 1961; Leickley and Buckley, 1986). Analysis of patients born before 1950 in a large Dutch pedigree revealed that there was a 90% probability for children with XLA to die between 2 months and 8 years of age (Mensink *et al.*, 1984). With the advent of antibiotics and gamma globulin replacement therapy the prognosis improved considerably (Lederman and Winkelstein, 1985) and it seems as if this process is still ongoing as Hermaszewski and Webster (1993) report that there is a trend toward better survival in patients recently diagnosed as having XLA. This trend is likely to continue as future therapies, such as gene replacement, even have a curative potential.

TABLE I
INCIDENCE OF XLA

Study	Geographic Region	Incidence/10^6	Deficiency in Males
Squire, 1960	UK	$10/10^6$	Congenital agammaglobulinemia
MRC Working Party, 1969	UK	$15/10^6$	Hypogammaglobulinemia + SCID
Hayakawa *et al.*, 1981	Japan	56 Cases in Japan	XLA
Koch *et al.*, 1981	Denmark	$6/10^6$	XLA
Fasth, 1982	Sweden	$6/10^6$	Congenital agammaglobulinemia
Luzi *et al.*, 1983	Italy	33 Cases in Italy	XLA
Hosking and Roberton, 1983	Australia	$7/10^6$	XLA
McCluskey and Boyd, 1991	Northern Ireland	$6/10^6$	XLA and hyper IgM syndrome

VII. Clinical Manifestations in XLA

Onset of symptoms in patients with XLA is normally within the first year of age with mean age of diagnosis around 2½ years in familial cases and 3½ in nonfamilial (Lederman and Winkelstein, 1985; Hansel *et al.*, 1987). However two out of five affected boys remain asymptomatic in their first year of life and one out of five present between the ages of 3 to 5 years (Hermaszewski and Webster, 1993). A generalized frequent usage of antibiotics is likely to influence the time of onset.

The clinical observations in patients with XLA have been of great importance for the understanding of the role of antibodies in the immune defense. However, several clinical findings in patients with XLA remain unexplained. The symptoms and signs can be subdivided into those directly associated with an infection and into those where an association is not proven. However, history has clearly taught us that seemingly noninfectious symptoms or signs may be late manifestations of an infection or be caused by a microorganism not yet identified.

One way of studying these phenomena is to compare various forms of immunodeficiencies. Although most manifestations are similar in patients with different immunoglobulin deficiencies there are several examples of those being more as well as less frequent in XLA. It is apparent that the virtual absence of both immunoglobulins and B lymphocytes in the majority of patients distinguishes XLA from most other deficiencies. This defect results in a typical feature of XLA, namely, the lack of an antibody response and the failure to clear microbial antigens, such as bacteriophage ϕX174, as demonstrated by experimental immunizations (Ochs *et al.*, 1971).

A. Bacterial Infections

The hallmark of immunoglobulin deficiencies is an increased susceptibility to pyogenic bacterial infections (Good and Zak, 1956; Lederman and Winkelstein, 1985, Stiehm *et al.*, 1986; Spickett *et al.*, 1991). This can be exemplified by a comment made by Janeway (1954): "I think anybody in the bronchiectasis clinic now should go through their patients and screen them this way [screen for lack of isoagglutinins] to find out what the story is." As presented in Table II, the most frequent sites of infections in both the 209 patients with XLA and the 401 with CVID were the respiratory and gastrointestinal tracts and the skin. The variation in the frequency of manifestations among these studies is likely to be technical in origin, reflecting differences in the classification of infections rather than being true divergencies. Similar figures were obtained in other clinical surveys (Goddard and Beatty,

TABLE II
INFECTIONS IN PATIENTS WITH XLA

Presenting Infection	Percentage of Patients				
	Study				
	Lederman and Winkelstein, 1985	Hermaszewski and Webster, 1993		Hansel *et al.*, 1987	
	XLA (n = 96)	XLA (n = 44)	CVID (n = 240)	XLA (n = 69)	CVID (n = 161)
Ear, nose, and throat infections	75	52	3	22	22
Pneumonia	56	32	63	67	90
Gastrointestinal infections	35	11	12	16	20
Bacterial skin infection	28	14	11	dc[a]	dc
Meningitis	10	5	7	17	5
Septicemia	10	7	2	dc	dc
Osteomyelitis	3	5	3	dc	dc

[a] dc, Different classification used, preventing a direct comparison.

1989). Few investigators have found an apparently increased rate in genito-urinary infections (Thompson and Rees-Jones, 1979) albeit infections with mycoplasma species may be occurring (Hermaszewski and Webster, 1993). Bacterial infections are seen in all forms of immunoglobulin deficiency and are also found in complement defects and often in granulocyte abnormalities and demonstrate the role of antibodies in complement and phagocyte-mediated killing of microorganisms (Johnston, 1984).

B. ENTEROVIRAL INFECTIONS

Most viral infections run a normal course in XLA (Janeway *et al.*, 1953; Good and Zak, 1956; Gitlin *et al.*, 1959; Squire, 1962). However, patients with immunoglobulin deficiency are unduly susceptible to chronic enteroviral infection (Maller *et al.*, 1967; Linneman *et al.*, 1973; Ziegler and Penny, 1975; Lederman and Winkelstein, 1985; McKinney *et al.*, 1987; Hermaszewski and Webster, 1993). Chronic echoviral disease may be manifested as a dermatomyositis-like syndrome, edema, hepatitis, and meningoencephalitis, and already in the 1950s XLA patients with such symptoms were observed (Janeway *et al.*, 1956; Good *et al.*, 1957), later followed by several reports prior to the realization of an infectious cause (Page *et al.*, 1963; Gotoff *et al.*, 1972).

In the study of Lederman and Winkelstein (1985) one-half of the deaths observed in patients with XLA resulted from severe viral infections. In the review of McKinney *et al.*, (1987) 42 patients with agammaglobulinemia having chronic enteroviral meningoencephalitis are described, 32 of which had classical or probable XLA. Tedder *et al.*, (1985) suggested that the T cell immaturity they observed in XLA might account for the increased frequency of enteroviral disease. However, in CVID T cell abnormalities are frequently seen despite the lower incidence of enteroviral disease and in patients with predominant T cell defects enteroviral disease is not known as a major infection (McKinney *et al.*, 1987).

Echoviruses dominate but other enteroviral infections have also been reported (Saulsbury *et al.*, 1980; Cooper *et al.*, 1983; McKinney *et al.*, 1987). Patients with agammaglobulinemia are highly susceptible to poliovirus infection as reviewed by Wyatt (1973) and vaccine-associated poliomyelitis has been observed in XLA (Wright *et al.*, 1977). The increased risk for vaccine-associated poliomyelitis in hypogammaglobulinemics was estimated to be 10^4 (Wyatt, 1973).

It seems reasonable to believe that the virtual absence of immunoglobulins both as secreted products and at the cell level predisposes

to enteroviral disease in XLA. Furthermore, the fact that other viral diseases run a normal course in XLA may suggest that B lymphocytes are not critical as antigen-presenting cells to induce antiviral cellular immunity.

C. Miscellaneous Infections

Protozoal infections, such as *Giardia lamblia* and *Pneumocystis carinii*, may also be seen in XLA as in other immunoglobulin deficiencies (Ochs *et al.*, 1972; Lederman and Winkelstein, 1985; Spickett *et al.*, 1991). Mycoplasma species have sometimes caused infections including arthritis (Stuckey *et al.*, 1978; Lederman and Winkelstein, 1985; Roifman *et al.*, 1986). The finding that the pathogenesis in experimental mycoplasma infection differs between athymic and SCID mice further substantiates the role of antibodies in this infection (Evengård *et al.*, 1994).

D. Neutropenia

Neutropenia is frequently observed in patients with agammaglobulinemia, including XLA (Janeway, 1954; Laski *et al.*, 1954; Good and Zak, 1956). In the study of Lederman and Winkelstein (1985), 10% of the XLA patients were found to have neutropenia in association with infections. The neutropenia resolves after replacement therapy and is likely to be caused by increased neutrophil destruction, secondary to an ongoing infection, and not by depressed production (Kozlowski and Evans, 1991).

E. Arthritis

In the 1950s a high incidence of rheumatoid arthritis was reported in agammaglobulinemia, including XLA (Good *et al.*, 1957). In later studies this polyarthritis was referred to as rheumatoid arthritis-like and was observed in lower frequency (Squire, 1962; McLaughlin *et al.*, 1972), especially in the acquired form of agammaglobulinemia (Wollheim *et al.*, 1964). Lederman and Winkelstein (1985) found arthritis, including polyarthritis, in 20% of their XLA cases, and slightly less than half were caused by acute pyogenic infections. The arthritis often responds to immunoglobulin treatment (Webster *et al.*, 1976).

F. Autoimmunity

There are only few reports on autoimmunity in XLA. Crohn's disease has been documented in an increased frequency in XLA (Eggert *et al.*, 1969). Rare cases of autoimmune hemolytic anemia in XLA have

also been published, whereas a pernicious anemia-like condition is found in one out of three with CVID but rarely in XLA (Rosen, 1987). A higher frequency of autoimmunity in CVID patients was also reported by others (Cunningham-Rundles *et al.*, 1987; Hermaszewski *et al.*, 1991). Thus, in the main, these findings are compatible with the idea that complete loss of the humoral immune system, as seen in XLA, does not predispose to autoimmunity.

G. Cancer

There are a large number of studies reporting tumors in patients with XLA (Table III). In the study of Kinlen *et al.*, (1985) 7/220 (3%) of CVID patients developed stomach cancer (47-fold increase) compared to 1/65 (1.5%) with XLA. An even higher frequency of lymphoid tumors in CVID was reported by Cunningham-Rundles *et al.*, (1987). Due to the low risk of tumor development compared to several other immunodeficiency disorders (Spector *et al.*, 1978), as well as the limited number of patients with XLA, accurate risk frequencies for different tumors are difficult to give. However, from the studies presented in Table III, despite the fact that the diagnosis may not be unquestionable in every single case, an increased risk for tumor development in XLA at a young age seems apparent.

The underlying mechanisms of the seemingly increased tumor incidence in XLA remain elusive, but an infectious origin is not unlikely. Thus, stomach cancer may be related to an abnormal bacterial flora, and as XLA patients are prone to infections with enteroviruses, the existance of tumorogenic forms would constitute another mechanism. We have so far been unable to detect expression of the *Btk* gene in cell lines from nonlymphoid tissues known to form tumors in XLA patients (Smith *et al.*, 1994a). Furthermore, as chromosomal abberations in the Xq22 region have not been noted in B cell tumors, as expected, alterations in the *Btk* gene were not observed in hematopoietic tumors (Katz *et al.*, 1994; Vorechovsky *et al.*, 1994b, C. Kinnon, personal communication).

H. Miscellaneous Manifestations

A number of other less frequent manifestations, such as amyloidosis and thrombocytopenia, have been reported in XLA, as reviewed by Lederman and Winkelstein (1985) and Hermaszewski and Webster (1993). Apart from the differences already described when comparing XLA and CVID, another manifestation of CVID that distinguishes it from XLA is the frequent granuloma formation often seen in the spleen and adjacent lymph nodes (Prasad and Koza, 1954; Hermaszewski and Webster, 1993).

TABLE III

TUMORS IN PATIENTS WITH XLA

Study	No. of Patients	Age at Tumor Diagnosis	Malignancy
Page *et al.*, 1963	2	3,4	Acute leukemia, malignant lymphoma
Reisman *et al.*, 1964	1	—	Chronic myelogenous leukemia (CML)
Miller, 1969	1	—	Leukemia
Gellman and Vietti, 1970	1	11	Hodgkin's disease
Gatti and Good, 1971	2	—	Acute lymphoblastic leukemia (ALL), thymoma with leukemia
Kersey *et al.*, 1973		(Lists five of the above six cases)	
Spector *et al.*, 1978	14/274	1,1–20	12 Mesenchymal, 2 cancer
Kinlen *et al.*, 1985	1/65	25	Stomach cancer
Lederman and Winkelstein, 1985	2/96	10,17	Reticulum cell sarcoma of the bowel, B cell lymphoma
Adachi *et al.*, 1992	1	22	Multiple colorectal malignancies
Lavilla *et al.*, 1993	1	23	Stomach adenocarcinoma
van der Meer *et al.*, 1993	3	15,19,35	Colorectal cancer
Hermaszewski and Webster, 1993	1/44	—	Pituitary adenoma
Smith and Hammarström, unpublished results	1	20	Colorectal adenocarcinoma

VIII. Agammaglobulinemia with Growth Hormone Deficiency

In 1980 a family with four affected males that had isolated growth hormone deficiency as well as clinical and laboratory findings suggestive of XLA was described (Fleisher *et al.*, 1980). This entity has been given MIM No. 307200. However, the presence of some tonsillar tissue seen in these males is an unusual feature in XLA. Furthermore, recent studies in this family have demonstrated recombination with the DNA marker DXS178 (see Section XIII) as well as nonrandom X-chromosome inactivation (see Section XII,A), distinguishing this kindred from XLA (Stewart *et al.*, 1994; Nelson, personal communication). Additional cases have been described and selective use of one of the X chromosomes in obligate carriers has been identified when investigated (Sitz *et al.*, 1990; Monafo *et al.*, 1991; Conley *et al.*, 1991; Buzi *et al.*, 1994; Duriez *et al.*, 1994). These findings are compatible with a contiguous gene deletion syndrome and evidence also exists in favor of an X-linked form of growth hormone deficiency (Phillips and Vnencak-Jones, 1989). However, as described in more detail under Section XVII the study of patients with an XLA phenotype and concomitant growth hormone deficiency has so far ruled out contiguous deletions in the investigated cases and instead demonstrated mutations confined to the *Btk* gene (Vihinen *et al.*, 1994b; Vorechovsky *et al.*, 1994a). The possibility of growth hormone deficiency secondary to enteroviral meningoencephalitis has also been advocated (A.D.B. Webster, personal communication), and one of the boys in the original report has been described as having contracted enteroviral disease (Wagner *et al.*, 1989).

IX. Female XLA

Female carriers of XLA are healthy and demonstrate no abnormalities of the immune system as B cells expressing the mutated gene are selected against (Conley *et al.*, 1986; Fearon *et al.*, 1987). However, females might show signs of XLA as they could have a translocation inactivating one of the XLA loci in conjunction with a mutation in the other. Alternatively, they could have a mutation in another gene, the product of which influences the protein affected by XLA. Other more remote possibilities involve extreme lyonization (inactivation of the nonmutant X), inheritance of two mutant X chromosomes, Turner's syndrome (a single X and no Y chromosome) with an XLA mutation, or uniparental disomy (two copies of the same X) (Conley and Sweinberg, 1992). Several female cases with congenital hypogammaglobulinemia

and the absence of B lymphocytes have been described (Aiuti *et al.*, 1973; Hoffman *et al.*, 1977; Luzi *et al.*, 1983; Buckley, 1986; McKinney *et al.*, 1987; Conley and Sweinberg, 1992). Conley and Sweinberg (1992) estimated that approximately 10% of patients with XLA are females. The identification of the underlying defect in these patients may shed light on the Btk signaling pathway.

X. Treatment

A. Antibiotics and Immunoglobulin Substitution Therapy

Since the original description of Bruton (1952) the treatment of XLA has been the combination of antibiotics and gamma globulin replacement therapy. The concept of Janeway (1954) still prevails: "You have to get them out of their acute episodes with antibiotics; you can keep them out of further acute episodes with gamma globulin." Bruton used subcutaneous administration of gamma globulin. This was later followed by intramuscular injections (Council of Farmacy and Chemistry, 1956; Medical Research Council Working Party, 1969). The result of the development of gamma globulin for intravenous use was that increased doses could be administered (Clinical Immunology Committee of the International Union of Immunological Societies, (1982) and this treatment regimen as well as rapid subcutaneous infusions (Ochs *et al.*,1986; Gardulf *et al.*, 1991) have resulted in safer administration allowing home treatment. The frequency of bacterial infections decreases after initiating gamma globulin treatment, whereas the effect on existing viral infections, such as the enteroviral-induced meningoencephalitis, is weak (Mease *et al.*, 1981,1985; McKinney *et al.*, 1987; Hermaszewski and Webster, 1993). However, it is possible that high dose gammaglobulin substitution therapy has a prophylactic effect on enteroviral infections (Liese *et al.*, 1992).

As with other patients with immunoglobulin deficiency receiving substitution therapy, XLA patients have contracted hepatitis C following intravenous administration of contaminated batches (Lever *et al.*, 1984; Williams *et al.*, 1989; Yap *et al.*, 1994). The hepatitis C seems to affect patients with CVID more severely compared to those with XLA, possibly secondary to the defect in cellular immunity seen in these patients (Hermaszewski and Webster, 1993).

B. Gene Therapy

Despite the fact that most XLA patients respond well to current treatments, it is obvious that passive administration of gamma globulin is inferior to an active immune response to foreign antigens. Bone

marrow transplantation has been applied for XLA (Good, 1987), but due to the often good prognosis of XLA and the hazards involved in bone marrow transplantation this treatment form is rarely used.

Gene therapy is the obvious future choice for hereditary disease although many obstacles still need to be addressed before this regimen can be attempted in XLA. Thus, this treatment form is mentioned in several papers including the two articles describing the successful cloning of the disease gene (Vetrie *et al.*, 1993b; Tsukada *et al.*, 1993). In the case of XLA one putative obstacle is that the gene encodes a PTK, as certain PTKs have been implicated in tumor formation when mutated or overexpressed (Heisterkamp *et al.*, 1990; Cantley *et al.*, 1991; Abraham *et al.*, 1991). Given the fact that current therapy in XLA is quite successful, this potential hazard remains an important obstacle for introducing at least certain forms of gene therapy. A more comprehensive understanding of the Btk signaling pathway as well as the control of Btk gene expression is therefore warranted. Technologies based on repairing or replacing the endogenous mutated gene would presumably also be acceptable, as normal regulation is likely to ensue.

XLA shares an important feature with adenosine deaminase deficiency, which constitutes the current prototype for hematopoietic cell gene therapy. Thus, in adenosine deaminase deficiency there is a natural selection of cells that have been gene corrected, as naive T cells lacking this enzyme will die from toxic metabolites. Gene therapy for this disease has been attempted without any apparent side effects (Culwer *et al.*, 1991). In XLA a similar selective advantage would be anticipated as only corrected cells are likely to mature into fully competent B lymphocytes.

XI. B Cell Development

A. Hematopoietic and B Lymphocyte Progenitors

All the cells of the hematopoietic system are derived from a common multipotential population of stem cells that differentiate and gradually generate precursors that are more restricted in terms of their capacity to generate the different blood lineages (Wu *et al.*, 1968; Abramson *et al.*, 1977). Progenitors that can give rise to B lymphocytes are first found in the embryonic part of the placenta and later in the omentum and fetal liver (Tyan and Herzenberg, 1968; Paige *et al.*, 1979; Melchers, 1979; Godin *et al.*, 1993; Medvinsky *et al.*, 1993; Marcos *et al.*, 1994). Well before birth in humans, and around the time of birth in

mice, the bone marrow becomes the major source of hematopoietic stem cells and, consequently, of B cell precursors (Rolink *et al.*, 1993).

One can divide the early B cell developmental process in the primary lymphoid organs into two phases. The events that occur during the first phase are most likely responsible for the commitment of the early precursors to the B lineage developmental pathway. Cell to cell interactions between the hematopoietic stem cells and stroma cell components as well as signals delivered to the differentiating precursors by cytokines that are localy produced must play a crucial role during this phase (Ogawa *et al.*, 1988; Nishikawa *et al.*, 1988; Sudo *et al.*, 1989; Melchers *et al.*, 1992; Palacios and Nishikawa, 1992; Nishikawa *et al.* 1992).

Once the hematopoietic precursors become committed to the B cell lineage the events that occur during the second phase will determine which of their progeny will eventually enter the pool of newly generated B cells. This phase of B cell development is characterized by the successive rearrangement of immunoglobulin variable region segments (Alt *et al.*, 1984, 1987).

B. Immunoglobulin Gene Rearrangements

In the beginning the differentiating cells rearrange a D to a J_H segment, thus giving rise to a pro-B cell. Subsequently, a V_H gene is joined to the D_HJ_H complex and if the result is a functional $V_HD_HJ_H$ complex then a pre-B cell is generated. Finally, if the light-chain variable region, V_L and J_L segments, are productively rearranged, the cells will develop into surface IgM ($sIgM^+$) and eventually IgD ($sIgM^+/IgD^+$) bearing mature B cells.

The progression along this pathway is regulated by the sequential expression of productively rearranged Ig loci, namely, the $DJ_HC\mu$ proteins, μH chains, and the κ and λ light chains. Two other genes, the V_{preB} (Kudo and Melchers, 1987) and $\lambda5$ (Sakaguchi and Melchers, 1986), encode for proteins that can associate noncovalently to form a light-chain-like structure known as the surrogate light chain (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990; Melchers *et al.*, 1993). The surrogate light chain can interact covalently with the $DJ_HC\mu$, the μH, and with a p55 protein that has been speculated to be the forerunner of the $DJ_HC\mu$ to create Ig-like structures that can be deposited on the surface of committed progenitors and precursors of the B cell lineage (Tsubata and Reth, 1990; Karasuyama *et al.*, 1993). It is believed that in this way the variable-like regions of these chains can be exposed to, and interact with, fetal liver or bone marrow stroma cells. This interaction is thought to transduce signals to the developing B cell

precursors inducing the next round of Ig rearrangement and the expression of new B cell differentiation-related genes.

Over the past few years, dramatic progress in the understanding of the early events of B lymphocyte development has taken place. Work in several laboratories has helped to characterize cell populations that develop sequentially from the early committed precursors to mature B cells (Hardy *et al.*, 1982; Osmond, 1990; Hardy *et al.*, 1991; Rolink and Melchers, 1993; Era *et al.*, 1994). This was done primarily in the mouse system and was due to (a) the development of useful antibody reagents specific for surface molecules that were differentially expressed during lymphocyte ontogeny, (b) the development of *in vitro* culture systems for growing early B cells precursors (Whitlock and Witte, 1982), and (c) the utilization of gene targeting or "knockout" methodology to disrupt genes that are important for B cell development. The bone marrow, being the primary site of B lymphopoiesis in adult mice and humans, received the major part of attention.

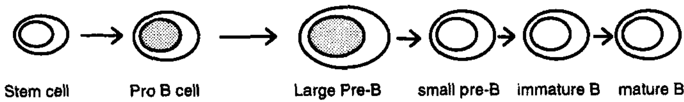
C. Subpopulations of B Cell Precursors

Using flow cytometry multiparameter analyses and a set of antibody reagents recognizing cell surface markers differentially expressed during B cell development bone marrows were analyzed and subpopulations of cells representing different stages in the B cell developmental pathway were defined on the basis of specific surface marker profiles (Hardy *et al.*, 1991). Using antibody reagents against the S7 leukosialin (CD43) (Baecher *et al.*, 1988; Gulley *et al.*, 1988), BP-1 (Wu *et al.*, 1989), and 30F1 anti-heat-stable antigen (Kay *et al.*, 1990) IgM, the $B220^{low}S7^{+}IgM^{-}$ B-lineage cells in the bone marrow were subdivided into four functionally distinct populations that were provisionally termed Fractions A, B, C, and C′, and the $B220^{+}S7^{-}$ cells were subdivided into another three populations provisionally termed fractions D, E, and F (Hardy *et al.*, 1991). The surface phenotypes of these subpopulations is summarized in Table IV. Development of B cell precursors was shown to procede from stage A to B to C to C′ to D to E to F (Hardy *et al.*, 1991). Fractions A and B were found to be enriched in pro-B cells, fractions C and C′ in early, large, actively proliferating pre-B cells, fraction D in small nonproliferating pre-B cells, fraction E in immature newly synthesized B cells, and fraction F in mature IgM/IgD-positive B cells (reviewed in Hardy *et al.*, 1994; Era *et al.*, 1994; Löffert *et al.*, 1994).

Elegant studies that utilized gene-targeted mice to analyze the *in vivo* requirements of B cell differentiation demonstrated that while B cell progenitors could go through the early stages of development

TABLE IV
ALTERATIONS IN THE EXPRESSION OF SURFACE MARKERS DURING THE DEVELOPMENT OF B LYMPHOCYTES

Fraction	A	B	C	C'	D	E	F
CD43	+	+	+	+	-	-	-
B220	+	+	+	+	+ +	+ +	+ + +
HSA	-	+	+	+ +	+ +	+ +	-
BP1	-	-	+	+	+	+	-
cIgM	-	-	-	+/-	+		
sIgM	-	-	-	-	-	+	+
TdT	(+)	+	+	+/-	-	-	-
ckit	(+)	+	+	+	-	-	-
IL-7R	(+)	+	+	+	+	-	-



CD19	+ +	+ +	+ +	+ +	+ +
CD34	+ +	+ +	-	-	-
CD10	+ +	+ +	+		
cCD22	+ +	+ +	+ +	-	-
cIgM	-	+	+ +		
sIgM	-	-	-	+ +	+ +
CD20	+	+	+	+ +	+ +
CD37	+	+	+	+ +	+ +
CD22	-	-	-	+ +	+ +
CD21	-	-	-	-	+ +
TdT	+ +	-		-	-
TdT XLA*	+ +	(+ +)		-	-
Ki67	+ +	+ +		-	-
Ki67 XLA*	+ +	(-)		-	-

[a] Fractions A–F represent the different stages in the development of mouse B cell precursors according to the nomenclature introduced by Hardy *et al.* (1991). The dissection of the human developmental pathway is not as well described as that in the mouse. The bottom of the table is based on the findings of Janossy *et al.* (1979,1980), Campana *et al.* (1985,1989,1990), and Loken *et al.* (1987). The gray shaded area indicates the period where particularly dramatic changes occur in both mouse and human B cell development and during which the defect in XLA could be manifested. Asterisks indicate peculiar phenotypes that are only seen in bone marrows from patients with XLA.

independententiy of Ig gene rearrangement, in order to reach the final stages of differentiation they needed to express appropriately rearranged Ig genes (reviewed by Löffert *et al.*, 1994). The majority of the B cell precursors that are generated in the bone marrow die without maturing into B cells. It has been calculated that almost 75% of the B cell precursors in the bone marrow are lost between the transition from stage C, large cytoplasmic μ-positive pre-B cells, to D, small cytoplasmic μ-positive pre-B cells (Obstelten and Osmond, 1983; Osmond, 1991). Interestingly, mice with a disrupted membrane exon of the μ-chain (Kitamura *et al.*, 1991), deleted the J_H locus (Chen *et al.*, 1990; Ehlich *et al.*, 1993), deficient for the RAG-2 gene (Shinkai *et al.*, 1992), and mice with the SCID mutation (Malynn *et al.*, 1988; Blackwell *et al.*, 1989) all have in their bone marrow early B cell precursors developing up to stage C. However, they are severely depleted of cells in fraction D, i.e., small pre-B cells. Large early pre-B cells, which accumulate in these mutant animals, can proceed further in the B cell developmental pathway only when, and if, they first express the pre-B cell receptor (μ-heavy chain associated with $\lambda 5$ and V_{preB} chains) and then the membrane-bound form of the μ-chain (Tsubata and Reth, 1990; Karasuyama *et al.*, 1990; Reichman Fried *et al.*, 1990; Reichman-Fried *et al.*, 1993). Apparently, the block in B cell development in the above mutants seems to coincide with the massive loss of the precursor B cells that are eliminated in normal mice because they carry nonfunctionally rearranged immunoglobulin genes (von Boehmer, 1994). This could imply that the expression of the so-called "pre-B cell receptor" could sustain survival of the large pre-B cells, induce their transition into the small pre-B cell stage, or sustain the survival of the small pre-B cells.

The majority of the light-chain genes appear to be assembled during, or at the transition to, the small pre-B cell stage (Coffman and Weissman, 1983; Ehlich *et al.*, 1993). Since the progression to the small pre-B cell stage depends on the expression of the pre-B cell receptor, it has been speculated that this receptor could actually upregulate light-chain gene rearrangements (Tsubata *et al.*, 1992). Interestingly, some of the early pre-B cell precursors can leave the $CD43^+$ compartment and develop directly into mature B cells, without going through the small pre-B cell stage, if a productive L-chain rearrangement takes place simultaneously with the productive heavy-chain rearrangement. As demonstrated in the $\lambda 5$-deficient animals this does not occur often and no more than 5% of the peripheral B cells in a normal mouse are expected to be generated in this way (Ehlich *et al.*, 1993).

Work from several laboratories has demonstrated that, during the

process of B cell development, the progressive commitment of B cell precursors to the expression of a particular H- and L-chain is associated with alterations in their growth requirements (Strasser *et al.*, 1989). Contact with stroma cells, interleukin-7 (IL-7), and the c-kit ligand has been implicated in the growth of early B cell precursors (reviewed in Cumano *et al.*, 1994; Era *et al.*, 1994; Hardy *et al.*, 1994; Löffert *et al.*, 1994). IL-7 receptors and c-kit are expressed at the stage of development represented by fraction B (according to Hardy *et al.*, 1991). While the former receptor remains expressed until surface IgM starts to appear, the latter receptor is turned off earlier around stage C′. The sequential downregulation of these receptors coinsides with major check points of B cell development, namely, the expression of cytoplasmic μ-chains, and the expression of the membrane IgM molecule. Additionally, while the developing precursors appear to require contact with stroma cells for their *in vitro* growth until stage C′, cells that develop further lose this contact dependency (Hayashi *et al.*, 1990; Rolink *et al.*, 1991).

The change in the growth requirements of the differentiating precursors at strategically chosen points reflects the change of the principles that govern their life and death. While in the early stages they should proceed with their development and commit themselves to a given cell lineage when they sense that they are within the correct microenvironment (by interacting with ligands on the surface of the appropriate stroma cells or responding to cytokines produced locally); at the later stages of development their life and death would entirely depend on whether they can actually perform the function they have been entrusted with, namely, to produce functional antibodies. It becomes almost self-evident that the quality of signals generated by either the mature Ig or the pro-B and pre-B receptors should trigger the intracytoplasmic processes that will eventually rescue the developing B cell precursors that have correctly rearranged their Ig loci (Rajewsky, 1992). B cell precursors start rearranging their Ig genes, and at the same time presumably shut down those mechanisms that sustained their growth until then. That gives them a sufficient, however, temporarily limited developmental window, during which they have to generate a functional new rescue devise by rearranging their Ig gene segments. This new rescue devise (receptor) will most probably have to rely on a new set of cytoplasmatic sensors that will be triggered to activate the downstream components of the "rescuing" signal transduction cascade. It is precisely these changes that take place on the surface and cytoplasm of the developing B cell precursors, during the early pre-B to late pre-B stage transition, that can account for the magnitude

of the phenotypic changes seen in the knockout animals that have been mentioned earlier and that could also provide some clues as to what might have gone wrong in the hematopoietic system of patients suffering from XLA. One could imagine B cell precursors as being amateur parachute jumpers, thrown from a relatively safe place in the airplane out into the empty space with a parachute that they have to master within the limited time they have on their way down. B cells in XLA could either fail to open it (reduced efficiency of recombination) or fail to maneuvre it correctly (inability to interpret rescuing stimulatory signals).

XII. B Cell Development in XLA

While the absence of gamma globulins in the serum of XLA patients and their inability to produce antibodies upon immunization were recognized almost from the beginning, it was not until 1969 that the observation of Naor *et al.* indicated the absence of surface Ig-bearing cells (Naor *et al.*, 1969). These authors demonstrated the lack of iodinated albumin binding to peripheral lymphoid cells in XLA and the presence of such cells in CVID and concluded "the ability of peripheral white cells of agammaglobulinemic patients to bind antigen strengthens our assumption that the binding is due to specific cell receptors." In the early 1970s a virtual explosion of papers demonstrated that the disorder was associated with a dramatic reduction of circulating mature B cells and reduced *in vitro* Ig synthesis (Grey *et al.*, 1971; Fröland *et al.*, 1971; Siegal *et al.*, 1971; Cooper and Lawton, 1972; Choi *et al.*, 1972; Frøland and Natvig, 1972; Yata and Tsukimoto, 1972; Preud'-Homme *et al.*, 1973; Geha *et al.*, 1973; Aiuti *et al.*, 1973; Buckley *et al.*, 1974). Whereas in normal individuals B cells comprise 5–15% of peripheral blood lymphocytes, in most patients with XLA, less than 1% are B cells (Conley, 1985). Occasionally, IgE and IgE-bearing lymphocytes have been observed in XLA (Polmar *et al.*, 1970; Gajl-Peczalska *et al.*, 1973a) in the absence of other B cells, but in the main IgD and IgE are decreased similar to the other isotypes (Newcomb and Ishizaka, 1968; Stites *et al.*, 1971; Buckley and Fiscus, 1975). Accordingly, XLA patients lack germinal center and follicles in their lymph nodes and appendices (Janeway *et al.*, 1953; Good, 1954; Porter, 1955; Gitlin and Craig, 1963; Gajl-Peczalska *et al.*, 1973b; Schiff *et al.*, 1974). The small numbers of B cells present in the peripheral blood of XLA patients were not normal and demonstrated an "immature" phenotype (Tsuchiya *et al.*, 1980; Conley, 1985; Golay and Webster, 1986). The defect could not be corrected after transfer of patient

cells to SCID mice (Saxon *et al.*, 1991). Based on the realization that lymphoid cells which contain cytoplasmic IgM, but lack stable surface IgM, are the direct precursors of B lymphocytes (Raff *et al.*, 1976; Hayward *et al.*, 1977; Gathings *et al.*, 1977; Burrows *et al.*, 1979), the presence of such cells in the bone marrow of XLA patients was investigated (Vogler *et al.*, 1976; Pearl *et al.*, 1978). While the former study demonstrated the presence of such cells in some, but not other patients Vogler *et al.*, 1976), the levels of pre-B cells in the 11 patients analyzed in the later report were variable; but always detectable and the mean frequency (3.8 ± 3.6) was not dramatically lower than that in normal samples (5.8 ± 5.7) (Pearl *et al.*, (1978). The presence of some pre-B cells in XLA patients was interpreted to suggest that in this disorder, B cell lymphopoiesis proceeds undisturbed up to the pre-B cell stage and that the XLA mutation affects the transition of B cell precursors from the pre-B to the mature B cell stage (Pearl *et al.*, 1978; Fu *et al.*, 1980; McCune and Fu, 1981). It was also demonstrated in these studies that B cell precursors from XLA bone marrows had reduced proliferative capacity in *in vitro* cultures in comparison to normal bone marrow cells (Pearl *et al.*, 1978). These extremely important observations had a significant impact on the development of the field. They demonstrated that the XLA gene must play a critical role in the early events of B cell development and indicated that the defect could reside in the capacity of early precursors to grow.

When bone marrows from a larger collection of patients were analyzed and more antibody reagents that could further subdivide the pre-B cell compartment into subcompartments were utilized, it was slowly realized that the picture in the XLA affected bone marrows was more heterogenous than earlier recognized. The heterogeneity was primarily appreciated by Conley who provided evidence supporting the notion that the XLA defect could interfere not only with the pre-B to B cell transition but also with other stages of B cell differentiation (Conley, 1985). This conclusion was in agreement with other studies demonstrating phenotypic heterogeneity in patients presumably carrying the same mutation (Wedgwood and Ochs, 1980; Krantman *et al.*, 1981; Landreth *et al.*, 1985; Leickley and Buckley, 1986) and was further supported by the careful analysis of XLA bone marrow phenotypes that was carried out by Campana and colleagues (Campana *et al.*, 1990). These investigators utilized antibody reagents to analyze the phenotype and proliferative activity of immature B cell lineage cells in eight XLA patients. Their studies demonstrated that (a) the frequency of pre-B cells in the XLA patients analyzed varied; (b) in those cases in which pre-B cells were seen, they were small and

failed to express the nuclear Ki67 marker, in contrast to the vigorously proliferating normal $c\mu^+$ cell (a conclusion compatible with that earlier reached by Pearl *et al.*, 1978); and (c) in one case all pre-B cells detected were terminal deoxynucleotidyl transferase positive (TdT^+), a phenotype that is extremely rare in the bone marrow. Because of these changes the ratio of pro-B to pre-B cells in the majority of the cases was markedly increased (>100 compared to <10 in normal bone marrow) in bone marrows of XLA patients.

The dissection of the B cell development process in the human system has not been as detailed and sophisticated as that in the mouse. Nevertheless, using different sets of antibody reagents the surface profiles of the different B cell subpopulations from the pro-B to the mature B cell stage have been characterized as illustrated in Table IV (Janossy *et al.*, 1979, 1980; Campana *et al.*, 1985, 1989; Loken *et al.*, 1987). The critical period within which the XLA defect becomes apparent must occupy the part of B cell development that lies before the stage when IgM and CD22 molecules are deposited on the cell membrane, the expression of CD37 and CD20 is increased, and after the stage at which TdT is supposed to be turned off (Table IV, shaded area) (Campana *et al.*, 1990).

A. The XLA Defect Is Intrinsic to the B Cell Lineage

Due to the random inactivation of one X chromosome that occurs early in the embryogenesis of the female, in women that are heterozygous for an X-linked allele, half of their cells on average express either of the two alleles (Lyon, 1966). Analysis of X-chromosome mosaicism in females heterozygous for a given X-chromosome-linked mutation has been used in order to assess the effect of the mutation on the development of the different cell lineages. Cell populations that, for their survival, are directly affected by the mutation should not express the mutated allele, whereas those that are not affected, or affected indirectly, should express either of the two alleles randomly. This type of analysis was used to study the effect of hypoxanthine–guanine phosphoribosyl tranferase deficiency in the Lesch–Nyhan syndrome (Lesch and Nyhan, 1964; Nyhan *et al.*, 1970). Similarly, carriers of the Wiskott–Aldrich syndrome, an X-linked recessive disease involving immunodeficiency and thrombocytopenia, have unbalanced mosaicism of glucose-6-phosphate dehydrogenase (G6PD, an X-linked enzyme) among platelets and T lymphocytes in particular (Gealy *et al.*, 1980b; Prchal *et al.*, 1980). In 1983, Nahm and colleagues demonstrated unbalanced X-chromosome mosaicism in B cell populations of the CBA/N mouse strain known to be the carrier of the X-linked

immunodeficiency (Xid) mutation (Nahm *et al.*, 1983) and emphasized the importance of such analysis in the study of X-linked immunodeficiencies. In their own words ". . . attention should be called to the utility of X-linked isoenzyme expression in the dissection of X-linked diseases, particularly immunodeficiencies. . . . The determination of the pattern of X chromosome expression in these diseases should be particularly informative" (Nahm *et al.*, 1983).

The failure of early B cell precusor to develop into mature B in XLA was suspected to be due either to their intrinsic inability to process and interpret signals generated during the critical transition stages between pro-B, early pre-B, and late pre-B cell stages (intrinsic defect), or due to an abnormality in another cell type necessary for normal B cell development, such as stroma cells in the bone marrow microenvironment or cells that produce essential growth and differentiation cytokines (extrinsic defect). To distinguish these two possibilities Conley *et al.*, (1986) adopted the earlier-mentioned strategy and investigated the pattern of X-chromosome inactivation in females that were heterozygotes for X-linked agammaglobulinemia and the G6PD gene (Conley *et al.*, 1986). By analyzing the expression of the A and B alleles of the G6PD gene in neutrophils, T cells, and B cells from XLA heterozygous females, these authors demonstrated that, while both alleles were active in neutrophils and T cells, only one was expressed in B cells (Conley *et al.*, 1986; Conley and Puck, 1988a). The biased X-chromosome inactivation that was observed in these elegant studies did not only demonstrate that XLA B cells were inherently defective, i.e., had an inherent survival disadvantage, but also for the first time provided a tool that allowed the detection of carriers among women at risk for carrying the gene for XLA. Based on the same principle of X-chromosome inactivation, but analyzing other parameters such as the differences in the methylation patterns of selected genes on the active or inactive X chromosomes, several procedures detecting the biased inactivation of "XLA" X chromosomes were described. This allowed an increased number of carriers to be analyzed as the frequency of G6PD heterozygotes is very low.

These studies clearly demonstrated that B lineage cells carrying a mutated XLA gene had a selective growth disadvantage and suggested that other hematopoietic cells did not. While some reports suggested that other lymphoid functions were impaired in XLA patients (Waldmann *et al.*, 1975; Siegal *et al.*, 1976; Edwards *et al.*, 1978; Herrod and Buckley, 1979; Edwards *et al.*, 1979; Thompson *et al.*, 1979b, 1980; Krantman *et al.*, 1981; Tedder *et al.*, 1985; Rozynska *et al.*, 1989; Crockard *et al.*, 1992; Richards *et al.*, 1992), other reports demonstrated

that T cell, natural killer cell, and macrophage functions were normal (Hirschhorn, 1963; Gotoff, 1968; Geha *et al.*, 1973; Griscelli, 1975; Pandolfi *et al.*, 1982; Messina *et al.*, 1986; Eskola *et al.*, 1989; Nilssen *et al.*, 1993). This suggests, as was also stated by some of the authors, that the former findings either represented exceptional cases, indirect effects due to the absence of Ig and B cells, or represented phenomena unrelated to the intristic B lymphocyte defect.
The demonstration of Ig synthesis in the B cell lineage in XLA is more difficult to reconcile, but could be secondary to the phenotypic heterogeneity of XLA or be technical in origin (Cooperband *et al.*, 1968; Dosch *et al.*, 1977; Percy *et al.*, 1977).

B. Recombination of Ig Genes in XLA

The inability of early B cell precursors to develop and generate normal numbers of antibody-producing cells seen in most patients with XLA was attributed to different reasons. Synthesis of functional Ig molecules is the hallmark of B cell development. Therefore, abnormal or inefficient rearrangement of the gene segments that are involved in the production of Ig molecules was considered a possible explanation for the defect in XLA. Initiating from the observation that B cell lines developed from the bone marrow of a family with a "minor form" of XLA could express truncated IgM heavy chains, which apparenly lacked V_H sequences, Schwaber and collegues suggested that B cell differentiation in XLA is blocked due to a failure of early B cell precursors to rearrange V_H region genes (Schwaber and Rosen, 1978; Schwaber *et al.*, 1978; Schwaber, 1983; Schwaber *et al.*, 1983; Ichihara *et al.*, 1988; Schwaber and Chen, 1988; Schwaber *et al.*, 1988; Schwaber, 1992). However, analyses of B cell lines that were generated by immortalizing with EBV the few sIgM-positive cells (~1% of the normal) that were usually present in the peripheral blood of XLA patients demonstrated that the clonal diversity of these populations was not limited and that the Ig rearrangement events that had occurred in these cells during development were not abnormal (Mensink *et al.*, 1986a; Anker *et al.*, 1989; Mortari *et al.*, 1991; Timmers *et al.*, 1991a, 1993a,b; Schiff *et al.*, 1993; Milili *et al.*, 1993). Additionally, the demonstration by Alt and colleagues that a common recombinase machinery catalyses the rearrangements in the V, D, and J segments of the Ig and T cell receptor loci (Yancopoulos *et al.*, 1986) and that the Dμ protein is a normal constituent of early B cell progenitors (Reth and Alt, 1984) clearly indicated that the recombinase machinery in XLA patients is not deficient in any qualitative aspect. Recent studies have demonstrated that the synthesis of IgH germline transcripts, which

represent an early step in the isotype switch process, are absent in XLA (Smith *et al.,* 1993), presumably secondary to the absence of peripheral lymphocytes.

The two opposing views need not be mutually exclusive. While the XLA mutation does not seem to affect any qualitative aspect of the recombination process, it could affect quantitative aspects by interfering with the efficiency of the mechanism that renders the genomic segments participating in the process accessible to the recombinase machinery (Blackwell and Alt, 1989). Since the products generated at each stage of the rearrangement process are essential for the further differentiation and survival of the developing B cell precursors, efficient rearrangement and further expansion/survival of the cells could be very tightly linked to each other. Alternatively, the XLA gene could independently regulate quantitative aspects of the recombination process as well as qualitative aspects of the growth-promoting signal transduction pathways.

XIII. The Geneticists' Approach to the XLA Problem

A. Hypotheses on the Origin of XLA

Prior to the cloning of the gene defective in XLA, several hypotheses on its nature were advocated by various authors. Persistance of fetal pattern of serum proteins was suggested by Martin (1954) and an enzyme affecting protein synthesis by Henley (1959). The absence of cells expressing surface Ig made Siegal *et al.* (1971) suggest that a gene functioning in the control of intracellular transport and secretion is present on the X chromosome, whereas Aiuti *et al.* (1973) interpreted this observation as lending some support to the idea of a genetical perturbation of the microenvironment. On the basis that XLA is inherited as a single gene defect, Hayward and Greaves (1975) proposed a single protein, presumably an enzyme, possibly in the inductive environment and not in the B cell itself. When interpreting their findings on 5′-nucleotidase, Edwards *et al.* (1979) suggested that T lymphocytes may be responsible.

Defective V_H recombinase genes, or genes regulating their expression, were advocated by Schwaber *et al.* (1983). Deficient expression of a B cell lineage-specific growth factor was hypothesized (Hendriks and Schuurman, 1991). Female agammaglobulinemia, indistinguishable from XLA, led Conley and Sweinberg (1992) to suggest that the XLA gene product may be part of a receptor–ligand-type relationship, participate in dimer formation, or perhaps be part of a cascade phenom-

enon. Another proposition was that the genes for several X-linked immunodeficiency diseases could be related (Conley, 1992).

B. Mapping of the XLA Gene—The Early Phase

Without having any clear idea concerning the nature and function of the defective gene product that was responsible for the development of XLA, the efforts of investigators were primarily focused on mapping the genomic region within which the XLA gene locus could lie. It was anticipated that on the basis of the obtained information, genes encoded within this region, and eventually the XLA gene, could be cloned and further analyzed. The inheritance of the disease with the typical X chromosome-linked inheritance pattern narrowed the search for the XLA locus to one chromosome. The earliest studies demonstrated that XLA was not linked to the Xg blood group determinants (Sanger and Race, 1963; Rosen *et al.*, 1965; Adam *et al.*, 1971; Mensink *et al.*, 1984) or to G6PD (Prchal *et al.*, 1980), located at the end of the short arm and long arm, respectively.

C. Mapping of the XLA Gene to Xq21.3–q22

The first breakthrough in the mapping efforts came when genetic linkage studies performed using the previously isolated polymorphic markers DXS3, DXS17, and DXS94 demonstrated that the gene for XLA was localized in the midportion of the X chromosome (Kwan *et al.*, 1986; Ott *et al.*, 1986; Mensink *et al.*, 1986b; Malcolm *et al.*, 1987). The DXS3 marker 119-2 was originally identified by screening of a human DNA panel (Aldridge *et al.*, 1984) using X-chromosome-specific DNA fragments previously assigned to a particular region of the chromosome (Kunkel *et al.*, 1982). The DXS17 probe S21 constituted a single-copy DNA sequence mapping to the Xq21.3–Xq22 region (Drayna *et al.*, 1984) also obtained using the flow-sorted X-chromosome-specific library described by Kunkel *et al.* (1982).

The finding of a recombination in a single family was initialy interpreted as a second XLA locus but was later shown to be due to X-chromosome mosaicism in the father (Mensink *et al.*, 1986b,1987). In 1989–1990, another breakthrough discovery was the tight linkage of the XLA locus to the DXS178 marker p212, previously described as one of eight unique probes mapping to different subregions of the X chromosome (Cooke *et al.*, 1985), setting the order of the genes: centromer–DXS3–(XLA,DXS178)–DXS17–telomer (Guioli *et al.*, 1989; Kwan *et al.*, 1990). In 1993, Lovering *et al.* demonstrated that two other polymorphic markers, namely DXS442 and DXS101, flanked the XLA locus and, thus, narrowed the genomic interval within which

resided the XLA locus to approximately 2 cM (Lovering *et al.*, 1993a). New polymorphic markers were continuously included in the linkage analysis and in the beginning of 1993 a combination of genetic linkage analysis and physical mapping studies utilizing pulsed-field electrophoresis indicated the order, centromer–DXS3–DXS366–DXS442–(PLP, DXS101, DXS382, DXS178–DXS265, DXS178CA complex, XLA)–(DXS87, DXS94)–DXS327–(DXS350, DXS362)–telomer, as illustrated in Fig. 1 (Arveiler *et al.*, 1987; MacDermont *et al.*, 1987; Cremers *et al.*, 1988; Barker *et al.*, 1991; Parolini and Conley, 1993;

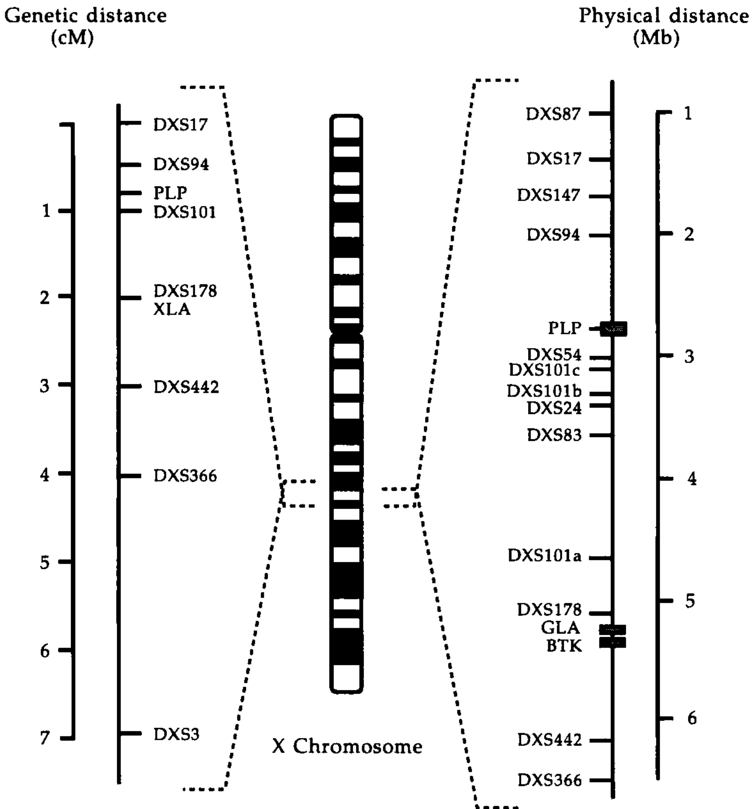


FIG. 1. Genetic and physical maps of the human XLA locus. The genetic map indicates the state-of-the-art at Xq22 just before the cloning of the *Btk* gene and is based on the findings of Lovering *et al.* (1993) and Parolini *et al.* (1993). The physical map of the human regions surrounding the *Btk* locus was deduced by Vetrie *et al.* (1993b) and after the isolation of the *Btk* gene reached the form shown here (Vetrie *et al.*, 1994).

Parolini *et al.*, 1993; O'Reilly *et al.*, 1993; O'Reilly *et al.*, 1993b; Lovering *et al.*, 1993b, 1994; Parkar *et al.*, 1994). The existence of CpG islands in the vicinity of DXS178 further indicated the existence of novel genes in this region (O'Reilly *et al.*, 1992; Lovering *et al.*, 1993b; O'Reilly *et al.*, 1993a; Parolini *et al.*, 1993; Vetrie *et al.*, 1993a).

XIV. Cloning of the XLA Gene

A. The Positional Cloning Strategy for the XLA Gene

In the beginning of 1993 the genetic map at Xq21.3–q22 was further refined to the degree that is illustrated in Fig. 1 (Lovering *et al.*, 1993a; Parolini *et al.*, 1993). In parallel with the genetic linkage studies, attempts were made to actually physically map the genomic region around the XLA locus. Limited information was provided by pulsed-field gel analyses (Lovering *et al.*, 1993b; Parolini *et al.*, 1993; Vetrie *et al.*, 1993a) until Vetrie *et al.*, (1993b), using already existing polymorphic markers and a panel of selected X-chromosome-specific yeast artificial chromosome clones (YAC), deduced the physical map of the chromosomal region flanking the XLA locus. Initially a map covering 5.2 Mb of genomic sequences that extended from DXS87 to a few hundred kb centromerically of DXS178 was constructed and YAC clones covering these regions were isolated (Vetrie *et al.*, 1993b). One of them, the y178-3, was positive for the DXS178 marker and the α-galactosidase-A gene and extended further toward the XLA locus. The y178-3 clone was considered a good candidate to contain the XLA locus and was therefore chosen by Vetrie and his collaborators at the Karolinska Institute, Stockholm, Sweden and the University of Umeå, Umeå, Sweden to become the basis for their strategy to isolate the XLA gene (Fig. 2).

As already mentioned, the DXS178 marker had been found to cosegragate with the XLA locus. The strategy followed was primarily based on the "direct cDNA selection" method that was described in late 1991 and which allowed the enrichment of region-specific cDNA populations up to 10^2- to 10^4-fold (Lovett *et al.*, 1991; Parimoo *et al.*, 1991).

DNA from the YAC y 178-3 was immobilized on nylon filter, which subsequently was hybridized to cDNA inserts amplified from a cDNA library constructed with mRNA from a mature B cell line, the Burkitt's lymphoma BL-29, or from a library constructed with mRNA from a pro-B cell like the fetal liver-derived EBV-immortalized FLEB 14 (Sideras *et al.*, 1992) (step 1a). The inclusion of a mature B cell library in this analysis was based on evidence that was provided earlier,

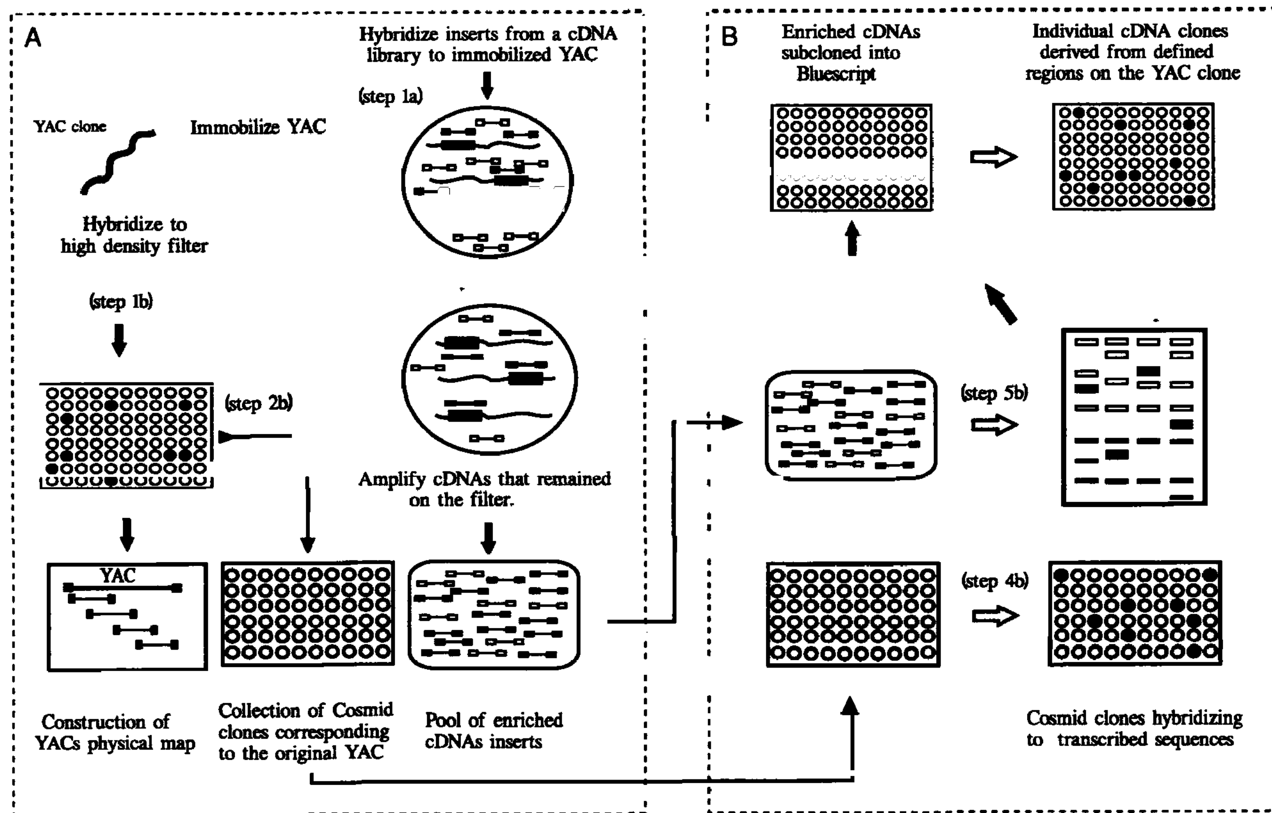


FIG. 2. Strategy that was developed in order to clone the XLA gene. (A) Preparation of yac-specific cosmid sublibrary and direct cDNA selection. (B) Utilization of enriched cDNA pool for the isolation of region-specific cDNAs.

suggesting that the gene may be expressed throughout B lymphocyte development (Conley, 1985; Conley *et al.*, 1986). A single round of selection was performed and the cDNA clones that bound to the immobilized y178-3 DNA were reamplified (step 2a) and either used as probe or subcloned and gridded in high-density arrays (step 3a) (Bentley *et al.*, 1992). In parallel, cosmid clones that hybridized to the y178-3 clone was also isolated (step 1b) and gridded on high-density filters (step 2b). The partially enriched cDNA population was used as a probe to hybridize the latter filters (step 3b). In this way cosmid clones corresponding to the transcribed portions of the YAC y178-3 were isolated (step 4b). DNA from the positive cosmid clones was digested with restriction enzymes, electrophoresed, and after transfer hybridized (step 5d) to the same pool of enriched cDNA clones (from step 2a). This time cosmid clone restriction fragments containing coding segments of genes located in the area covered by the y178-3 clone were isolated. Each of them was used to hybridize filters (step 6b) that contained the subcloned pool of the enriched cDNA clones (from step 3a). Positive clones in each case thus represented cDNAs derived from genes located within the region covered by the corresponding cosmid clone.

The cDNA clones hybridizing to cosmid fragments were arranged into 12 mapping groups and clones representative for each one of them were used to screen Southern blots from XLA patients. cDNAs from two of these groups, namely clones 6G11 and 7C11, detected DNA rearrangements in one out of seven Swedish XLA families (patient A) and one British family (patient B) strongly suggesting that the gene from which they were derived could be the XLA gene (Vetrie *et al.*, 1993c).

Using the same cDNA probes, the original cDNA library was screened and larger, putative full-length clones were isolated. Both 6G11 and 7C11 probes hybridized to the same set of "full-length" cDNA clones and were later shown by direct sequencing analysis, to correspond to the 5′ and 3′ ends of the same gene, respectively. Nucleotide sequencing analysis demonstrated that the full-length cDNA clones contained an open reading frame that encoded a putative cytoplasmic tyrosine kinase. The gene was designated *Atk* (for agammaglobulinemia tyrosine kinase) and it soon became clear that it was the XLA gene.

Four additional new genes identified by this enrichment procedure were also encoded by the genomic segment corresponding to the y178-3 clone (Fig. 3) (Vorechovsky *et al.*, 1993a, 1994a). The physical map around the locus defective in XLA was further refined and in 1994 a

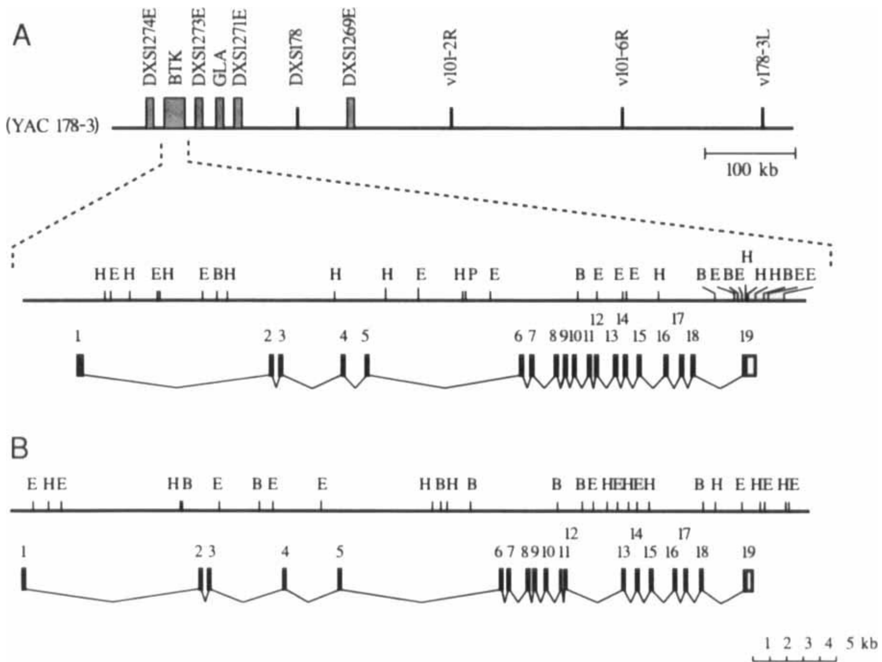


FIG. 3. (A) Physical map of the human genomic regions proximal to the *Btk* locus and restriction maps of the mouse and human *Btk* genes. The YAC 178-3 clone was used to select cDNA clones from B cell libraries with the direct selection method (Vetrie *et al.*, 1993c). These clones were mapped by Vorechovsky *et al.* (1994a). The locations of the *Btk* gene as well as of the other four new genes (DXS1274E, DXS1273E, DXS1271E, DXS1269E) that were isolated in the process (Vorechovsky *et al.*, 1994a) and the location of the DXS178, v101-2R, v101-6R, and v178-3L markers are indicated. (B) A complete restriction map of the human and mouse *Btk* genes for *Bam*HI (B), *Hin*III (H), and *Eco*RI (E) (Reproduced from Sideras *et al.*, 1994).

6.5-Mb YAC contig that incorporated 33 DNA markers and covered the sequences from DXS366 to beyond DXS87 was completed (Vetrie *et al.*, 1994) and included the genes for XLA, Fabry disease (Bernstein *et al.*, 1989), and Pelizaues–Merzbacher disease (Willard and Riordan, 1985; Fahim and Riordan, 1986) (Fig. 1). This physical map did not differ dramatically from the one predicted by the early genetic linkage analyses based on the various DXS probes except that the distances between some of the markers and genes investigated as slightly overestimated in the genetic linkage analysis (Fig. 1).

In retrospect, there were three crucial markers contributing to the positional cloning of the XLA gene, namely DXS3, 17, and 178. The

first two identified the correct region on the X chromosome, and DXS178 was the marker used to select the YAC that was applied in the isolation of the *Btk* cDNA (Vetrie *et al.*, 1993c). The isolation of the XLA gene was the first time a cDNA-enrichment procedure was successfully employed in the cloning of a novel gene. According to Dr. F. S. Collins, director of the Human Genome Project, *Btk* became the 17th disease gene, isolated by positional cloning (personal communication).

B. The XLA Gene Encodes a Novel PTK

In parallel with the studies of Vetrie and colleages, following a different strategy, another collaborative effort independently succeeded in isolating the XLA gene (Tsukada *et al.*, 1993). These investigators, led by O. N. Witte, were primarily interested in isolating novel tyrosine kinases, expressed in early B cell precursors, anticipating that they would regulate important aspects of the differentiation process. Using the kinase domain of Ltk, a previously isolated tyrosine kinase (Ben-Neriah and Bauskin, 1988; Bernards and de la Monte, 1990), as a probe they screened a mouse B-lineage progenitor cDNA library using reduced stringency conditions. A gene that contained homologous, but still different sequences, was isolated and designated *Bpk* (for B-cell progenitor kinase). A corresponding human cDNA was isolated and fluorescence *in situ* hybridization was combined with X-chromosome somatic cell hybrid analysis, and expression studies in patients suggested that this was very likely to be the XLA gene. The nucleotide sequences of *Atk* and *Bpk* were identical and, therefore, in order to avoid confusion, a commonly agreed name was designated *Btk* (for *B*ruton's agammaglobulinemia *t*yrosine *k*inase) for the XLA gene. According to kinase nomenclature (Hanks *et al.*, 1988), *Btk* denotes nucleic acid (DNA and RNA) and Btk protein.

The simultaneous isolation of the gene by two laboratories that followed different strategies and had different primary interests was very fortunate. The two reports published only 8 days apart complemented and supported each other. Together, the mutations identified by Vetrie *et al.* (1993c), the biochemical data of Tsukada *et al.* (1993), and the mapping data provided in both reports demonstrated beyond any doubt that XLA was caused by mutations in gene encoding a novel cytoplasmic PTK.

C. Structure and Expression of Mouse and Human *Btk* Loci

Using a human *Btk* cDNA as a probe, corresponding mouse cDNAs were isolated from a PMA plus ionomycin-stimulated mouse spleen

cell cDNA library (Sideras *et al.*, 1994). The mouse *Btk* sequences were highly homologous to the human (99.3% conservation at the amino acid level). The genomic organization of mouse and human *Btk* loci and the intron/exon boundaries in both species were deduced using mouse 129SV genomic lambda and P1 phage clones and human cosmid clones (Sideras *et al.*, 1994). The coding regions were divided into 19 exons covering approximately 43.5 kb in the mouse and 37.5 kb in the human genomes, respectively (Fig. 3). The last exon encoded the 23 carboxy teminal amino acids as well as the 3′-untranslated region (3′-UTR). Single exons (exon 1) located almost 9 kb upstream from the translation initiation codon (exon 2) encoded for the 5′-untranslated regions (5′-UTR) of the *Btk* messages. Exons ranged in size from 55 to ~560 bases. With the exception of the exons encoding for the 5′-UTR and the 3′-UTR (referred to as exons 1A and 18) all the other exons had identical lengths in both mouse and human genes. The size of the introns varied significantly, ranging from 164 bp (mouse intron 10) to over 9 kb (mouse and human introns 1A and 4). The sequences surrounding all the exon boundaries fulfilled the AG/GT rule (Mount, 1982; Ruskin *et al.*, 1984; Horowitz and Krainer, 1994). The location of the splice borders for the exons encoding the PH domain of the mouse *Btk* gene are identical to those previously described (Rawlings *et al.*, 1993). The organization of the human *Btk* locus was characterized in parallel by other investigators with similar results (Hagemann *et al.*, 1994; Ohta *et al.*, 1994). Originally, we had named the 5′-UTR encoding exon 1A and the coding exons 1 to 18 (Sideras *et al.*, 1994). However, since a different nomenclature was adopted by the other two groups, in order to avoid any future confusion we adopted their system, thus naming the 5-UTR encoding exon as exon 1 and the 3′-UTR encoding exon as exon 19 (Hagemann *et al.*, 1994; Ohta *et al.*, 1994).

PTKs have been grouped into several subfamilies on the basis of their sequence and structural homology (Bolen, 1993). The pattern of exon boundaries of *Src*, for example, resembles very much that of *Lck*, whereas that of *Csk* appears to be an intermediate between the patterns seen in *Src* and *Fer* (Bräuninger *et al.*, 1993). As shown in Fig. 4, the pattern of exon boundaries of the *Btk* gene is consistent with the classification of *Btk* in a separate subgroup which is more closely related to the *Fes*/*Fer* and less related to the *Csk* and *Src* families.

D. Expression of the *Btk* Gene

Some of the predictions regarding the expression of the XLA gene turned out to be true. *Btk* mRNA and protein was indeed found to be

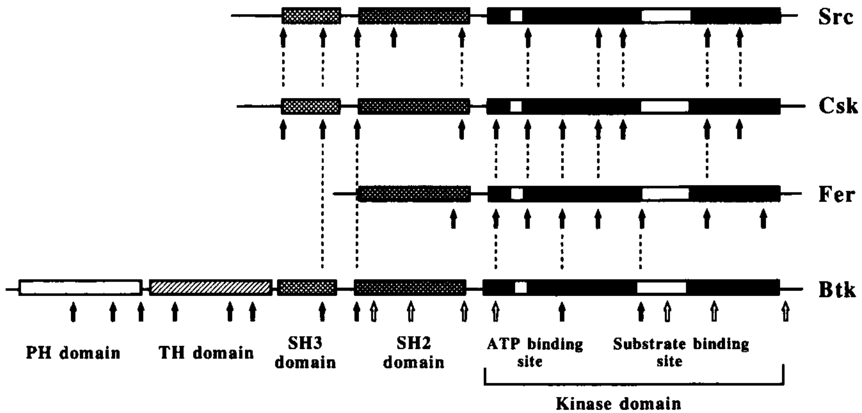


FIG. 4. Schematic comparison of the exon–intron boundaries of representative members of the four subfamilies of Src-related PTKs. The amino acid sequences of the Src, Csk, Fer, and Btk were aligned. Only the parts of the molecules where the intron–exon boundaries have been characterized are shown in the figure. Gaps were introduced for optimal alignment. Solid arrows indicate the location of exon boundaries. Open arrows indicate splice junctions that are characteristic for Btk. Vertical dotted lines indicate the boundaries that are found at the corresponding position in the kinases connected by the lines. The PH, TH, SH2, SH3, kinase (ATP-binding and substrate-specific regions) domains are indicated. (Reproduced from Sideras *et al.*, 1994.)

expressed in cell lines representing most stages of B cell development. However, contrary to the original expectations, *Btk* was found to be expressed in many non-B cells as well. Using different cell lines and organ samples, it was found that *Btk* mRNA and protein is expressed in all hematopoitic lineages with the exception of T lymphocytes and the most mature form of antibody-producing lymphocytes, the plasma cells (de Weers *et al.*, 1993; Smith *et al.*, 1994a,b; C. Kinnon and W. Khan, personal communications). The expression pattern of *Btk* in different hematopoitic lineages is illustrated in Fig. 5. It is still unclear at which stage during hematopoiesis the *Btk* gene is turned on but both the erythroleukemia line K562 and the $CD34^+$ myeloid progenitor KG-1 express *Btk* message (Smith *et al.*, 1994a). Interestingly, the human pro-T cell line P30/Okubo, which does not demonstrate any T cell antigen receptor (TCR) rearrangements but expresses germline TCRα transcripts, synthesizes *Btk* message (Smith *et al.*, 1994b). Also, purified double-negative thymocytes from RAG-2-deficient mice express low but detectable levels of *Btk* message (W. Khan, personal communication). This could indicate that the *Btk* gene is silenced in T cell precursors after their entry into the thymus and

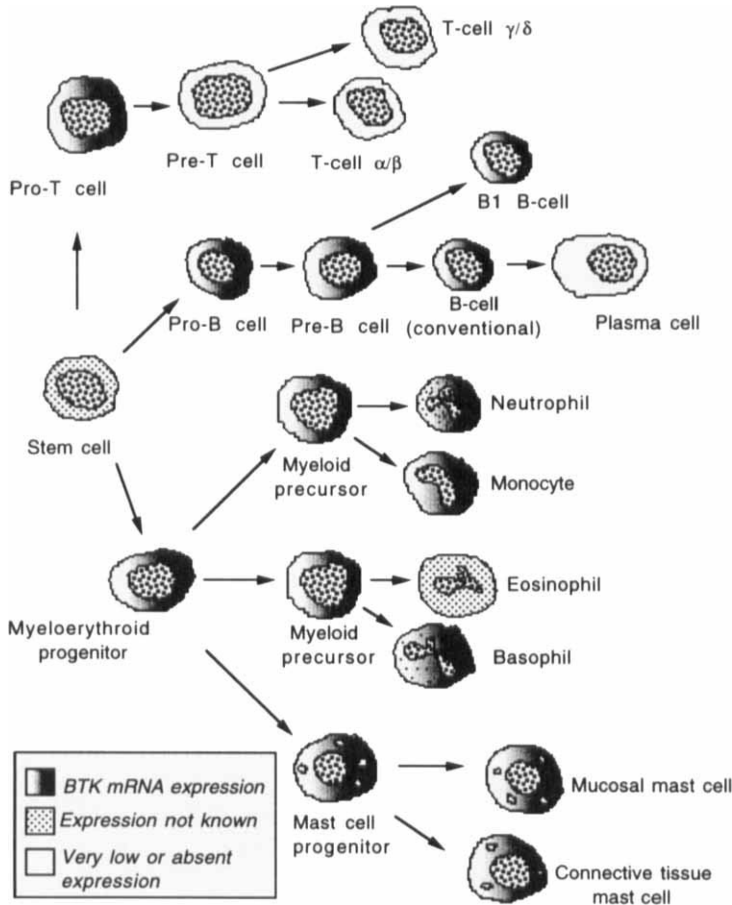


FIG. 5. Schematic representation of *Btk* mRNA expression in hematopoietic cells. (Reproduced from Smith *et al.*, 1994b.)

only after they start expressing their own specialized kinase molecules such as *Lck*. Not much is known about the molecular elements that regulate the *Btk* gene expression. Putative transcription start sites have been mapped immediately upstream of the noncoding exon 1A (Fig. 6) (Sideras *et al.*, 1994). The sequences upstream of the putative start sites are very conserved between mouse and human but do not contain a TATAA-like element. Interestingly, the nucleotide sequences surrounding the major transcription start site (CTCAGACT) resemble the "initiator" (Smale and Baltimore, 1989) consensus sequence

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                                   PuF                                       PuF
mBtk  GAGGTCCCAGGCAAATGTCGGGGGGTGGGGGTAGAGAGTTGGGGGTGGGGAGTGGAATAGGGACAGCAGCTCGAAATCAG  -96
hBtk  GAGGTCC AAGCAAATGAAGGGCGG------------GGACAGTTGAGGGGGTGGAATAGGGACGGCAGCAGGGAACCAG  -93

                                                             PEA-3               Zeste
mBtk  ATACCATATTGCTAAGGAGAAAAAAAAAGGGCAATGGTTTAAGTCAGGAAACAAAAAAAGGGAACTGAGTGGCTGCGAAA  -16
hBtk  ATAGCATGCTGCTGAGAAGAAAAAAAGA---CATTGGTTTAGGTCAGGAAGCAAAAAAAGGGAACTGAGTGGCTGTGAAA  -16

        PuF
mBtk  GGGTGGGGTTTGCTCAGACTGTCCTTCCTCTCTGGACTGTAAGAATATGTCTCCAGGTCCAGAGTCTTCAGAGATCAA  +63
hBtk  GGGTGGGGTTTGCTCAGACTGTCCTTCCTCTCTGGACTGTAAGAATATGTCTCCAGGGCCAGTGTCTGCTGCGATCGA  +65
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FIG. 6. Alignment of putative mouse and human promoter sequences. Asterisks above the mouse and below the human sequences indicate ends of independent cDNA clones generated using the RACE system (Sideras *et al.*, 1994.) Putative recognition sites for known transcription factors were identified using the "tfsite" database and the FINDPATTERN program in GCG (Madison, WI).

(CTCANACT) in seven out of the eight nucleotide residues. Computer-assisted analysis of sequences upstream of the transcription initiation sites of the mouse and human *Btk* loci demonstrated the presence of characteristic sequences of a PEA-3 motif (Martin *et al.*, 1988) and a binding site similar to that of the Drosophila factor zeste (Benson and Pirotta, 1988). In addition, three putative binding sites in the mouse and two in the human for the recently described, c-*myc*-regulating factor PuF (Postel *et al.*, (1989) were identified. A 364-bp fragment that includes the putative start sites can confer promoter activity in an orientation-dependent manner when linked upstream of a promoterless reporter gene. However, this minimal promoter segment is active not only in B and myeloid cell lines, but also in the T cell line Jurkat (S. Müller, personal communication). This could indicate that other, silencer-like elements, downregulating *Btk* transcription in mature T cells, reside in the *Btk* locus. While *Btk* is expressed in most of the hematopoietic cells but mature T cells, the only obvious defects associated with mutations affecting this molecule are confined to the B lymphocyte compartment (Conley, 1985; Conley *et al.*, 1986).

E. The Xid Phenotype is due to a Mutation in the *Btk* Gene

The CBA/N mouse strain was isolated during a series of breeding experiments from a single litter of a CBA/Harwell mouse at the National Institute of Health. In the early 1970s, it was shown that CBA/N mice made abnormal immune responses to polysaccharide antigens (Amsbaugh *et al.*,1972; Scher *et al.*, 1973). The pattern of inheritance of this abnormality was consistent with that of an X-linked recessive gene controlling unresponsiveness. The gene was designated XID and by mapping analysis was located between the *Tabby* and *Hypophosphatemia* genes on the X chromosome (Berning *et al.*, 1980).

Animals homozygous for the Xid mutation do not produce antibodies in response to immunization with polysaccharides or hapten–polysaccharide conjugates and have low levels of serum IgM and IgG3 (Perlmutter *et al.*, 1979; Slack *et al.*, 1980). Additionally, Xid mice have moderately reduced numbers of B cells that present a high surface IgM to IgD ratio, fail to form colonies in soft agar, and have abnormal responses to a variety of activation signals including surface Ig receptor cross-linkage, IL-5, IL-10, and CD38 (Go *et al.*, 1990; Hitoshi *et al.*, 1993; M. Howard, open citation). The absence of cells expressing surface markers, such as the Lyb-3 (Huber *et al.*, 1977), Lyb-5 (Ahmed *et al.*, 1977), and others (Subbarao *et al.*, 1979; Kung *et al.*, 1982) and the immature B cell-like high surface IgM^{high}/IgD^{low} profile of the Xid B cells triggered a debate on the origin of these cells and their

relationship to other normal B cell subpopulations which lasted for several years. While some investigators have argued that Xid B cells represent an immature B cell subpopulation that is normally present in small numbers in normal cells, others have argued that Xid B cells are abnormal in many aspects and do not relate to any normal B cell subpopulation (Hardy *et al.*, 1983; Sprent and Bruce, 1984; Chung *et al.*, 1992).

The Xid gene was mapped more precisely between the markers DXSWas17 and DXSmh43 and was closely linked to the simple sequence repeat marker DXMit3 (Thomas *et al.*, 1993). In over 1114 backrosses performed by these investigators, the Xid phenotype cosegregated with the DXMit3 marker, thus setting the distance between these two loci to less than 0.25 cM. The above mapping data suggested that the Xid gene was located in a region of the mouse genome homologous to the region syntenic to the XLA locus, raising, the possibility that Xid and XLA could be mutations either of the same or of two closely linked genes. Despite the apparent differences in the phenotype of XLA and Xid, B lymphocytes affected by these two disorders share a number of characteristics. The very few B cells found in XLA patients and the B cells found in Xid mice have a surface marker phenotype which resembles that of immature B lymphocytes and could be the result of abnormal maturation (Conley, 1985). In both disorders the only cell lineage that is obviously affected is that of the B cell (Nahm, 1983; Conley *et al.*, 1986).

The isolation of a mouse *Btk* cDNA clone allowed Thomas *et al.*, (1993) to study the relationship between Btk (XLA) and Xid. Analysis of the same 1114 backcrosses demonstrated that the Xid phenotype, the DXMit3 marker, and the *Btk* gene cosegregated and, therefore, indicated that the maximum distance between them was 0.25 cM. Finally, direct sequencing analysis demonstrated that the *Btk* gene in Xid mice carried a mutation converting the arginine residue at position 28 into a cysteine (Thomas *et al.*, 1993; Rawlings *et al.*,1993). This mutation did not affect the catalytic capacity of the Btk molecule since cells from Xid mice had normal levels of Btk protein and Btk kinase activity (Thomas *et al.*, 1993; Rawlings *et al.*, 1993). The initial interpretation of this finding was that Xid represents a mutation of the *Btk* gene that has a moderate effect on the function of this molecule therefore resulting a milder phenotype. Since Arg28 is located in one of the several noncatalytic domains of Btk that mediate protein–protein interactions, it was thought, and reasonably so, that only some of the interactions involving Btk would be impaired in Xid mice (see below for further description of Btk structure).

The absence of an Xid-like mutation in the first 14 XLA families analyzed was thought to be compatible with the previous conclusions (Vorechovsky *et al.*, 1993b). However, during the characterization of mutations in more XLA families, de Weers *et al.*, (1994) came across a family with a severe form of XLA that carried a mutation converting Arg28 into a histidine. While many investigators, including ourselves, wanted to believe that these surprising findings were due to the differences in the amino acid substitutions, gene targeting experiments clearly demonstrated that the Btk mutation is indeed the cause of the Xid phenotype and, furthermore, that mice do not develop a phenotype as severe as the human XLA even when their kinase domain is completely disrupted (W. Khan, F. W. Alt, and P. Sideras, unpublished results).

The demonstration that the Xid phenotype is the result of a mutation in the *Btk* gene vindicated those that correctly argued that Xid cells do not represent any normal lymphocyte subpopulation (Hardy *et al.*, 1983; Sprent and Bruce, 1984; Chung *et al.*, 1992). It is not unreasonable to guess that the Xid mutation is one of the best-studied single-point mutations that has ever occurred in nature. An enormous amount of data has been accumulated over more than 20 years that the mouse Xid mutation has been studied. These data include the effect of the Xid mutation on B cell development and growth, isotype switching, tolerance and memory induction, susceptibility to different infections, and many other aspects of lymphocyte physiology (reviewed by Scher, 1982; Wicker and Scher, 1986). Hopefully, once we understand the differences between mouse and human B cell physiology or development, accounting for the dramatic differences in the phenotypes caused by mutations in *Btk* in these two species, then this invaluable source of ideas and information gathered in the mouse system could be of great help in elucidating the role of Btk in human B cell development.

XV. PTKs Are Important Regulators of Lymphocyte Functions

Precursors of immune cells (both B and T cells) go through developmental switches that are genetically, temporarily, and geographically well defined (Perlmutter *et al.*, 1993; Weissman, 1994). Antigen-specific receptor components generated at each step and coreceptor molecules than can contribute to cell activation must interact in a harmonious way with ligand molecules on the surface of neighboring accessory cells (von Boehmer, 1994). Signals generated during this molecular communication are integrated by the cellular machinery

and, depending on the developmental stage of the differentiating cells or the cellular context of the interaction, signals that ensure the survival of the cell are generated and passed to the nucleus. Otherwise, the internal program of self-elimination, known as apoptosis, is triggered and the cells that failed are removed from the stage.

Protein–tyrosine phosphorylation has been strongly implicated in the initiation of cellular responses in both B and T cells (Perlmutter *et al.*, 1993; Weiss and Littman, 1994). Triggering of their antigen-specific receptors, for example, has been shown to initiate a cascade of biochemical events, the earlier of which is an increase in the phosphorylation of proteins on tyrosine residues (Reth *et al.*, 1991; Cambier, 1994). Since neither the B cell antigen-specific receptor (BCR) nor the TCR subunits have intrinsic PTK activity, signal transduction via these receptor complexes seems to be initiated by the activity of cytoplasmic PTKs that are delivered to and/or activated by the BCR or TCR complexes (Veillette *et al.*, 1989; Campbell and Sefton, 1990; Burkhardt *et al.*, 1991; Hutchcroft *et al.*, 1991; Campbell and Sefton, 1992; Bolen, 1993; Watling *et al.*, 1993; Fearon, 1993; Silvennoinen *et al.*, 1993; Mustelin and Burm, 1993; Muller *et al.*, 1994; Iwashima *et al.*,1994; Takata *et al.*, 1994).

Cytoplasmic PTKs are thought to play important roles in several cellular signaling pathways that regulate cell proliferation and differentiation (Bolen, 1993). They appear to mediate their function in different ways, thereby interacting either constitutively or upon activation with the cytoplasmic tails of receptors that lack signaling devices. The sequential interaction of Lck and ZAP-70 with the TCR complex (Iwashima *et al.*, 1994) provides an excellent example of such molecular interplay. Lymphocyte antigen-specific receptors, coreceptors, as well as lymphokine receptors have been shown to interact in a well-orchestrated manner with cytoplasmic PTKs. Lyn, Blk, Syk, and Lck kinases have all been reported to associate with the BCR (Campbell and Sefton, 1990; Burkhardt *et al.*, 1991; Campbell and Sefton, 1992; Takata *et al.*, 1994). Coreceptors in both B and T cells appear to interact with cytoplasmic PTKs and probably deliver them to the antigen-specific receptors. The CD4 and CD8 coreceptors in T cells interact with Lck and deliver it to the TCR complex (Barber *et al.*, 1989; Veillette *et al.*, 1989), whereas, the CD19/CD21 coreceptor complex in B cells has been proposed to interact with Lyn and deliver it to the BCR complex (Fearon, 1993). Studies on the interaction between several lymphokine receptors, such as the interleukin-2, interleukin-3, and interferon receptors, with their corresponding lymphokines have implicated Lck, Lyn, and Fyn, and the recently described Jak

family of cytoplasmic kinases in the lymphokine-induced signaling pathways (Watling *et al.*, 1993; Silvennoinen *et al.*, 1993; Muller *et al.*, 1994). Additionally, cytoplasmic PTKs can modulate cellular responses not only by interacting directly with surface receptors but also by regulating the activity of other kinases that do so. This has been very clearly illustrated by the interaction of Csk with other members of the Src family (Imamoto and Soriano, 1993; Nada *et al.*, 1993). The importance of cytoplasmic PTKs has been demonstrated by either overexpressing them in transgenic animals or by inactivating their endogenous genes by homologous recombination (Heisterkamp *et al.*, 1990; Tybulewicz *et al.*, 1991; Soriano *et al.*, 1991; Abraham *et al.*, 1991; Molina *et al.*, 1992; Stein *et al.*, 1992; Appleby *et al.*, 1992). The transgenic model systems have demonstrated that the deregulated expression of these molecules may contribute to malignant transformation (Heisterkamp *et al.*, 1990; Abraham *et al.*, 1991a). The expression pattern of cytoplasmic PTKs varies considerably among these genes. Some show spatially and temporarilly restricted pattern of expression whereas others are ubiquitously expressed. Targetted inactivation of ubiquitously expressed kinases, such as Src or Lyn, may impair the function of a single cell lineage without any direct correlation to the level of expression (Soriano *et al.*, 1991; Appleby *et al.*, 1992; Stein *et al.*, 1992).

As the complexity in PTK-induced signal transduction is enormous, kinases with defined functions may be critical in deciphering these processes. Despite the fact that the investigation of the Btk signaling pathway has just begun, its successive unraveling may thus have a great impact on the general understanding of these phenomena.

XVI. The Btk Family of Cytoplasmic PTKs

A. Classification of Kinases

Molecules involved in signal transduction frequently contain various domains believed to have independent functions. When the *Btk* cDNA was isolated and the amino acid sequence was deduced, it was apparent that the corresponding protein was a PTK belonging to the Src superfamily of cytoplasmic kinases as it contained a catalytic domain with certain characteristics (Vetrie *et al.*, 1993; Tsukada *et al.*, 1993; Desiderio, 1993).

Kinases catalyze the transfer of a phosphate goup from ATP to serine, threonine, or tyrosine residues and are subdivided into the protein–serine/threonine type (PSKs) and PTKs that phosphorylate tyrosine residues (Hanks *et al.*, 1988). PTKs are further subdivided into receptor

PTKs, such as the PDGF and insulin receptors, and cytoplasmic (nonreceptor) kinases lacking a membrane-spanning portion as reviewed by (Hanks *et al.*, 1988; Bolen, 1993). Btk differs in many respects from the protooncogene Src and forms a new family together with Tec (Mano *et al.*, 1990,1993) and the recently described PTK Itk/Tsk (Siliciano *et al.*, 1992; Heyeck and Berg, 1993 (Table V). Both the *Btk* gene and the *Itk/Tsk* gene were subsequently also reported by Yamada *et al.*, (1993) who called them *Emb* and *Emt*. A fourth member, *Bmx*, has recently been identified in man (Tamagnone *et al.*, 1994). Sequence alignment revealed that this gene is most related to *Btk*.

B. The Catalytic Domain (SH1)

The linear structure of Btk is outlined in Fig. 7. The kinase domain, which harbors the catalytic function of PTKs, is located in the C terminus and encompasses about 250 amino acids. This region interacts with the putative substrate for the enzyme and is also referred to as the Src homology 1, or SH1, domain, as the protooncogene v-Src was the first PTK to be cloned (Czernilofsky *et al.*, 1980). The catalytic core of the large family of protein kinases, including Btk, contains 9 invariant and 15 highly conserved residues involved in ATP binding and catalysis (Hanks *et al.*, 1988; Vihinen *et al.*, 1994c).

The catalytic subunit of the cAMP-dependent PSK, cAPK, was the first kinase domain for which a 3-dimensional structure was solved (Knighton *et al.*, 1991). Btk and cAPK sequences have sufficient similarity to allow reliable modeling, and a putative structure of the Btk catalytic domain has recently been obtained (Vihinen *et al.*, 1994c) as reproduced in Fig. 8c. This represents the first model of the SH1 in a cytoplasmic PTK.

When the Btk and the Src family kinase domains are compared there is one characteristic difference. Src family members contain a C-terminal tyrosine residue that has a regulatory function (Cantley *et al.*, 1991). Phosphorylation of this residue in Src impairs the catalytic activity, presumably secondary to binding to the SH2 domain (see below), whereas, conversely, site-directed mutagenesis exchanging this residue for tryptophan activates the kinase resulting in transforming capability. This critical tyrosine residue is absent in the Btk family.

C. SH2 and SH3 Domains Are Involved in Protein–Protein Interactions

N-terminal of the SH1 domain in Btk are two regions called SH2 and SH3 that are also found in Src. These modules are found in a large number of signaling molecules as reviewed by Pawson and Gish

TABLE V

CHARACTERISTICS OF BTK PTK FAMILY MEMBERS[a]

Name	Expression pattern	Species	Size (a.a.)	MW (kDa)	Chromosomal Location	Reference for Cloning of the Gene
Btk	Hematopoietic cells, not in T or plasma cells [1,2]	Human	659	77	Xq22	Vetrie *et al.*, 1993; Tsukada *et al.*, 1993
		Mouse	659	77	X[3,4]	Tsukada *et al.*, 1993; Yamada *et al.*, 1993; Sideras *et al.*, 1994
Bmx	Bone marrow, endothelial cells	Human	675	80	Xp22.2	Tamagnone *et al.*, 1994
Itk/Tsk	T and mast cells	Mouse	625	72	11[5]	Siliciano *et al.*, 1992; Heyeck and Berg, 1993; Yamada *et al.*, 1993
		Human	621	74	5q31–32	Gibson *et al.*, 1993; Tanaka *et al.*, 1993
Tec	Myeloid and T cells	Mouse	630	74	5	Mano *et al.*, 1990; Mano *et al.*, 1993
		Characteristics of Btk-Related PTKs				
Dsrc28C	Embryonic imaginal discs and oocytes	Drosophila	529	66	2, Position 29A	Gregory *et al.*, 1987
Txk	T and myeloid cells	Human	527	61	4p12	Haire *et al.*, 1994

[a]Numbered footnotes denote the report describing the characteristic. Features without a footnote are described in the cloning article given as a reference in the table. [1]de Weers *et al.*, 1993; [2]Smith *et al.*, 1994a,b; [3]Thomas *et al.*, 1993; [4]Rawlings *et al.*, 1993; [5]Janis *et al.*, 1994.

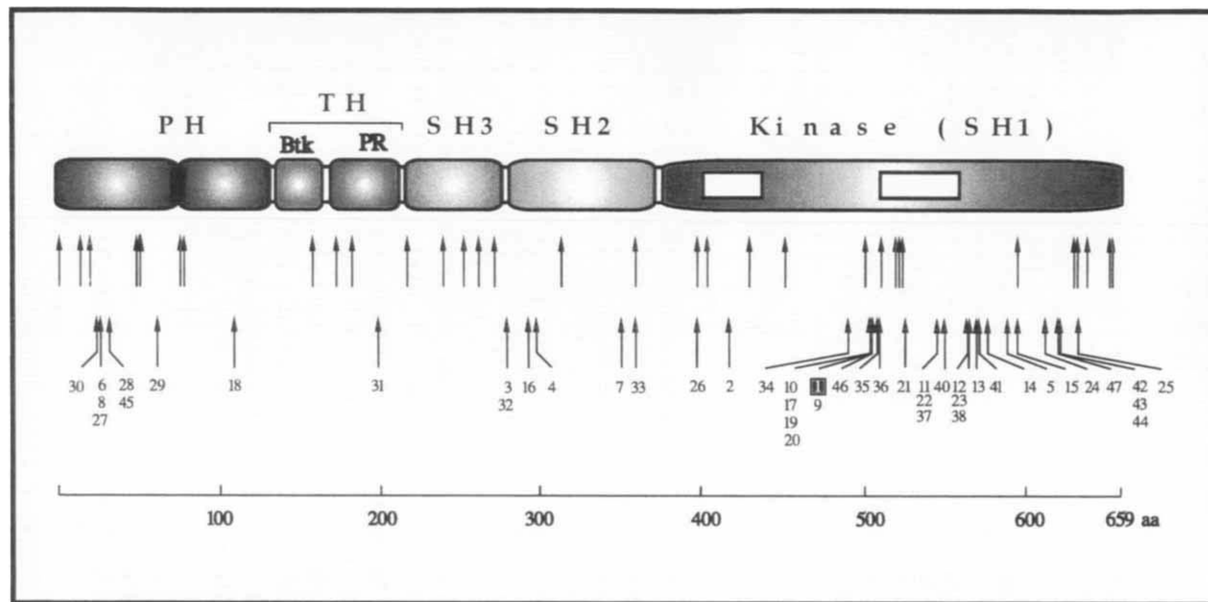


FIG. 7. Structure of the Btk molecule and location of different point mutations. The different interactive domains, PH, TH, (Btk–PR), SH3, SH2, and the kinase domain (SH1), with the ATP binding site and the catalytic site (left) (open boxes) are shown. Black numbered arrows indicate the position of amino acid substitutions, red (nonnumbered) arrows indicate the position of mutations that result in the creation of stop codons, and black (nonnumbered) arrows indicate the position of frameshift mutations. The numbers under amino acid substitutions correspond to those of Table VI. Mutation 1 is boxed since this was the first XLA mutation to be described (Vetrie *et al.*, 1993c). For further description of the mutations see Tables VI and VII. The estimated sizes of the various domains, based on the available sequence alignments are: PH: amino acids 1–138 (Vihinen *et al.*, 1995); TH: 139–215 (Vihinen *et al.*, 1994a); SH3: 216–280 (Zhu *et al.*, 1994a); SH2: 281–377 (Vihinen *et al.*, 1994b); and kinase domain: 378–659 (Vihinen *et al.*, *1994c).*

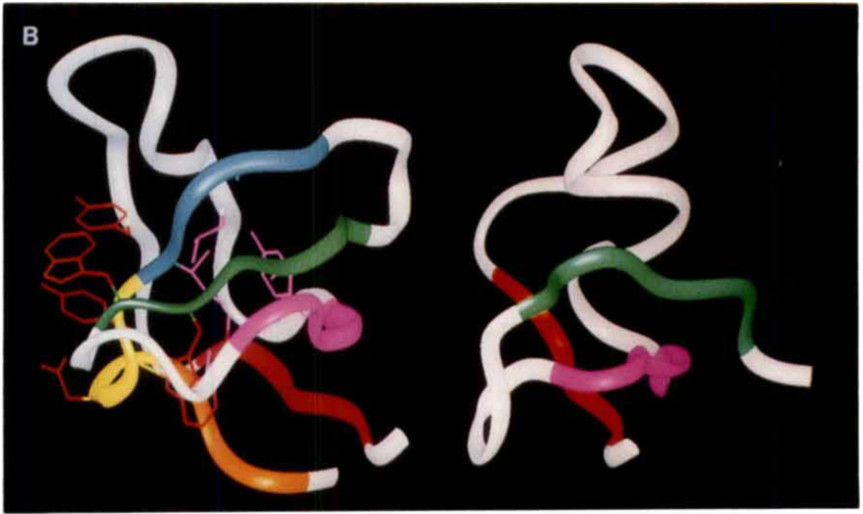
FIG. 8. (A) Sequence alignment of Fyn and Btk SH3 regions. The secondary structure elements are indicated with colors. The residues constituting the hydrophobic core in Fyn are denoted by asterisks. Residues corresponding to those perturbed by ligand binding in Src are indicated by open circles if identical to Btk and by closed circles if different. The residues deleted in the SH3 mutant are underlined. (B) Ribbon model of the backbone of the normal BTK SH3 (left) and the deletion mutant (right). The secondary structure elements are colored as in the alignment. The magenta side chains are for the residues forming the hydrophobic core and the red side chains are for putative ligand-binding residues identical to those in Src. (Reproduced from Zhu *et al.*, 1994.) (C) Ribbon representation of Btk kinase domain. The model was based on the cAPK structure. Residues corresponding to those preceding deletions in cAPK are blue, insertions are white, ATP is in green, and Mg^{2+} ions are red spheres. The residues substituted by mutations causing the disease XLA are in yellow; from top, K430, R526, R565, R520, A582, E589, G594, and G613. (Reproduced in a different view from Vihinen *et al.*, 1994c.)

(1992). SH2 domains contain about 100 amino acids, whereas the SH3 domains are smaller and comprise about 60 amino acids. SH2 domains interact with phosphorylated tyrosine residues in a specific fashion (Songyang *et al.*, 1993) and have been shown to modulate enzyme activity or target proteins to certain cellular locations (Cantley *et al.*, 1991; Waksman *et al.*, 1992). A model of the Btk SH2 domain has recently been constructed (Vihinen *et al.*, 1994b).

SH3 domains have been found to bind peptides rich in proline and hydrophobic residues (Ren *et al.*, 1993; Pleiman *et al.*, 1994). Their function is also likely to be targeting of signaling molecules to specific locations in the cell. As there is a proline-rich stretch located N terminal of the SH3 domain in Btk, interactions between these regions could have a regulatory function (Smith *et al.*, 1994b). Solving of the SH3 domain structure revealed that it was composed of two β sheets forming a compact β barrel (Musacchio *et al.*, 1992; Yu *et al.*, 1992). A model of the Btk SH3 domain has recently been constructed (Zhu *et al.*, 1994) as reproduced in Fig. 8b.

D. PH CONSTITUTES A NOVEL DOMAIN FREQUENTLY FOUND IN SIGNALING MOLECULES

In the two articles describing the cloning of the gene defective in XLA, the N-terminal region was referred to an "unique" (Vetrie *et al.*, 1993; Tsukada *et al.*, 1993), analogous to the Src family members which differ in this region. In 1993, several groups identified a novel, about 100 amino acids long, protein motif referred to as the pleckstrin homol-



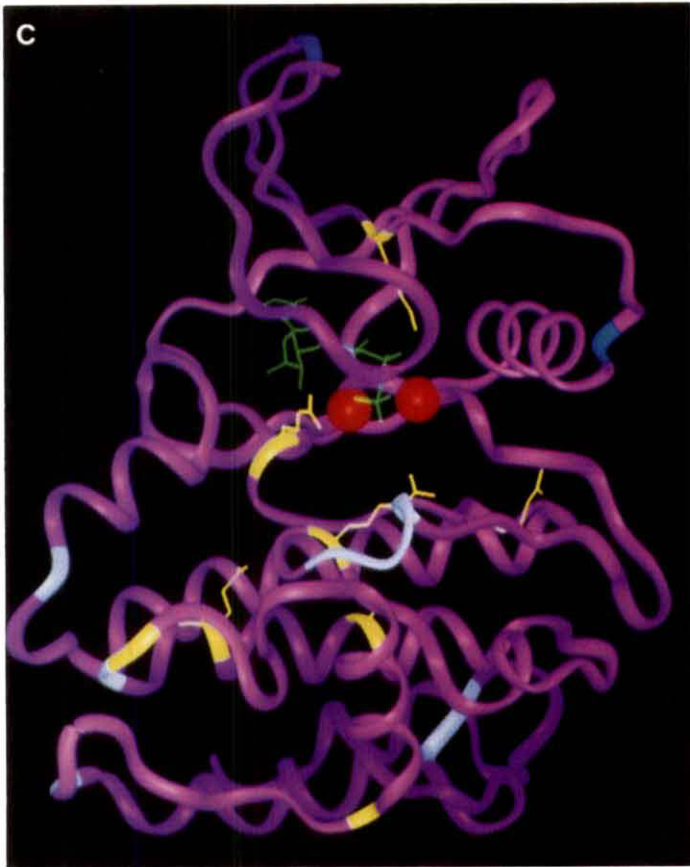


FIG. 8—*Continued*

ogy, or PH, domain (Clark and Baltimore, 1993; Haslam *et al.*, 1993; Mayer *et al.*, 1993; Musacchio *et al.*, 1993; Shaw, 1993). The domain was identified as it occurred twice in the protein kinase C substrate pleckstrin. Originating from work on the β-adrenergic receptor kinase (Koch *et al.*, 1993), the idea that PH domains interact with βγ subunits of heterotrimeric G proteins has been advocated (Musacchio *et al.*, 1994; Shaw, 1994). It is possible that this interaction brings Btk to the cell membrane carrying out a similar function as myristylation of Src family kinases.

The identification of a mutation in the PH domain of Btk in the mouse defect Xid was the first genetic evidence for the functional importance of this domain (Thomas *et al.*, 1993; Rawlings *et al.*, 1993). Recently, direct interactions between the Btk PH domain and βγ subunits have been demonstrated (Touhara *et al.*, 1994; Tsukada *et al.*, 1994). However, functional evidence identifying this interaction as critical in the Btk signaling pathway is still missing.

The recent determination of three NMR structures of PH domains show that they all have the same fold, consisting of a β barrel formed by two β sheets and a C-terminal α helix [Yoon *et al.*, (1994), Macias *et al.*, (1994), Downing *et al.*, (1994)].

E. The TH Domain—A Unique Feature of the Btk Family

Between the PH domain and the SH3 domain in Btk there is still a stretch of 60–80 amino acids (Fig. 7). Sequence analysis of this region demonstrated a highly conserved motif of about 25 amino acids located C terminal of the PH domain followed by a proline-rich stretch. As this region was conserved among Btk family members, and as Tec was the first kinase to be identified in this family, the region has tentatively been called the Tec homology (TH) domain (Smith *et al.*, 1994b).

A more thorough analysis of this region demonstrated that the TH domain contains a stretch of 27 conserved residues (7 being invariant) which immediately follow the PH domain and which are found in two other molecules, a putative RasGTPase activating protein, as also noted by Rawlings *et al.* (1993), and an interferon-γ-binding protein (Vihinen *et al.*, 1994a). The recently identified kinase Bmx also carries this conserved stretch (Tamagnone *et al.*, 1994). This motif was originally called the TH motif (Smith *et al.*, 1994b), but as the whole 60–80 amino acid domain was named TH, the 27 amino acid stretch (amino acids 139–166 in Btk) was renamed the Btk motif to avoid confusion (Vihinen *et al.*, 1994a).

As mentioned under Section XVI,C on SH3 domains, a binding between the Btk SH3 domain and the proline-rich region in the TH

domain could have a regulatory function (Smith *et al.*, 1994a). As these subregions are located in the same molecule this may favor intra-versus intermolecular interactions. A complete TH domain, containing both the Btk and the proline-rich motifs, has so far not been found in proteins outside the Btk family and may be a hallmark of these cytoplasmic PTKs.

F. Expression Pattern, Size, and Chromosomal Location

The *Btk* gene family constitutes one of eight defined subfamilies of cytoplasmic PTKs as reviewed by Bolen (1993). Table V summarizes certain characteristics of the different members. All members of the Btk family identified to date are expressed in hematopoietic tissues. However, Btk, Bmx, and Tec all seem to be expressed in nonhematopoietic tissues as well (Vetrie *et al.*, 1993; Tamagnone *et al.*, 1994; Mano *et al.*, 1990), even if expression of Btk in nonhematopoietic cell lines has not been observed so far (Smith *et al.*, 1994a,b).

All current members of the Btk family, with the possible exception of Txk and Dsrc28C (Table V and see below), are more than 600 amino acids long with corresponding MWs of more than 70 kDa. Owing to their extended N-terminal regions containing PH and TH domains they are thus considerably larger than Src family kinases.

Both the *Btk* and the *Bmx* genes are located on the X chromosome. However, they are on different arms, indicating that this chromosomal colocalization most likely is fortuitous. Based on the chromosomal position in mouse it has been predicted that the human *Tec* gene is localized in the syntenic region 4q11–13 (Mano *et al.*, 1993). In the mouse, the gene is closely linked to the *Kit* gene and the gene encoding the A-chain of the PDGF receptor (Mano *et al.*, 1993).

A novel human gene, designated *Txk*, has recently been isolated using a *Btk* probe (Haire *et al.*,1994). However, despite that the protein resembles Btk, there are some major differences; human Txk lacks a PH domain and has only the proline-rich C-terminal part of the TH region (lacks the 27 amino acid-long Btk motif), resulting in a size of less than 600 amino acids. In *Drosophila melanogaster* one gene, designated *Dsrc28C*, also demonstrates a high degree of similarity to the Btk family (Gregory *et al.*, 1987; Vetrie *et al.*, 1993b). Similar to Txk, this protein lacks a typical PH domain. Furthermore, with the introduction of a gap, alignment of residues 52–57, SttNs–Qfs, in Dsrc28C to amino acids 4–12, SsyNtiQsv, in Txk reveals 3 identical and 3 related residues indicating that these two proteins may share a common short motif. However, in other regions the sequences diverge.

Dsrc28C and Txk have been included under a separate heading in Table V.

G. The Role of Btk in Signal Transduction

The signaling pathway of Btk is still virtually unknown. Affinity isolation followed by an *in vitro* kinase analysis revealed that the Btk SH3 domain interacts with a phosphorylatable ~140-kDa protein in both stimulated and unstimulated mouse B lymphocytes, whereas no binding was seen using an SH2 or a fusion protein containing the PH and the N-terminal TH region (Aoki *et al.*, 1994). Using this approach, these investigators were unable to identify any Btk subdomain protein interactions in a mouse pre-B cell line.

Certain interactions between Btk and proteins known as signal transducers have also been revealed. Baltimore and his associates have recently identified binding between the SH3 domains of the Src family members Fyn, Lyn, and Hck and the proline-rich stretch in the TH domain of Btk (Cheng *et al.*, 1994).

Other interactions concern the PH domain. As mentioned earlier, the recognition of the PH domain resulted in the idea that the function of this domain may be to target molecules to the cell membrane via an interaction with $\beta\gamma$ subunits (Musacchio *et al.*, 1993; Shaw, 1993) as shown for βARK (Koch *et al.*, 1993). Subsequent studies have demonstrated that such an interaction may also be seen using fusion proteins containing the C-terminal region of the Btk PH domain (Touhara *et al.*, 1994; Tsukada *et al.*, 1994). Recently, the PH domain was shown to interact with different isoforms of protein kinase C *in vitro* and *in vivo*. Cross-linking of the FcεRI on bone marrow-derived mouse mast cells induced phosphorylation of tyrosine as well as serine and threonine residues and enzymatic activation of Btk (Kawakami *et al.*, 1994). Tyrosine phosphorylation was 1.5- to 3-fold and occurred within 1 min after cross-linking. However, coimmunoprecipitation between Btk and FcεRI subunits β and γ was not seen, possibly indicating that there are intermediate steps.

When the kinetics of activation of three classes of cytoplasmic PTKs were investigated in a murine cell line following anti-μ treatment characteristic differences were found (Saouaf *et al.*, 1994). Tyrosine phosphorylation of the Src family members Blk and Lyn took place first, followed by Btk and later by Syk. However, there was no indication of a direct interaction between components of the antigen-receptor complex and Btk. Using the same cell line (WEHI 231) other investigators were unable to convincingly demonstrate that Btk becomes phosphorylated after activation by anti-IgM antibody (Cheng *et al.*, 1994).

XVII. Mutations Are Found in Different Domains of Btk

A. Kinase Domain (SH1) Mutations

As described under Section XIV, the first two mutations found in patients with XLA identified critical residues in the kinase domain (Vetrie *et al.*, 1993). Patient A was a Swedish patient identified by a mutation causing loss of a *Taq*I site resulting in substitution of an arginine residue (R525) by a glutamine. The mutation in the British patient B created a novel *Taq*I site in the *Btk* cDNA, altering a lysine residue (K430), invariant in all kinases, to glutamic acid. Mutations of the invariant K430 residue in other kinases have always resulted in loss of catalytic activity (Hanks *et al.*, 1988; Smith *et al.*, 1994c) and *in vitro* kinase analysis revealed that the R525Q mutation also lacked catalytic activity (Vihinen *et al.*, 1994c). All mutants so far reported, including patients A and B, are depicted in the linear (not 3D) model of Btk (Fig. 7; Tables VI and VII).

Both the K430E and the R525Q mutations are readily explained in a 3D model of the Btk catalytic domain, resulting in the elimination of interactions with the α- and β-phosphates (K430E) and γ-phosphate (R525Q) of ATP, respectively (Vihinen *et al.*, 1994c) as reproduced in Fig. 8a. Six additional, novel amino acid substitutions in the kinase domain were also studied in a structural context. Interestingly, the model indicates that all eight mutations are located on one face of the catalytic subunit, presumably critical for substrate binding and catalysis (Vihinen *et al.*, 1994c). Mutations on the reverse face have recently been identified and the model indicates that they exhibit their effect through the interaction with the catalytic face (Vorechovsky *et al.*, 1995).

Highly conserved residues are also found in the very C-terminal end of the catalytic region (Vetrie *et al.*, 1993b). This is compatible with the finding of mutations, including amino acid substitutions (Conley *et al.*, 1994; Vorechovsky *et al.*, 1995; Jin *et al.*, 1995), located near the C terminus (Table VI, Fig. 7).

B. SH2 Domain Mutations

Five mutations in XLA patients resulting in amino acid substitutions have been found in the SH2 domain: R288W, G302E, R307G, Y361C, and I370M (Bradley *et al.*, 1994; Hagemann *et al.*, 1994, Saffran *et al.*, 1994; de Weers *et al.*, 1994; Vorechovsky *et al.*, 1995). Analysis of the Y361C mutation revealed that the kinase activity was intact, but that the $t_{1/2}$ of Btk was shortened (Saffran *et al.*, 1994). Both of the arginine residues are predicted to be critical for the interaction with

the phosphotyrosine binding to the SH2 domain, whereas the functions of Y361 and I370 are less clear as interpreted in a 3D model of the Btk SH2 domain (Vihinen *et al.*, 1994b; Vorechovsky *et al.*, 1995).

C. SH3 Domain Mutations

Exon 9 of Btk encompasses 63 nucleotides and encodes the C-terminal portion of the SH3 domain (Sideras *et al.*, 1994; Hagemann *et al.*, 1994; Zhu *et al.*, 1994a). As a deletion of this exon is in frame, the recent identification of splice site mutations deleting this exon in a case of XLA is informative regarding the function of SH3 (Zhu *et al.*, 1994a; Jin *et al.*, 1995). The deletion did not affect kinase activity nor the stability of Btk. Based on available SH3 structures a 3D model was used to interpret the consequence of this mutant as reproduced in Fig. 8b.

D. TH Domain Mutations

So far mainly frameshift mutations have been identified in the TH region (Table VI; Fig. 7). The exception is a patient with a mutation in exon 8, E205D; however, this male also carries a second mutation causing a frameshift (Vorechovsky *et al.*, 1995). This may indicate that the C-terminal proline-rich subregion is functionally resistant to single amino acid substitutions, or alternatively, that there are very few critical residues. Similar conditions are valid for the N-terminal Btk motif that only encompasses 27 amino acids, 7 of which are invariant. The mere fact that this is a short stretch is, from a statistical point of view, compatible with a considerably lower total number of mutations compared to, e.g., the catalytic domain.

E. PH Domain Mutations

Mutations in a PH domain were first described for the mouse Btk, where a single amino acid substitution R28C caused the mouse defect Xid (Thomas *et al.*, 1993; Rawlings *et al.*, 1993). Although mutations of this critical residue initially were not found in humans (Vorechovsky *et al.*, 1993b), the missense mutation R28H has subsequently been demonstrated (de Weers *et al.*, 1994; Ohta *et al.*, 1994; Vihinen *et al.*, 1995). Interestingly, this mutation causes the more severe phenotype XLA in man. As histidine is more related to arginine compared to cystein, this finding is compatible with the species differences seen in mice with targeted deletions of *Btk* having a milder phenotype than XLA (see Section XIX). A model of the Btk PH domain has recently been developed to interpret the structural basis of mutations in this novel domain (Vihinen *et al.*, 1995).

TABLE VI
CHARACTERIZED AMINO ACID SUBSTITUTIONS AND MUTATIONS IN THE C TERMINUS OF THE KINASE DOMAIN CAUSING XLA

Patient		Amino Acid Change	Nucleotide Change[a]	Affected Domain	Reference	Same Mutation in Patient No.[b]
No.	Name					
1	Patient A	Arg525Gln	G1706 → A	Kinase	Vetrie *et al.*, 1993c	9
2	Patient B	Lys430Glu	A1420 → G	Kinase	Vetrie *et al.*, 1993c	
3[c]	276	Arg288Trp	C994 → T	SH2	Bradley *et al.*, 1994	32
4	JP	Arg307Gly	A1051 → G	SH2	Bradley *et al.*, 1994	
5[c]	TF	Ala607Asp	C1952 → A	Kinase	Bradley *et al.*, 1994	
6	M76	Arg28His	G215 → A	PH	de Weers *et al.*, 1994	8, 27
7[c]	—	Tyr361Cys	A1214 → C	SH2	Saffran *et al.*, 1994	
8	P05	Arg28His	G215 → A	PH	Ohta *et al.*, 1994	6, 27
9	P08	Arg525Gln	G1706 → A	Kinase	Ohta *et al.*, 1994	1
10	—	Arg520Gln	G1691 → A	Kinase	Vihinen *et al.*, 1994c	17, 19, 20
11	—	Arg562Pro	G1817 → C	Kinase	Vihinen *et al.*, 1994c	
12	—	Ala582Val	C1877 → T	Kinase	Vihinen *et al.*, 1994c	38
13	—	Glu589Gly	A1898 → G	Kinase	Vihinen *et al.*, 1994c	
14	—	Gly594Glu	G1913 → A	Kinase	Vihinen *et al.*, 1994c	

15[c]	—	Gly613Asp	G1970 → A	Kinase	Vihinen *et al.*, 1994c	
16	23	Gly302Glu	G1037 → A	SH2	Hagemann *et al.*, 1994	
17	50	Arg520Gln	G1691 → A	Kinase	Hagemann *et al.*, 1994	10, 19, 20
18	6	Val113Asp	T470 → C	PH	Conley *et al.*, 1994	
19	15	Arg520Gln	G1691 → A	Kinase	Conley *et al.*, 1994	10, 17, 20
20	16	Arg520Gln	G1691 → A	Kinase	Conley *et al.*, 1994	10, 17, 19
21	19	Leu542Pro	T1757 → C	Kinase	Conley *et al.*, 1994	
22	21	Arg562Trp	C1816 → T	Kinase	Conley *et al.*, 1994	
23	22	Trp581Arg	T1873 → C	Kinase	Conley *et al.*, 1994	
24	24	Met630Lys	T2021 → A	Kinase	Conley *et al.*, 1994	
25	25	Leu652Pro	T2087 → C	Kinase	Conley *et al.*, 1994	
26	7	Leu408Pro	T1355 → C	Kinase	Zhu *et al.*, 1994b	
27	JN	Arg28His	G215 → A	PH	Vihinen *et al.*, 1995	6, 8
28	SG	Thr33Pro	A229 → C	PH	Vihinen *et al.*, 1995	45
29	—	Val64Phe	G322 → T	PH	Vihinen *et al.*, 1995	
30	10/41	Phe25Ser	T206 → C	PH	Vorechovsky *et al.*, 1995	

(*continues*)

TABLE VI—*Continued*

Patient		Amino Acid Change	Nucleotide Change[a]	Affected Domain	Reference	Same Mutation in Patient No.[b]
No.	Name					
31	5/29	Glu205Asp	G747 → T	TH	Vorechovsky *et al.*, 1995	
32	11/37	Arg288Trp	C994 → T	SH2	Vorechovsky *et al.*, 1995	3
33	17/5	Iso370Met	A1242 → G	SH2	Vorechovsky *et al.*, 1995	
34	19/10	Met509Val	A1657 → G	Kinase	Vorechovsky *et al.*, 1995	
35	15/20	Arg525Pro	G1706 → C	Kinase	Vorechovsky *et al.*, 1995	
36	2/19	Asp526Lys	C1710 → G	Kinase	Vorechovsky *et al.*, 1995	
37	7/15	Arg562Trp	C1816 → T	Kinase	Vorechovsky *et al.*, 1995	
38	18/13	Ala582Val	C1877 → T	Kinase	Vorechovsky *et al.*, 1995	12
39	20/17	Gly594Arg	G1912 → A	Kinase	Vorechovsky *et al.*, 1995	
40	40	Glu567Lys	G1831 → A	Kinase	Jin *et al.*, 1995	
41	39	Met587Leu	A1891 → C	Kinase	Jin *et al.*, 1995	
42[c]	23	Arg641His	G2054 → A	Kinase	Jin *et al.*, 1995	43, 44
43	35	Arg641His	G2054 → A	Kinase	Jin *et al.*, 1995	42, 44

44	20	Arg641His	G2054 → A	Kinase	Jin *et al.*, 1995	42, 43
45	107	Thr33Pro	A229 → C	PH	Kinnon, personal communication	28
46	FM	Arg520Trp	G1691 → A	Kinase	Kinnon, personal communication	
47	SO	Arg641Cys	C2053 → T	Kinase	Kinnon, personal communication	
	AP	Glu636Stop	G2038 → T	Kinase	Bradley *et al.*, 1994	
	Go3	Glu636Stop	Ins2041A	Kinase	de Weers *et al.*, 1994	
	14	Ile629Frsh	Ins2019C	Kinase	Zhu *et al.*, 1994b	
	6/26	Ile652Stop	del2084T	Kinase	Vorechovsky *et al.*, 1995	

[a]Nucleotide numbering according to Vetrie *et al.* (1993c).
[b]Underlined number denotes mutation affecting CpG doublet.
[c]Patients with mild phenotype.

TABLE VII
MUTATIONS CAUSING XLA

Type	No. of Patients (%)	Reference
Amino acid substitution	47(40)	See Table VI
Stop codons	21(18)	Bradley *et al.*, 1994; de Weers *et al.*, 1994; Ohta *et al.*, 1994; Hagemann *et al.*, 1994; Conley *et al.*, 1994; Zhu *et al.*, 1994b; Vorechovsky *et al.*, 1995; Jin *et al.*, 1995
Deletions (all with frameshifts)	21(18)	Ohta *et al.*, 1994; Conley *et al.*, 1994; Zhu *et al.*, 1994b; Vorechovsky *et al.*, 1995; Jin *et al.*, 1995; Kinnon, personal communication
Splice	13(11)	
With frameshift	11	Duriez *et al.*, 1994; Ohta *et al.*, 1994; Hagemann *et al.*, 1994; Conley *et al.*, 1994; Vorechovsky *et al.*, 1995; Jin *et al.*, 1995; Zhu *et al.*, 1994a
Without frameshift	2	
Insertions	12(10)	
With frameshift	11	de Weers *et al.*, 1994; Hagemann *et al.*, 1994; Conley *et al.*, 1994; Zhu *et al.*, 1994b; Vorechovsky *et al.*, 1995; Jin *et al.*, 1995; Kinnon, personal communication; Bradley *et al.*, 1994
Without frameshift	1	
Start sites	3(3)	Bradley *et al.*, 1994
Insertion + deletion	1(1)	Conley *et al.*, 1994
Total	118	

F. The Spectrum of Mutations in XLA and Their Implications

To date, several types of mutations have been found in XLA. Thus, missense, nonsense, splice site, insertions, and small and large deletions have all been found (Tables VI and VII; Fig. 7). Several methods have been employed for the analysis of mutations. Initially, cDNA-based techniques were used (Vetrie *et al.*, 1993c). The recent characterization of the genomic organization of the *Btk* locus (Sideras *et al.*, 1994; Hagemann *et al.*, 1994; Ohta *et al.*, 1994) permitted the development of DNA-based analysis, thus enabling the identification of splice site mutations (Conley *et al.*, 1994; Vorechovsky *et al.*, 1995; Jin *et al.*, 1995). It is clear from studies in XLA families performed many years prior to the cloning of the defective gene that there is a phenotypic variation among family members supposedly carrying the same mutation (Buckley and Sidbury, Jr., 1968; Goldblum *et al.*, 1974; Wedgwood and Ochs, 1980; Leickley and Buckley, 1986). This phenotypic variation could be caused by the influence of other genes or originate from other mechanisms (Bowman and Maynard Smith, 1963; Romeo and McKusick, 1994).

However, interpretation of the mutations in functional and structural terms also indicates that certain regions of the Btk molecule can tolerate missense mutations without dramatic effects on the activity of the PTK (Bradley *et al.*, 1994; Saffran *et al.*, 1994; Vihinen *et al.*, 1994c).

Extrapolating these findings, it seems likely that there will be Btk variants that could be referred to as "allelic," not having a dramatic effect on function. Individuals carrying such alterations would not be easily recognizable as they would not show any clinical symptoms. To some extent the effect of such substitutions could be foreseen from sequence alignments identifying conserved and, therefore, presumably critical, residues. However, the A607D mutation identified by Bradley *et al.*, (1994) and the A582V mutation reported by Vihinen *et al.*, (1994b) did not affect amino acids previously recognized as conserved (Vetrie *et al.*, 1993b). Such mutations may therefore provide insight into residues unique to Btk function, or alternatively, represent regions being functionally tolerant in kinases in general. The observation that the phenotype of the A582V mutation seems to be severe and that of the A607D was reported as mild is compatible with the idea that both these alternatives are valid. Using site-directed mutagenesis, the implication of the A582V mutation has recently been further studied (Maniar *et al.*, 1995).

Btk is likely to soon become the best characterized of all kinases in terms of mutations, as a very large number of patients are being analyzed worldwide. Several factors influence the mutation rate and, in particular, the CpG doublet content must be taken in account (Duncan

and Miller, 1981). This is exemplified by the four independent mutations affecting the R28 residue in the PH domain (Thomas *et al.*, 1993; de Weers *et al.*, 1994; Ohta *et al.*, 1994; Vihinen *et al.*, 1995). In fact, $\frac{14}{18}$ mutations occurring in more than one patient affect a CpG doublet (Table VI). From the collection of different mutations in Table VI and Fig. 7, it is already apparent that certain patterns exist. Thus, frameshift mutations are evenly spread out over the molecule, whereas certain areas are more frequently affected by in-frame mutations, presumably affecting functionally critical regions.

It could be anticipated that revertants of certain mutations could occur. Thus, a point mutation is expected to be corrected at a certain frequency. However, the fact that Epstein–Barr virus-derived B cell lines from patients with XLA are more easily obtained in young versus old individuals (Fu *et al.*, 1980; Wedgwood and Ochs, 1980; Leickley and Buckley, 1986) may indicate that revertants are rare.

G. Mutations in XLA with Growth Hormone Deficiency and Other Complex Phenotypes

XLA with growth hormone deficiency was a candidate for a contiguous gene deletion. However, analysis of patients with XLA and growth hormone deficiency excluded major deletions (Vorechovsky *et al.*, 1994a) and point mutations within the *Btk* gene have recently been characterized in such patients (Vihinen *et al.*, 1994c; Conley *et al.*, 1994; Duriez *et al.*, 1994). In the original family with this disease entity (Fleisher *et al.*, 1980) a full-length *Btk* transcript with a normal sequence has been identified (Stewart *et al.*, 1994). A deletion extending from the *Btk* gene into the centromerically located gene designated DXS1274E was found in a patient with torsion dystonia and cosegregating X-linked deafness compatible with an involvement of this gene (Vorechovsky *et al.*, 1994a). Furthermore, the absence of cytogenetically detectable deletions in Xq22 indicates the presence of gene(s) flanking the *Btk* locus whose defects are not compatible with survival.

XVIII. Carrier Detection and Prenatal Diagnosis

In female embryogenesis one of the X chromosomes becomes inactivated in each cell and this inactivation will remain in the subsequent progeny of this cell (Lyon, 1966). Prior to detailed information on the location of the XLA gene on the X chromosome, the analysis of X-chromosome inactivation made it possible to identify carriers (Conley *et al.*, 1986). Isoenzymes of G6PD were first used followed by the

identification of methylation differences and human–rodent hybrids to isolate the active human X chromosome (Conley *et al.*, 1986; Conley and Puck, 1988a; Puck, 1993; Fearon *et al.*, 1994).

Contingent upon the mapping of the XLA gene to a subregion of the X chromosome (Kwan *et al.*, 1986) the continued refinement of the map has resulted in improvements in carrier detection owing to an increased number of polymorphic markers (Lau *et al.*, 1988; Timmers *et al.*, 1991b; Schuurman *et al.*, 1988, Parolini and Conley, 1993; Lovering *et al.*, 1993b; Sweatman *et al.*, 1993; Parolini *et al.*, 1993; Tsuge *et al.*, 1993; Vorechovsky *et al.*, 1994a. These markers could also be employed in prenatal diagnosis based on chorionic villous analysis (Lau *et al.*, 1988; Journet *et al.*, 1992), obviating the need for fetal blood sampling later during gestation (Durandy *et al.*, 1982). When a number of polymorphic markers are combined, such as repeat polymorphisms, essentially all subjects are likely to have informative haplotypes. However, gonadal mosaicism, uniparental disomy (two copies of a single paternal X chromosome), and other phenomena, such as autosomal recessive inheritance, must be taken into consideration (Hendriks *et al.*, 1989; Conley and Sweinberg, 1992; Parolini *et al.*, 1993).

Novel methods to identify inactivated versus noninactivated X chromosomes make use of PCR-based techniques to identify methylation-sensitive sites in close proximity to nucleotide repeats on the X chromosome (Allen *et al.*, 1992; Hendriks *et al.*, 1992). Using more than one methylation-sensitive site in the PCR template and employing magnetic bead separation to obtain purified B and non-B cell populations further increases the applicability of this technique (Allen *et al.*, 1992). This method has also been successfully adopted by others (Alterman *et al.*, 1993) and in a recent article Allen *et al.* (1994) applied this technique to genetic counseling and were able to assess carrier status for mothers of isolated affected males in 10 of 11 families.

With the advent of the isolation of the *Btk* gene mutation analysis can now be directly employed unambiguously identifying the defective gene. However, of note is that the sensitivity of different mutation scanning techniques available, varies among different genes.

XIX. Development of Animal Model Systems for the Analyses of Btk Function

In order to generate an animal model system for XLA, the *Btk* locus of mouse embryonic stem cells was modified using homologous recombination (W. Khan, F. W. Alt, and P. Sideras, unpublished results).

Two types of mutated ES cells were generated using this approach. In the first type, exons 2 and 3, which contain the translation initiation site and most of the pleckstrin domain (PH domain mutant), and in the second type-exons 13 and 14, containing a small part of the SH2 domain and the ATP-binding region of the kinase domain (kinase mutant), were replaced with the neomycin-resistance gene. Both types of ES cells were injected into RAG-2-deficient blastocysts and chimeric animals were generated. The kinase mutant ES cells were also injected into B6-derived blastocysts and animals carrying the mutated allele in their germline were obtained (W. Khan, F. W. Alt, and P. Sideras, unpublished results). Preliminary analyses of the above animals demonstrated that the B cell development in all of them was abnormal. However, in none of these animals was a defect as dramatic as the one seen in the human XLA patients observed. All types of animals lacked CD5-positive B cells, thus veryfing that the absence of these cells in the *xid* mice is a direct consequence of the *Btk* mutation. The animals could be ordered according to the severity of the B cell development defect as *xid* < (PH domain mutant/chimera-kinase domain mutant/chimera-kinase domain mutant germline) < human XLA. The more severe phenotype of the PH domain mutant compared to the Xid mouse, is probably due to the fact that while this mutation probably affects the efficiency of protein–protein interactions mediated only be the first of the two subdomains of the PH domain, the PH mutant animals completely lack both of them. The kinase mutant animals are particularly interesting. Significant numbers of IgM-positive cells that have an "immature B cell" phenotype, i.e., IgM^{high}/IgD^{low}, are found in the peripheral organs of the $RAG\text{-}2^{-}$ chimeras as well as the germline homozygous males and females (approximately 30–50% compared to the wild-type males or the heterozygous female litermates). However, preliminary analysis has indicated that these cells are deficient in their capacity to be activated (W. Khan, F. W. Alt, and P. Sideras, unpublished results). The above animals might not be the very much expected animal model system for XLA; however, they provide unique starting material for biochemically analyzing the role of Btk during B cell activation and its interaction with other cellular signaling molecules. Crossing of these animals to other kinase-deficient mice that have been generated or will be generated in the near future could facilitate the identification of the other kinase molecules that compensate the defective Btk activity. More detailed analyses of these as well as other mutant animals to be generated in the close future will certainly provide extremely important information.

XX. Concluding Remarks

As already mentioned, not more than a year and a half has passed since the *Btk* gene was recognized as the primary genetic locus defective in XLA. Despite the limited time that has passed a quite impressive amount of information has been gathered. XLA, by being the first human disease caused by a defect in the function of a cytoplasmic tyrosine kinase, has attracted various investigators with interest in cellular immunology, clinical immunology, genetics, gene therapy, structural biochemistry, and signal transduction. This is already reflected in that several scientific papers from investigators with a background in signal transduction have appeared in the literature and the continuous cross-talk between these different disciplines is likely to be most fruitful.

It is obvious that the present understanding of the mechanism of action of this novel kinase is merely fragmentary. However, for the clinician it has now become possible to unambiguously identify mutations and thereby detect carriers and perform prenatal diagnosis. To this end, the likely prospect of a gene therapy approach in the not too distant future may influence counseling.

From its original description by Bruton in 1952, XLA has had a major impact on the development of several areas in biology. Looking into the crystal ball, formed by the previously mentioned prospects, makes us confident that in the foreseeable future this influence is not likely to come to a halt.

Acknowledgments

The authors thank Drs. O. Witte and C. Kinnon for providing unpublished information. The work of P. Sideras has been supported by the Swedish Medical Research Council and the Swedish Cancer Foundation (Cancerfonden). The work of C. I. E. Smith has been supported by the Swedish Medical Research Council, the Swedish Cancer Society, the European BIOMED concerted action "PL1321," The Axel and Margaret Ax:son Johnson Foundation, and the Dagmar and Ragnar Salen Foundation.

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This article was accepted for publication on 23 January 1995.

ADVANCES IN IMMUNOLOGY, VOL. 59

The Common γ-Chain for Multiple Cytokine Receptors

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I. Introduction

The immune system is regulated by transmembrane various extracellular stimuli, such as antigens, cytokines, and cell adhesion molecules. IL-2 is a cytokine molecularly identified during the early stages of cytokine studies, and this was initially detected as a T cell growth factor supporting a long-term culture of T cells (Morgan *et al.*, 1976; Gillis *et al.*, 1977). Biological functions of IL-2 have been extensively investigated after cloning of the IL-2 gene (Taniguchi *et al.*, 1983). They are involved in multiple biological responses of various types of cells, such as growth promotion of T cells (for a review, see Smith, 1988), B cells (Loughnan and Nossal, 1989), natural killer (NK) cells (Suzuki *et al.*, 1983), and glioma cells (Benveniste *et al.*, 1986), and also activation of Ig production of B cells (Waldmann *et al.*, 1984), and cytotoxicities of NK, LAK (Henney *et al.*, 1981; Trinchieri *et al.*, 1984), and monocytes/macrophages (Herrmann *et al.*, 1985; Malkovsky *et al.*, 1987). In some cases, however, IL-2 shows suppressive effects on T cell growth (Sugamura *et al.*, 1985) and causes T cell anergy (Boussiotis *et al.*, 1994), and may have costimulatory effects on T cell apoptosis (Lenardo, 1991).

These IL-2-responsive cells express specific receptors for IL-2 on

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their surface. IL-2 receptors are classified into three types differing in their affinities to IL-2—the high-, intermediate-, and low-affinity receptors—and there are at least three distinct subuits, the α-, β-, and γ-chains, constituting these receptors (Takeshita *et al.*, 1992a). The α-chain (IL-2Rα) was first identified by a monoclonal antibody (mAb) recognizing Tac antigen, a 55-kDa cell surface molecule specifically expressed on human T cell leukemia virus type I (HTLV-I)-induced T cell leukemic cells and activated T cells; anti-Tac mAb specifically inhibited IL-2-dependent cell growth and IL-2 binding to the receptor (Uchiyama *et al.*, 1981; Leonard *et al.*, 1982). Cloning of the IL-2Rα gene revealed that the IL-2Rα is composed of the low-affinity receptor which has no ability to transduce intracellular signals, suggesting the possible existence of the second component, the β-chain (IL-2Rβ), of the IL-2 receptor (Leonard *et al.*, 1984; Nikaido *et al.*, 1984; Cosman *et al.*, 1984). Prior to the molecular cloning of the IL-2Rβ gene, a few mAbs were established which recognized 75-kDa molecules on the surface of IL-2 receptor-positive cells and significantly inhibited IL-2-dependent cell growth, suggesting that these mAbs are specific for the human IL-2Rβ (Takeshita *et al.*, 1989; Tsudo *et al.*, 1989). The mAbs contributed to the successful expression cloning of a complete cDNA clone encoding the 75-kDa molecule, which was then confirmed to be the IL-2Rβ (Hatakeyama *et al.*, 1989a). The reconstitution study of the IL-2 receptor with IL-2Rα and IL-2Rβ genes indicated that the lymphoid transfectants with both genes or the β gene alone expressed functional high-affinity or intermediate-affinity IL-2 receptors for intracellular signal transduction, respectively, but the fibroblastoid transfectants with the genes or the β gene alone expressed nonfunctional incomplete high-affinity or undetectable affinity IL-2 receptors, respectively (Hatakeyama *et al.*, 1989a; Minamoto *et al.*, 1990). These findings suggested the possibility that another component present in lymphoid cells is required for formation of the functional complete high-affinity and intermediate-affinity IL-2 receptor complexes. Prior to these studies, another mAb, TU11, specific for the human IL-2Rβ, which did not block IL-2 binding, coprecipitated a 64-kDa molecule distinct from IL-2Rα and IL-2Rβ on the surface of lymphoid cells expressing IL-2 receptors (Takeshita *et al.*, 1990). A complete cDNA coding for the 64-kDa molecule was successfully achieved by immunoisolating the 64-kDa molecule along with the β-chain, eventually demonstrating that it is the third component, the γ-chain (IL-2Rγ), of the IL-2 receptor that is indispensable for functional high- and intermediate-affinity receptor complexes (Takeshita *et al.*, 1992a).

Studies of primary immunodeficiencies have contributed much to

our understanding of the mechanisms of development of T and B cells as well as the regulation of their functions. X-linked severe combined immunodeficiency (XSCID) is a disease characterized by a marked reduction or the absence of T cells, and its causative gene was mapped on X chromosome (Xq13) (for a review, see Conley *et al.*, 1992). Chromosome mapping of human IL-2Rγ ensued from the molecular identification of the gene for human IL-2Rγ. Interestingly, the human IL-2Rγ gene was shown to be mapped on the same locus (Xq13) as the putative gene responsible for XSCID; moreover, point mutations of IL-2Rγ derived from patients with XSCID were indeed discovered (Noguchi *et al.*, 1993). XSCID is now understood to be caused by mutations of IL-2Rγ. However, a line of evidence suggested that IL-2 dysfunction caused by IL-2Rγ deficiency cannot account for the mechanism of XSCID occurrence emanating from impairment of early T cell development. In this regard, IL-2Rγ was found to be used as a common receptor subunit for multiple cytokine receptors, similar to the common β-chain of IL-3, IL-5, and GM-CSF (for a review, see Miyajima *et al.*, 1992), and gp130 of IL-6, LIF, OSM, CNTF, and IL-11 (for a review, see Kishimoto *et al.*, 1994). Such cytokines interacting with IL-2Rγ should be implicated in the early T cell development that is impaired in XSCID.

Since no intrinsic effector function has been seen with cytokine receptors sharing IL-2Rγ, signal transducers are assumed to be associated with the cytoplasmic domains of the receptor subunits. It can be speculated that the signal transducers are composed of both common and specific molecules for the cytokines, resulting in overlapping and pleiotropic functions on various target cells in similar situations to other cytokines that share the common receptor subunits. Elucidation of the modes of signal transduction from IL-2Rγ together with the accompanied subunits will lead to the demonstration of regulatory mechanisms of early T cell development as well as cellular responses to the ligands.

II. Structure and Function of IL-2 Receptor Subunits

A. Molecular Identification of IL-2 Receptor Subunits

1. IL-2Rα and IL-2Rβ

Molecular characterization of IL-2 receptors commenced with gene cloning of IL-2Rα. A mAb recognizing the Tac antigen expressed on human leukemic cell lines carrying HTLV-I (Uchiyama *et al.*, 1981) significantly suppressed radiolabeled IL-2 binding and IL-2-

dependent cell proliferation, suggesting the possibility that the Tac antigen is the IL-2 receptor (Leonard *et al.*, 1982). The Tac antigen was purified by immunoaffinity column, and based on its partial amino acid sequences, a complete cDNA clone encoding the Tac antigen was isolated (Leonard *et al.*, 1984; Nikaido *et al.*, 1984; Cosman *et al.*, 1984). The Tac antigen was expressed on various types of cells negative for functional IL-2 receptor, exhibiting only low affinities [dissociation constant (k_d) = ~10 nM] to IL-2 binding. The Tac antigen was then identified to be a 55-kDa cell surface glycoprotein (p55), currently referred to as the α-chain of the IL-2 receptor (IL-2Rα), comprising the low-affinity IL-2 receptor. The gene for IL-2Rα is organized into eight exons and seven introns, spanning more than 35 kb on chromosome 10p14–15 (Leonard *et al.*, 1985a,b; Ishida *et al.*, 1985). The peptide backbone of IL-2Rα consists of 251 amino acid residues with no significant homology to known cytokine receptors, and the extracellular domain contains critical amino acid residues for interaction with IL-2 and IL-2Rβ (Robb *et al.*, 1988). The cytoplasmic domain of IL-2Rα contains only 13 amino acid residues, which seems insufficient to harbor a signal transducing ability. In fact, the low-affinity receptors composed of Tac antigen were nonfunctional in terms of IL-2 internalization and transduction of IL-2 mediated intracellular signals. On the contrary, most of the functional IL-2 receptors for intracellular signal transduction had high (k_d = ~10p*M*) and intermediate (k_d = ~1 n*M*) affinities, indicating another component necessary for formation of the high- and intermediate-affinity receptors.

The second component, IL-2Rβ, was initially identified by affinity cross-linking experiments with radiolabeled IL-2 (Sharon *et al.*, 1986; Tsudo *et al.*, 1986; Teshigawara *et al.*, 1987; Robb *et al.*, 1987; Dukovich *et al.*, 1987) and subsequently by mAbs specific for the human IL-2Rβ to be a 75-kDa cell surface glycoprotein (p75) (Takeshita *et al.*, 1989; Tsudo *et al.*, 1989). Treatment of cells with an anti-IL-2Rβ mAb together with anti-IL-2Rα resulted in marked inhibition of IL-2-dependent cell proliferation, and such anti-IL-2Rβ mAbs also reduced affinities to IL-2 binding from the high to the low, suggesting that IL-2Rβ is an essential subunit for functional IL-2 receptors (Takeshita *et al.*, 1989; Tsudo *et al.*, 1989). The complete cDNA clone encoding the human IL-2Rβ was isolated by expression cloning with the mAbs (Hatakeyama *et al.*, 1989a). The human IL-2Rβ gene is partitioned into 10 exons and nine introns, spanning 24 kb on chromosome 22q11.2–12 (Shibuya *et al.*, 1990; Gnarra *et al.*, 1990). The mature form of IL-2Rβ deduced from the nucleotide sequence consists of 525 amino acid residues and was characterized to belong to the cytokine

receptor superfamily with the common features of two pairs of the conserved cysteine residues near the amino terminal and a tryptophan–serine–X–tryptophan–serine (WSXWS, WS motif) in the extracellular domain (Bazan, 1990). The WS motif is thought to be important for ligand binding and signal transduction, features for the formation of the functional IL-2 receptor, because point mutations at the sites of WS motif of IL-2Rβ induced loss of its IL-2-binding ability (Miyazaki *et al.*, 1991). The cytoplasmic domain of IL-2Rβ, which consists of 286 amino acid residues, is long enough to contribute to intracellular signal transduction. Since no catalytic motif like kinase consensus sequences was seen in the cytoplasmic domain, signal transducers are assumed to be associated with it. As discussed later, the cytoplasmic domain of IL-2Rβ contains unique regions that are involved in signal transduction, such as the box 1, serine-rich, acidic, and proline-rich regions. Transfection of the IL-2Rβ gene alone into lymphoid cells induced the functional intermediate-affinity IL-2 receptor for intracellular signal transduction, but no functional IL-2 receptor for IL-2 binding was seen in fibroblastoid transfectants with the IL-2Rβ gene alone; besides, lymphoid transfectants with both IL-2Rβ and IL-2Rα induced the functional high-affinity receptor for intracellular signal transduction, but fibroblastoid transfectants with both IL-2Rβ and IL-2Rα expressed the high-affinity receptor that has no signal-transducing ability (Hatakeyama *et al.*, 1989a). These observations suggested the possible existence of another lymphoid-specific component that is required for the formation of the functional IL-2 receptor complex along with IL-2Rβ.

2. *IL-2Rγ*

Prior to the gene cloning of IL-2Rβ, two unique mAbs, TU11 and TU27, specific for the human IL-2Rβ, were established; TU27 showed significant reducing activities on IL-2-binding affinities, from high to low affinities and from intermediate to undetectable affinities, and TU11 had no interfering activity with IL-2 bindings (Takeshite *et al.*, 1989; Suzuki *et al.*, 1990). TU11 precipitated IL-2Rβ in lysates of cells untreated with IL-2, while it precipitated a 64-kDa cell surface molecule distinct from IL-2Rα together with IL-2Rβ in lysates of lymphoid cells but not fibroblastoid cells expressing IL-2Rβ in the presence of IL-2 (Takeshita *et al.*, 1990). Furthermore, the numbers of IL-2Rβ molecules on lymphoid transfectants with the IL-2Rβ gene usually exceeded sites of the intermediate-affinity receptor, and the amount of the 64-kDa molecule coprecipitated with IL-2Rβ correlated well with the level of the intermediate-affinity IL-2 binding sites

(Takeshita *et al.*, 1992b). Moreover, the mouse mutant IL-2 with aspartic acid 141 showed a high-affinity interaction with the $\alpha\beta$ complex on fibroblastoid transfectant cells but antagonized the biological activity of IL-2 (Zurawski *et al.*, 1990). These findings suggested the possibility that the 64-kDa molecule might be the lymphoid-specific third component of the IL-2 receptor which contributes to the formation of the high- and intermediate-affinity IL-2 receptors. The 64-kDa molecule was purified by immunoaffinity column with TU11, and its amino-terminal amino acid residues were determined. Based on the amino acid sequence, the complete cDNA clone encoding the 64-kDa molecule was isolated, demonstrating that the 64-kDa molecule is the third component, γ-chain (IL-2Rγ), of the IL-2 receptor (Takeshita *et al.*, 1992a).

The schematic structure of IL-2Rγ deduced from its nucleotide sequence is shown in Fig. 1. The mature form of IL-2Rγ consists of 347 amino acid residues with sequences typical of the cytokine receptor superfamily such as IL-2Rβ. The extracellular domain of IL-2Rγ contains a leucine zipper-like region, but it is still unknown whether this region is significantly implicated in any function of the receptor. Its cytoplasmic domain, consisting of 86 amino acid residues, contains the so-called SH2-like region which is homologous to the last two

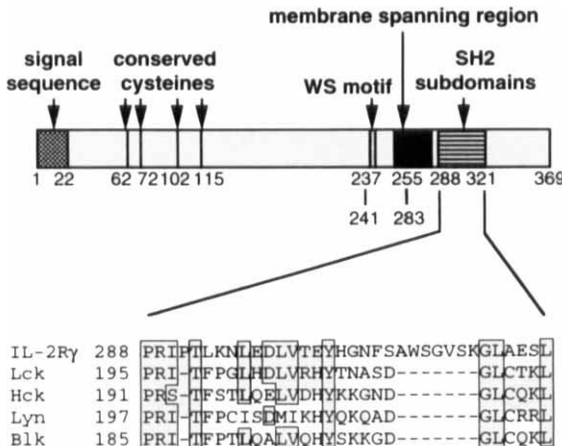

FIG. 1. Schematic structure of human IL-2Rγ. The possible signal sequence, conserved cystein residues, WS motif, transmembrane region, and the SH2 subdomain region are shown with their amino acid positions (top). Sequence alignment of the SH2 subdomains of IL-2Rγ and Src-related kinases (bottom). (Modified from Takeshita *et al.*, 1992a.)

subdomains of the Src homology region 2 (SH2) composed of the conserved five subdomains (Koch *et al.*, 1991). The SH2 domain is known to bind phosphotyrosine residues of some phosphoproteins, which is considered to be important for association with upstream or downstream events of the signal-transducing pathway present in the Src family tyrosine kinases (Mayer and Hanafusa, 1990). Although the last two subdomains of the SH2 domain present in IL-2Rγ are thought to be insufficient for binding to phosphotyrosine residues, the SH2-like domain of IL-2Rγ appears to be important for intracellular signal transduction as discussed below.

Mouse cDNA clones encoding IL-2Rγ were isolated based on cross-hybridization techniques using human IL-2Rγ cDNA (Kumaki *et al.*, 1993; Cao *et al.*, 1993). The predicted figure of mouse IL-2Rγ is identical to that of human IL-2Rγ except for one amino acid difference in both the extracellular and the cytoplasmic regions; the mouse IL-2Rγ extracellular region is one amino acid longer, whereas the cytoplasmic region is one amino acid shorter than the human IL-2Rγ. The homology between the two species at the amino acid level is 66% in the extracellular domain and 83% in the cytoplasmic domain including the SH2-like region.

The genomic structure of human IL-2Rγ was shown to consist of eight exons and seven introns spanning 4.2 kb on chromosome Xq12–13, where the putative gene for human XSCID was previously mapped. These findings led to the demonstration of the causative relationship between mutations of IL-2Rγ and human XSCID as described under Section IV (Noguchi *et al.*, 1993a).

B. Reconstitution of IL-2 Receptors

Expression studies of IL-2Rγ together with IL-2Rα and/or IL-2Rβ clearly showed that IL-2Rγ is an indispensable subunit of the functional IL-2 receptor complexes (Fig. 2). Mouse fibroblastoid L929 cells were stably transfected with various combinations of the α-, β-, and γ-chains of the IL-2 receptor. The transfectant clones were first examined for their association, dissociation rate constants, and affinities for IL-2 binding (Matsuoka *et al.*, 1993). Expression of IL-2Rα alone or both IL-2Rα and IL-2Rγ showed low affinities ($k_d = 10^{-8}$ M) to IL-2 binding, and either IL-2Rβ or IL-2Rγ alone possessed undetectable affinities ($k_d > 10^{-7}$ M) to IL-2 binding. The association rate constant with the αβγ heterotrimer complex was fourfold larger than that with the αβ heterodimer complex, and the dissociation rate constant was one-fifth of that with the αβ complex, resulting in two different types of high-affinity receptors with k_d of 10^{-11} and 10^{-10} M, respectively. The

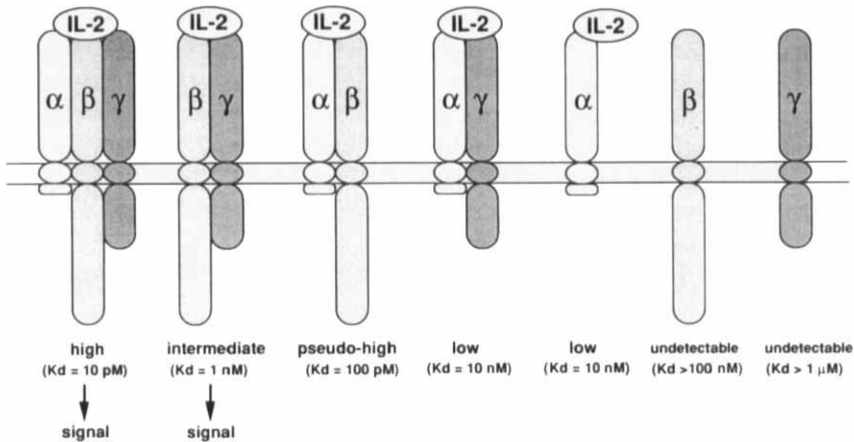

FIG. 2. Affinities and function of various IL-2 receptor subunit complexes. IL-2 receptor complexes reconstituted on the surface of fibroblastoid cells by transfection with three subunit genes were examined for their IL-2-mediated signal-transducing abilities and IL-2-binding affinities.

latter, consisting of the $\alpha\beta$ complex and nonfunctional for intracellular signal transduction, is referred to as the pseudo-high-affinity IL-2 receptor (Minamoto *et al.*, 1990; Takeshita *et al.*, 1992a). These results indicate that IL-2Rγ is involved in mechanisms by which IL-2 associates with and dissociates from receptors, resulting in the generation of the high-affinity IL-2 receptor ($k_d = 10^{-11}\ M$) together with IL-2Rα and IL-2Rβ, and the intermediate-affinity IL-2 receptor ($k_d = 10^{-9}\ M$) together with IL-2Rβ. The dissociation rate constants of the $\alpha\beta\gamma$ and $\beta\gamma$ complexes on lymphoid cells were generally much slower than those on fibroblastoid cells, suggesting that there is a significant difference between these two types of cells in regulating the dissociation of IL-2 from the receptors.

Since IL-2Rα formed the low-affinity receptor by itself and IL-2Rβ or IL-2Rγ alone possessed undetectable affinity to IL-2 binding, an affinity conversion model has been proposed such that IL-2Rα first binds IL-2 after which IL-2Rβ and IL-2Rγ associate with the IL-2-bound IL-2Rα to constitute the high-affinity receptor (Kondo *et al.*, 1986). Since any interaction among these three subunits is not fixed via covalent bonds, their interactions are expected to be dynamic. However, much weaker associations among the three receptor subunits than that of the IL-2 interaction with receptors have been detected in the absence of IL-2, suggesting that the specific interaction

among the subunits may contribute to the complex formation of the IL-2 receptor along with the affinity conversion model (Takeshita *et al.*, 1990).

As described later, both αβγ and βγ complexes of the IL-2 receptor have the ability to transduce intracellular signals even in the fibroblastoid transfectants, although the αβ heterodimer complex on the fibroblastoid cells, which also comprises the pseudo-high-affinity receptor, has inadequate signal transduction, indicating that not only IL-2Rβ, but also IL-2Rγ is required for intracellular signal transduction. Other cytokine receptor systems are also known to include two types of receptors, the high- and low-affinity receptors. The α-chains of the receptors for IL-3, IL-5, and GM-CSF have low affinities to ligand binding, and although their common β-chain is unable to bind the ligands on its own, the αβ heterodimers form high-affinity receptors (for a review, see Miyajima *et al.*, 1992). Similarly, the α-chains of IL-6 and CNTF have low affinities, but the heterodimers of the α-chains and the gp130 subunit form their high-affinity receptors, although gp130 itself has no ability to bind these ligands (for a review, see Kishimoto *et al.*, 1994). IL-2Rα is in a situation akin to that of the α-chains of other cytokine receptors described previously, and both IL-2Rβ and IL-2Rγ, which by themselves have undetectable affinities to IL-2 binding, contribute to constitute the high-affinity IL-2 receptor together with the α-chain.

Reconstitution studies of mouse IL-2 receptors provided evidence of some differences in the IL-2 receptor systems between human and mouse, although the homologies between mouse and human at the amino acid level are 61, 58, and 70% for IL-2Rα, IL-2Rβ, and IL-2Rγ, respectively (Shimizu *et al.*, 1985; Miller *et al.*, 1985; Kono *et al.*, 1990; Kumaki *et al.*, 1993; Cao *et al.*, 1993). Mouse IL-2 receptors were reconstituted on various types of mouse cell lines by stable transfection with mouse IL-2Rα, IL-2Rβ, IL-2Rγ genes. It was found that there is a critical difference between the two species in the IL-2-binding affinity of the βγ heterodimer complex. The mouse αβγ heterotrimer complex expressed on either lymphoid or fibroblastoid tranfectants comprised the high-affinity receptor for both human and mouse IL-2, while, in contrast to the human system, the mouse βγ heterodimer complex exhibited undetectable affinity to IL-2 binding (Kumaki *et al.*, 1993). Mouse splenic $CD8^+$ T cells, expressing significant amounts of IL-2Rβ and IL-2Rγ but not IL-2Rα, had no IL-2-binding ability and were unresponsive to IL-2 for proliferation (our unpublished data), whereas not only transfectant cells with both human IL-2Rβ and IL-2Rγ, but also human $CD8^+$ T cells and NK cells,

which express both IL-2Rβ and IL-2Rγ but not IL-2Rα, were able to respond to IL-2 for proliferation and activation of their cytotoxicities (Siegel *et al.*, 1987; Phillips *et al.*, 1989; Yagita *et al.*, 1989). These results suggest that IL-2Rα is necessary for the formation of the functional IL-2 receptor in the mouse system.

C. Expression of IL-2 Receptor Subunits on Various Hematopoietic Cell Populations

1. *Human Peripheral Blood*

Clonal expansion of antigen-specific T cells is known to be mediated by the interaction of IL-2 and the functional IL-2 receptor, of which expression was initially induced by antigen stimulation. The functional IL-2 receptors are composed of complexes consisting of the αβγ or βγ subunits in human and of the αβγ subunits in murine. IL-2Rγ is a common receptor subunit for other cytokines as described previously; therefore, regulation of IL-2Rγ expression is considered to be different from that of the other subunits. Expressions of IL-2Rα, IL-2Rβ, and IL-2Rγ on various populations of human peripheral blood cells were examined by two-color flow cytometry (Ohashi *et al.*, 1989; Tsudo *et al.*, 1989; Ishii *et al.*, 1994a). IL-2Rγ expression was seen on all of the cell populations including $CD4^+$T, $CD8^+$T, $CD20^+$B, $CD56^+$NK cells and $CD14^+$ monocytes. Most of the granulocytes also expressed IL-2Rγ. IL-2Rα and IL-2Rβ were differentially expressed on these cell populations, although their expressions were enhanced by extracellular stimuli such as antigens and mitogens. $CD8^+$T and $CD56^+$NK cells significantly expressed IL-2Rβ, but little of IL-2Rα, while $CD4^+$T cells expressed little or faintly expressed IL-2Rβ. The intermediate-affinity IL-2 receptor composed of IL-2Rβ and IL-2Rγ should be constituted on $CD8^+$T and $CD56^+$NK cells but not on $CD4^+$T cells. In fact, $CD8^+$ T cells freshly prepared from the peripheral blood showed a strong proliferative response to a high concentration of IL-2 (Yagita *et al.*, 1989). In contrast to $CD8^+$T cells, fresh $CD4^+$T cells revealed no significant IL-2 responsiveness, but they responded to IL-2 after stimulation with macrophages via MHC class II molecules, suggesting that antigen stimulation leads to expression of the functional IL-2 receptor on $CD4^+$T cells (Nakamura *et al.*, 1991). $CD56^+$ or $CD16^+$NK cells were also shown to respond directly to IL-2 for induction of their cytotoxicity and proliferation (Henney *et al.*, 1981; Suzuki *et al.*, 1983; Trinchieri *et al.*, 1984; Siegel *et al.*, 1987; Phillips *et al.*, 1989; Nishikawa *et al.*, 1990). On the other hand, $CD16^-CD56^+$NK cells in the human early pregnancy decidua ex-

pressed the high-affinity IL-2 receptor consisting of the $\alpha\beta\gamma$ heterotrimer complex, suggesting that these NK cells may be activated *in vivo* (Nishikawa *et al.*, 1991).

About 10% of the $CD14^+$ monocyte population expressed IL-2Rβ, and their stimulation with LPS induced expression of the high-affinity IL-2 receptor (Ishii *et al.*, 1994a). These results may explain the incompatible observations reported previously that activated monocytes or monocytic cell lines express the functional IL-2 receptor and show induction of proliferation and cytotoxicity in response to IL-2 (Malkovsky *et al.*, 1987), and that normal monocytes/macrophages and monocytic cell lines are negative for the IL-2 receptor without stimulation by IFNγ or LPS (Herrmann *et al.*, 1985; Holter *et al.*, 1987). On the other hand, TGF-β1 is known to suppress various immune responses including monocyte activation mediated by IL-2. However, the mechanism of the inhibitory effect of TGF-β1 on IL-2-induced monocyte activation was elusive, because TGF-β1 increased expression of IL-2Rβ in the presence of IL-2. Recently, TGF-β1 was shown to downregulate expression of IL-2Rγ in monocytes; hence, affording a hitherto adquate explanation of the inhibiting effect of TGF-β1 on IL-2 action (Espinoza-Delgado *et al.*, 1994).

2. *Mouse Thymus*

As described below in detail, mutations of IL-2Rγ are closely related to human XSCID, which is characterized by impairment of early T cell development. Therefore, it is of particular interest to see the expression of IL-2Rγ on thymocyte subsets in various differentiation lineages. Expressions of IL-2Rα, IL-2Rβ, and IL-2Rγ on various populations of mouse splenic cells were not substantially different from those on the human populations (Kondo *et al.*, 1994a). Mouse thymocyte cell populations including the double-negative ($CD4^-CD8^-$), double-positive ($CD4^+CD8^+$), and single-positive ($CD4^+$ or $CD8^+$) T cell subsets were also examined for their expressions of IL-2Rα, IL-2Rβ, and IL-2Rγ in comparison with those of IL-4Rα and IL-7Rα, because IL-2Rγ is shared with receptors for IL-4 and IL-7 (Fig. 3) (Kondo *et al.*, 1994a). Fetal thymus at Day 15 contained mostly immature thymocytes. The double-negative T cell subset, which is the most immature subset displayed high expression of IL-2Rγ, IL-2Rα, and IL-7Rα but little or no expression of IL-2Rβ and IL-4Rα, predicts that the double-negative T cells express functional IL-7 receptor but little of the functional IL-2 and IL-4 receptors. These findings are imperative for considering the role of IL-7 in the mechanisms of XSCID occurrence as discussed under Section III,B. The double-positive sub-

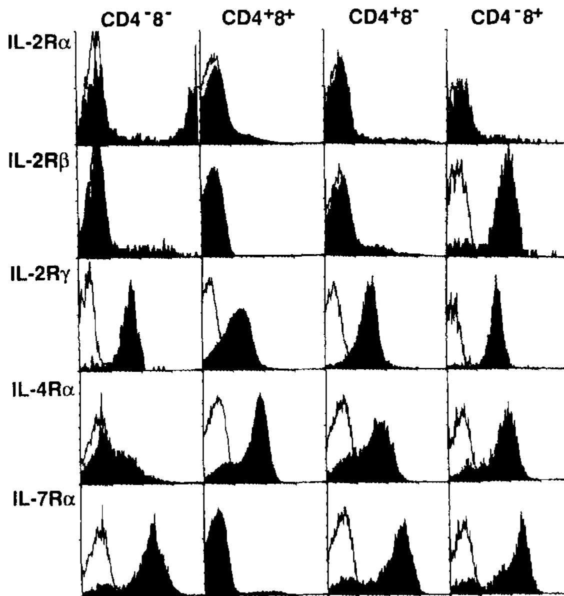

FIG. 3. Expression of the subunits of receptors for IL-2, IL-4, and IL-7 on adult mouse thymocytes. Expression of cytokine receptor subunits on thymocytes of C57BL/6 mice (~4 or 5 weeks old). Black lines represent staining with isotype control antibody and filled black fields represent anti-cytokine receptor subunit mAb-specific stainings. Monoclonal antibodies are AMT-13 (anti-IL-2Rα; Gibico BRL), TM-β1 (anti-IL-2Rβ; provided by Dr. Miyasaka, Osaka University), TUGm3 (anti-IL-2Rγ), M1 (anti-IL-4Rα; provided by Dr. Cosman, Immunex R & D), and A7R34 (anti-IL-7Rα; provided by Dr. Nishikawa, Kyoto University). This figure consists of the results in "*European Journal of Immunology*" **24**, 2026–2030, 1994, and our unpublished data. (Modified from Kondo *et al.*, 1994a.)

set contained a small population of IL-2Rγ-positive cells and a large population of IL-4Rα-positive cells, but was negative for IL-2Rα, IL-2Rβ, and IL-7Rα, predicting that a small population of the double-positive cells expresses the functional IL-4 receptor, and that most of the double-positive cells have no functional receptors for IL-2 and IL-7. $CD8^+T$ cells expressed IL-2Rβ, IL-2Rγ, IL-4Rα, and IL-7Rα, suggesting that they express the functional receptors for IL-2, IL-4, and IL-7. $CD4^+T$ cells also expressed IL-2Rγ, IL-4Rα, IL-7Rα, but little of IL-2Rα and IL-2Rβ, indicating that they express functional receptors for IL-4 and IL-7.

3. *Regulation of IL-2Rγ Expression*

Expressions of IL-2Rα and IL-2Rβ on peripheral blood leukocytes are differential among various cell populations. Their expressions were induced or enhanced within a day after antigen or mitogen stimulation (Ohashi *et al.*, 1989). On the other hand, IL-2Rγ expression was generally detected on the hematopoietic cell populations but scarcely detected on nonhematopoietic cells. To elucidate the regulatory mechanisms of IL-2Rγ expression, the promoter region of the IL-2Rγ gene has been identified (Ohbo *et al.*, 1995). The 633-bp fragment upstream of the initiation codon for IL-2Rγ showed the promoter activity in a human T cell line, Jurkat, and a human promonocytic cell line, THP-1, when linked to the luciferase gene. This fragment contained several sequences similar to those of previously identified transcription regulatory elements: four P.U.1 recognition sites, one PEA-3 site, three GT boxes, one TATA-like sequence, and one Ets-binding site. With a series of 5′-deletion mutants, basal promoter activity was found in a fragment from nucleotides 80 to 58 upstream from the RNA start site, including an Ets-binding sequence. 12-*o*-Tetradecanoylphorbol 13-acetate (TPA) or phytohemagglutinin (PHA), but not forskolin, significantly increased transcription from the IL-2Rγ gene promoter. In contrast, IL-2 treatment of cells induced suppression of transcription of the native IL-2Rγ gene as well as the IL-2Rγ promoter-driven luciferase gene. These results may explain the general phenomenon that normal human IL-2-dependent T cell lines are inclined to cessation of proliferation for a long period. On the other hand, HTLV-I-infected T cells are often established as IL-2-dependent cell lines, and this was thought to result from constitutive expression of the functional IL-2 receptor induced by HTLV-I infection (for a review, see Sugamura *et al.*, 1986). In fact, a *trans*-acting transcriptional activator, HTLV-I Tax, was found to act on the expression of the IL-2Rα gene (Inoue *et al.*, 1986). Although IL-2 suppressed expression of IL-2Rγ, an essential component of the functional IL-2 receptor, such a suppressive effect of IL-2 could not be observed in HTLV-I-infected T cells. Tax was found to augment expression of IL-2Rγ; moreover, Tax nullified the IL-2-mediated suppression of IL-2Rγ expression (Ohbo *et al.*, 1995). IL-2Rβ is another component required for the functional IL-2 receptor. Since the promoter region of IL-2Rβ has been shown to contain a TPA-responsive element and an Ets binding site like the IL-2Rγ promoter (Lin *et al.*, 1993), it can be speculated that HTLV-I Tax induces constitutive expression of the three subunits of IL-2 receptor in HTLV-I-infected T cells, resulting in the establishment of IL-2-dependent T cell lines.

In contrast to T cells, fresh human monocytes expressing a low level of IL-2Rγ were reportedly enhanced for IL-2Rγ expression by either IL-2 or IFNγ stimulation, but this enhancement was suggested to be due to the stabilization of IL-2Rγ mRNA (Bosco *et al.*, 1994).

III. Sharing of IL-2Rγ among Multiple Cytokine Receptors

The finding that the causative gene for XSCID is the IL-2Rγ gene revealed the indispensable role of IL-2Rγ in early T cell development. IL-2-deficient SCID patients and mice had normal phenotype of lymphocytes including T cell subsets, which differs from that observed in patients with XSCID. These observations allowed us to infer that IL-2Rγ is a common subunit of receptor complexes for IL-2 and other cytokines that may be necessary for early T cell development. Such possible involvement of IL-2Rγ in the formation of multiple cytokine receptor complexes has been substantiated by using two types of mAbs, TUGm2 and TUGm3, specific for IL-2Rγ; TUGm2 can block the specific interaction between IL-2 and IL-2Rγ, and TUGm3 can precipitate IL-2Rγ cross-linked with IL-2 (Kondo *et al.*, 1993). Candidate cytokines that share IL-2Rγ were thought to affect T cells and have so far been identified as one of their receptor subunits because most cytokine receptors are known to contain heterotrimer or heterodimer subunit complexes. In this context, IL-2Rγ was found to be shared as a common subunit among receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (Fig. 4). Thereby, IL-2Rγ is now called the common γ (γc)-chain for the multiple cytokine receptors.

A. IL-4/IL-4 Receptor System

1. Biological Activities of IL-4

IL-4 was initially detected as a B cell growth factor that costimulates with anti-IgM antibodies in short-term culture of resting B cells to induce polyclonal B cell proliferation (Howard *et al.*, 1982; Farrar *et al.*, 1983). In addition to a B cell growth factor, IL-4 was shown to possess a broader spectrum of biological activities, such as expression of MHC class II on resting B cells (Roehm *et al.*, 1984; Noelle *et al.*, 1984), expression of CD23 on both lymphocytes and monocytes (DeFrance *et al.*, 1987; Vercelli *et al.*, 1988), and enhancement of production and secretion of IgG1 and IgE in activated B cells (Vitetta *et al.*, 1985; Coffman *et al.*, 1986). Although such biological activities of IL-4 were somewhat elusive, successful preparation of recombinat IL-4 defined them to be involved in IL-4 action (Noma *et al.*, 1986;

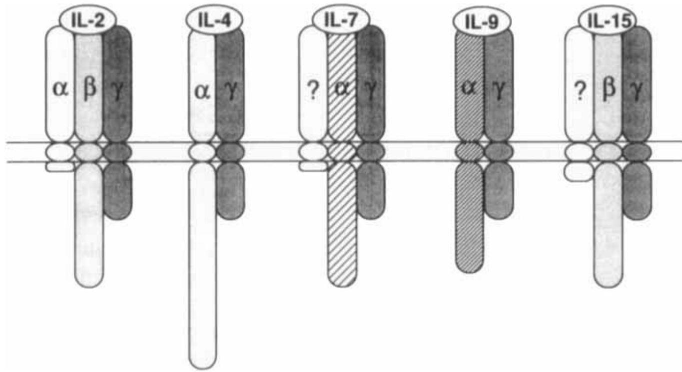

FIG. 4. Sharing of IL-2Rγ among receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. Utilization of IL-2Rγ as a common receptor subunit for IL-2, IL-4, IL-7, IL-9, and IL-15. The α-chains of the receptors for IL-2, IL-4, IL-7, and IL-9 are distinct from each other. The β-chain of the IL-15 receptor is the same as the β-chain of the IL-2 receptor.

Lee *et al.*, 1986). In addition, IL-4 was found to induce growth stimulation of the HT-2 T cell line and the MC/9 mast cell line (Mosmann *et al.*, 1986a; Smith and Rennick, 1986) and of resting T cells by costimulation with TPA (Hu-Li *et al.*, 1987). The role of IL-4 in helper T cell response to antigen was also investigated; some helper T cells responded to antigen by secreting IL-4, resulting in IL-4 autocrine growth (Fernandez-Botran *et al.*, 1986). Such IL-4-producing helper T cells are classified into the second type (TH2) of helper T cells, which provide help to B cells (Mosmann *et al.*, 1986b).

It has been difficult to define the *in vivo* functions of cytokines from *in vitro* studies because of their pleiotropic activities. Thus, transgenic mice for the IL-4 gene have been generated to assess the *in vivo* biological role of IL-4. Although their phenotypes varied with the transgene promoters, in general they showed reduced number of double-positive T cells and an increase of $CD4^{-}CD8^{+}$ thymocytes (Tepper *et al.*, 1990; Lewis *et al.*, 1991). The transgenic mice with Ig promoter exhibited a marked increase of serum IgE and allergic-like inflammatory disease, while the transgenic mice with lck promoter did not exhibit such phenotypes but induce osteoporosis resulting from decreased bone formation by osteoblasts (Lewis *et al.*, 1993). These observations suggested that IL-4 affects T cell development as well as mature T cell response to antigen stimulation for B cell help and nonhematopoietic osteoblasts. However, in IL-4-deficient mice with IL-4 gene knockout, T and B cell development was normal, but

serum levels of IgG1 and IgE were strongly reduced, suggesting that IL-4 may be required for Ig class switching but is not essentially involved in the T cell development (Kuhn *et al.*, 1991). $CD4^{+}$T cells from IL-4-deficient mice failed to produce TH2-derived cytokines, such as IL-4, IL-5, and IL-10, indicating that IL-4 is required for TH2-mediated immune responses (Kopf *et al.*, 1993). These *in vivo* studies correlated with some but not all of the *in vitro* biological activities of IL-4.

2. *IL-4 Receptor*

The biological activities of IL-4 are mediated by specific cell surface receptors expressed at low levels on these IL-4-responsive cells. To identify the IL-4 receptor, the mouse IL-2-dependent T cell line CTLL-2, which is also responsive to IL-4, was subcloned, resulting in the isolation of a subclone, CTLL 19.4, expressing IL-4 receptors at an extremely high level. IL-4 receptors were purified from lysates of CTLL 19.4 cells by affinity column with IL-4, the amino-terminal sequence was then determined, and according to the deduced nucleotide sequence, a complete cDNA clone encoding the IL-4 receptor was isolated (Mosley *et al.*, 1989). Expression of the cDNA clone in COS-7 cells resulted in an IL-4-binding molecule with a 140-kDa molecular mass, which is indistinguishable from the natural IL-4 receptor. The 140-kDa IL-4 receptor is now referred to as the α-chain of the IL-4 receptor (IL-4Rα). The mature form of IL-4Rα consists of 800 amino acid residues and belongs to the cytokine receptor superfamily. Its cytoplasmic domain with 553 amino acids in length contains some unusual sequence characteristics, including a high content of proline, serine, and acidic amino acids, but no known catalytic motifs of kinases.

Previous data regarding IL-4-binding affinities of receptors had been rather unclear; lymphoid cells expressing IL-4Rα exhibited higher affinities than those of nonlymphoid COS-7 transfectant with the IL-4Rα gene (Mosley *et al.*, 1989; Harada *et al.*, 1990). These observations suggested that the high-affinity IL-4 receptor consists of a complex composed of at least IL-4Rα and another subunit. Therefore, IL-2Rγ was first examined for its sharing with the IL-4 receptor complex. For this study, CTLL-2 cells showing dependency on both IL-2 and IL-4 were used. TUGm2 markedly suppressed the IL-4-dependent growth of CTLL-2 cells, while their IL-2-dependent growth was significantly inhibited only when TUGm2 and TM-β1 specific for IL-2Rβ were simultaneously added; however, TUGm2 or TM-β1 alone was not enough for IL-2-dependent growth inhibition (Kondo *et al.*, 1993). These observations suggested that IL-2Rγ contributes to not only IL-2-mediated but also IL-4-mediated cell growth signal transduc-

tion. The variable inhibitory effects of TUGm2 between IL-2 and IL-4 may be accounted for by the difference in binding affinities among IL-2, IL-4, and the mAbs and by the difference in numbers between the α-chains of IL-2 and IL-4 receptors expressed on CTLL-2 cells.

To assess the contribution of IL-2Rγ to the IL-4-binding of IL-4 receptor, TUGm2 was also examined for its effects on ligand binding to CTLL-2 cells using Scatchard plots (Kondo *et al.*, 1993). Treatment of cells with TUGm2 reduced the affinity of IL-4 binding from high (k_d = 130 pM) to intermediate (k_d = 850 pM) and the affinity to IL-2 binding from high (k_d = 130 pM) to pseudo-high (k_d = 370 pM). No significant change in the number of IL-4 binding sites was seen. IL-2Rγ itself bound little or no IL-4, indicating that IL-2Rγ itself does not have the ability to bind IL-4 directly.

Direct participation of IL-2Rγ in the IL-4 receptor complex was manifested by immunoprecipitation of IL-2Rγ after chemical cross-linking with IL-4 (Kondo *et al.*, 1993). Another IL-2Rγ-specific mAb, TUGm3, which has no inhibitory effects on the actions of IL-2, was used for this study. Cells were treated with radiolabeled IL-4 or IL-2, then with a chemical cross-linker, DSS, and their lysates were immunoprecipitated with TUGm3. TUGm3 apparently precipitated IL-2Rγ and IL-2Rβ cross-linked with IL-2. Additionally, IL-2Rγ cross-linked with IL-4 was also detected by TUGm3, although coprecipitation with IL-4Rα cross-linked with IL-4 was not observed.

All the results described above suggest a physical association of IL-2Rγ with the functional IL-4 receptor. Similar results were obtained from the reconstitution experiments of IL-4 receptors by cotransfection of IL-2Rγ and IL-4Rα genes (Russell *et al.*, 1993).

A close relationship between the receptors for IL-4 and IL-13 has been reported; IL-13 competitively inhibited binding of IL-4 to the functional IL-4 receptor expressed on a cell line responsive to both IL-4 and IL-13, and mutant IL-4 competitively antagonized both IL-4 and IL-13, but binding of IL-4 to an IL-4-responsive cell line that does not respond to IL-13 was not inhibited by IL-13 (Zurawski *et al.*, 1993; Aversa *et al.*, 1993). These observations suggested the possibility that the receptors for IL-4 and IL-13 share a common receptor subunit, but so far there has been no evidence of sharing IL-2Rγ with the IL-13 receptor complex.

B. IL-7/IL-7 Receptor System

1. *Biological Activities of IL-7*

A long-term bone marrow culture system provided evidence that a factor, currently referred to as IL-7, derived from stromal cells stimulates the proliferation of pre-B cells (B220$^+$, sIg$^-$). A mouse stromal

cell line, IXN/A6, known as a high-producer cell line for IL-7, was used for cDNA cloning of IL-7. The complete cDNA-encoding mouse IL-7 was cloned by the direct expression cloning in COS-7 cells (Namen *et al.*, 1988a). Mouse IL-7 was found to be significantly transcribed in spleen, thymus, and kidney, and to stimulate proliferation of both $B220^-$ and $B220^+$ cells, predicting that IL-7-responsive cell populations may include T cells and other lymphocytes other than pre-B cells. The responsiveness of T cells to IL-7 was first substantiated as a costimulatory activity with Con A (Morrissey *et al.*, 1989). When highly purified T cells were cultured with Con A and IL-7, they showed increased levels of IL-2 receptor expression, IL-2 production and proliferation. IL-6 was previously demonstrated to possess a costimulatory activity for Con A-activated T cells, but the costimulatory activity of IL-7 could not be inhibited by anti-IL-6. Furthermore, IL-7 was found to induce *in vitro* proliferation of T cells in combination with TPA stimulation, which proceeded in an IL-2-independent manner, and of T cells stimulated with anti-CD3 *in vivo* but not *in vitro* (Chazen *et al.*, 1989; Grabstein *et al.*, 1990; Armitage *et al.*, 1990). These observations suggest that, in addition to a potent B cell stimulatory activity, IL-7 may act on T cell growth and differentiation *in vivo*. Using thymus organ cultures, the effects of IL-7 on thymocytes were examined. IL-7 induced growth of double-negative thymocytes, but did not change their phenotype with respect to CD4 and CD8 expression, suggesting a significant effect of IL-7 on proliferation of double-negative thymocytes but little or no effect on their differentiation (Murray *et al.*, 1989). Indeed, adminstration of neutralizing mAb specific for IL-7 into mice resulted in marked inhibition of the development of B cell progenitors and in a substantial reduction of all major thymic subpopulations (Grabstein *et al.*, 1993; Sudo *et al.*, 1993). Thymic epithelial cells were shown to produce IL-7, which may regulate T cell development.

In vivo functions of IL-7 have been investigated with IL-7 transgenic mice and IL-7 receptor knockout mice. The IL-7 transgenic mice, of which the IL-7 transgene was regulated by the Ig promoter/enhancer, developed perturbation of T cell development in the thymus, characterized by reduction of double-positive T cells, and cutaneous lymphoproliferative disorder that induces B and T lymphomas (Rich *et al.*, 1993). These observations suggested *in vivo* functions of IL-7 in T cell development and in cutaneous immunity. Knockout mice for the α-chain of IL-7 receptor also provided critical information about the *in vivo* biological significance of IL-7. In these mice, the development of T and B cells was markedly inhibited in their early developmental

stages to induce a severe combined immunodeficiency (Peschon *et al.*, 1994). The biological significance of IL-7 in T cell development and its implication in XSCID are again discussed under Section IV.

Besides the effects of IL-7 on T and B cells, IL-7 has potent stimulatory effect on early myelopoiesis in synergy with CSF-1, IL-3, or GM-CSF. IL-7 enhanced CSF-induced myeloid colony formation from $Lin^-Sca\text{-}1^+$ murine bone marrow progenitor cells without affecting their ability to differentiate along the myeloid lineages (Jacobsen *et al.*, 1993).

2. *IL-7 Receptor*

Molecular and biochemical characterization of the IL-7 receptor commenced by cDNA cloning of the human and murine IL-7 receptors. A cDNA encoding the human IL-7 receptor was isolated by direct expression cloning strategy (Goodwin *et al.*, 1990). The mature form of the IL-7 receptor is 439 amino acids in length and has a calculated molecular weight of 49.5 kDa. The extracellular domain contains the features of the cytokine receptor superfamily. The amino acid sequence of the cytoplasmic domain consists of 195 amino acid residues and does not contain consensus sequences for protein kinases. The cloned IL-7 receptor is referred to as the α-chain of the IL-7 receptor (IL-7Rα). Transfection of the IL-7Rα gene into COS-7 cells induced expression of the high-affinity IL-7 receptor (k_d = 270 p*M*), the affinity of which is significantly lower than that of the high-affinity IL-7 receptor expressed on lymphoid cells. These results suggest that the high-affinity IL-7 receptor on lymphoid cells consists of a complex composed of IL-7Rα and another receptor component.

IL-2Rγ was suspected to be a common receptor subunit shared with the IL-7 receptor. First, TUGm2 was examined for its effect on IL-7 responsive cells such as Con A-stimulated splenic cells and a mouse pre-B cell line, IxN/2b. IL-7-dependent growth of the cells was significantly blocked by treatment with TUGm2, a mAb specific for mouse IL-2Rγ (Kondo *et al.*, 1994b). Treatment with TUGm2 alone induced inhibition of IL-7-mediated proliferation of both IxN/2b cells and Con A-stimulated splenic cells. The inhibition was most prominent at low concentrations of IL-7. Simultaneous addition of TUGm2 and A7R34, a mAb specific for mouse IL-7Rα, completely blocked the IL-7-dependent cell growth. Thus, IL-7-dependent growth required the function of IL-2Rγ.

TUGm2 also affected IL-7-binding affinities to the receptor (Kondo *et al.*, 1994b). The Scatchard analysis showed that IxN/2b cells have two phase-binding plots: a high affinity of 79 p*M* and a low affinity of

16 n*M*. Addition of TUGm2 reduced the affinity of the high-affinity receptor from 79 to 255 p*M* without affecting the number of binding sites, whereas no change in the low-affinity receptor was apparent. In contrast, treatment of cells with A7R34 (anti-IL-7Rα) alone or together with TUGm2, displayed only the low-affinity receptor. These results are similar to those of the IL-2/IL-2 receptor system, suggesting the possibility that the high affinity IL-7 receptor consists of a three-subunit complex composed of IL-7Rα, IL-2Rγ, and another unknown component which is expected to be involved in the formation of the low-affinity receptor. An IL-7 receptor complex without IL-2Rγ may comprise the intermediate-affinity IL-7 receptor with a k_d of 255 p*M*.

The chemical cross-linking experiments revealed the direct interaction between IL-2Rγ and IL-7. IxN/2b cells were treated with radiolabeled IL-7, then with a chemical cross-linker, DSS, and their lysates were immunoprecipitated with TUGm3 (Kondo *et al.*, 1994b). The precipitate contained IL-2Rγ cross-linked with IL-7. Although IL-2Rγ itself has no binding activity to IL-7, these observations indicate the direct participation of IL-2Rγ in the formation of the IL-7 receptor complex.

Similar to the IL-4 receptor, all the results concerning the effects of TUGm2 and TUGm3 suggest a physical association of IL-2Rγ with the functional IL-7 receptor. Similar results were obtained from reconstitution experiments of IL-7 receptors by cotransfection of IL-2Rγ and IL-7Rα genes (Noguchi *et al.*, 1993b).

C. IL-9/IL-9 Receptor System

IL-9 was originally described as a murine T cell growth factor, P40, produced by activated T cells and characterized by a narrow specificity for certain helper T cell clones (Uyttenhove *et al.*, 1988; Van Snick *et al.*, 1989) or a growth-enhancing factor for mast cells stimulated with IL-3 or IL-4 (Hültner *et al.*, 1990). Molecular cloning of IL-9 evidenced that IL-9 is responsible for mitogenic activity for erythroid progenitors (Donahue *et al.*, 1990), T cells (Houssiau *et al.*, 1993), B cells (Petitfrère *et al.*, 1993), and fetal thymocytes, but not for adult thymocytes (Suda *et al.*, 1990). The human IL-9 gene mapped onto the chromosome 5q31–35 region where various cytokines and a cytokine receptor gene, such as IL-3, IL-4, IL-5, CSF-1, and CSF-1 receptor, have been mapped (Kelleher *et al.*, 1991; Modi *et al.*, 1991).

Studies from molecular cloning illustrated that the IL-9 receptor belongs to the cytokine receptor superfamily (Renauld *et al.*, 1992). A functional IL-9 receptor was reconstituted on a mouse helper T cell clone, TS-1, by transfection of the human IL-9 receptor gene,

demonstrating that the transfectant cells become responsive to human IL-9 for their proliferation. The cloned IL-9 receptor is now referred to as the α-chain of IL-9 receptor (IL-9Rα). The mature form of human IL-9Rα contains 483 amino acid residues with 53% homology to mouse IL-9Rα. The cytoplasmic domain is 231 amino acids in length and contains the 33 amino acid residues proximal to the transmembrane domain, which is highly homologous to the cytoplasmic domain of IL-2Rβ. This observation suggested a functional similarity between IL-9Rα and IL-2Rβ. Since the IL-2Rβ can interact with IL-2Rγ, which is now known to be shared with receptors for at least IL-4 and IL-7, the possibility that IL-2Rγ is involved in the constitution of the functional IL-9 receptor was assessed.

A mouse IL-3-dependent mast cell line, MC/9, which is responsive to IL-9, was examined for the effect of TUGm2 on its proliferation in response to IL-9 (Kimura *et al.*, 1995). The IL-9-dependent growth of MC/9 cells was almost completely inhibited by its treatment with TUGm2, while its IL-3-dependent growth was unaffected. Similar inhibition of IL-9-mediated cell growth by TUGm2 was seen with a mouse IL-2-dependent T cell line, CTLL-2. These observations suggested that IL-2Rγ is functionally involved in transduction of cell growth signals mediated by IL-9. In contrast to the other cytokine receptor systems sharing IL-2Rγ, however, Scatchard analysis showed no effect of TUGm2 on the affinity of IL-9 binding (Kimura *et al.*, 1995). These results illustrate a different mode of participation of IL-2Rγ in the functional IL-9 receptor: that IL-2Rγ does not exert an influence on the affinity of IL-9 receptor but is essential for intracellular signal transduction.

The cross-linking experiments with radiolabeled IL-9 demonstrated that TUGm3 precipitated IL-9-cross-linked IL-2Rγ (99 kDa), probably IL-9Rα (86 kDa), and a 65-kDa molecule at a low level, indicating the physical association among IL-2Rγ, IL-9Rα, and IL-9 to form the functional IL-9 receptor (Kimura *et al.*, 1995). The 65-kDa molecule may be a complex containing IL-9 and an unidentified molecule (p43), which may also be responsible for the formation of the functional IL-9 receptor complex associated with IL-9Rα and IL-2Rγ because the interaction between IL-9 and p43 seems to coincide with the interaction of IL-9 with IL-9Rα and IL-2Rγ.

D. IL-15/IL-15 Receptor System

Recently, a novel cytokine, IL-15, was originally detected in supernatants from a simian kidney epithelial cell line, CV-1/EBNA, as an IL-2-like T cell growth factor mediating proliferation of IL-2-dependent

cells (Grabstein *et al.*, 1994). Using purified IL-15 from serum-free supernatants of CV-1/EBNA, its partial amino acid sequences were determined and, based on the amino acid sequences, a complete cDNA clone was isolated (Grabstein *et al.*, 1994). IL-15 was produced by a wide variety of cells and tissues. Recombinat IL-15, like IL-2, induced proliferation of CTLL-2 cells and PHA-stimulated $CD4^+$ T cells and $CD8^+$ T cells and generation of cytolytic effector cells such as cytotoxic T cell and LAK cells. Interestingly, anti-IL-2Rβ mAbs significantly inhibited the biological activities of IL-15, suggesting that IL-2Rβ is involved in the IL-15-mediated signal transduction (Grabstein *et al.*, 1994; Carson *et al.*, 1994). Analysis of IL-15 interaction with subunits of the IL-2 receptor revealed that not only IL-2Rβ but also IL-2Rγ are utilized as essential components of the IL-15 receptor complex (Giri *et al.*, 1994). IL-2 and IL-15 are known to function differentially on a number of cells vis-a-vis their bindings and responses, indicating the existence of another component of the IL-15 receptor complex like IL-2Rα (Giri *et al.*, 1994). Recently, the α-chain of the IL-15 receptor was molecularly identified to have a homologous structure to IL-2Rα (Cosman, personal communication).

IV. Causative Relationship between IL-2Rγ Mutations and Human XSCID

A. Classification of SCID

Primary immunodeficiency diseases, which are due to recessive gene defects, are categorized into several groups based on the characteristics of their respective immunodeficiencies. Among them, SCID features profound depression of both humoral and cell-mediated immunity, which is classified into several forms according to their genetic bases (Table I). Adenosine deaminase deficiency and purine nucleotide phosphorylase deficiency caused by autosomal gene defects result in the accumulation of nucleotide metabolites that are particularly toxic to developing T cells (Carson *et al.*, 1977; Cohen *et al.*, 1978). Patients with defective IL-2 production manifest SCID; one of these patients was shown to possess a mutation of a transcription factor, NF-AT, or its influencing factor, which is thought to be essential for the promotion of IL-2, IL-3, IL-4, and IL-5 gene expression (Chatila *et al.*, 1990; Castigli *et al.*, 1993). Bare lymphocyte syndrome is characterized by a lack of all MHC class II gene products on the cell surface, leading to impaired positive selection of $CD4^+$T cells in thymus and dysfunction of antigen-presenting cells (for a review, see Mach *et al.*, 1994). This syndrome was considered to be caused by a defect of any

TABLE I
HUMAN SCID SYNDROMES

Diseases	Causes
I. Severe Combined Immunodeficiency (SCID)	
Adenosine deaminase deficiency	ADA deficiency
Purine nucleotide phosphorylase deficiency	PNP deficiency
X-linked SCID	IL-2Rγ deficiency
Autosomal recessive SCID	?
Selective T cell defect	ZAP-70 deficiency
II. DiGeorge syndrome	Thymic aplasia
III. Bare lymphocyte syndrome	MHC II expression (−)
IV. Deficiency of cytokines	NF-AT dysfunction

one of the three or four complementary genes so far found, the products of which may be implicated in MHC class II transcription (Reith *et al.*, 1988; Hume and Lee, 1989; Bénichou and Strominger, 1991). In fact, the class II transactivator gene has been shown to be closely related to this syndrome (Steimle *et al.*, 1993). DiGeorge syndrome carries thymic aplasia resulting in profound T cell defect (Lischner and Huff, 1975). Another autosomal recessive SCID is accompanied with a defect of D–J recombination of the immunoglobulin genes (Schwarz *et al.*, 1991). Cells from such patients were abnormally sensitive to X-ray, suggesting a defect in double-stranded DNA repair like that in SCID mice (Cavazzana Calvo *et al.*, 1993), although a responsible gene(s) has not been identified yet. Recently, a mutation of the ZAP-70 tyrosine kinase molecule, which is a signal transducer associated with the CD3 complex of T cell receptor, has been documented to induce a selective T cell defect (STD) reminiscent of SCID (Arpaia *et al.*, 1994; Chan *et al.*, 1994; Elder *et al.*, 1994). Patients with STD have $CD4^+T$ cells that are unresponsive to antigens or mitogens. In addition the patients are defective in $CD8^+T$ cells, suggesting that ZAP-70 is indispensable for $CD8^+T$ cell development as well as for signal transduction of $CD4^+T$ cells.

As many as 50% of patients with SCID appear to have XSCID, which manifests complete or profound defect of T cells and NK cells, but carry normal or slightly increased numbers of B cells, leading to their death mostly within a year after birth if not treated with bone marrow transplantation (O'Reilly *et al.*, 1977; Buckley *et al.*, 1986). As described below, mutations of the IL-2Rγ gene have been demonstrated to cause XSCID.

B. Mutations of IL-2Rγ Result in XSCID

1. Various Loci of IL-2Rγ Mutations

Linkage analyses have resolved the loci of putative genes on the X chromosome responsible for X-linked immunodeficiency diseases, such as XSCID, Wiskott–Aldrich syndrome, X-linked lymphoproliferative syndrome, X-linked hyper IgM syndrome (XHM), and X-linked chronic granulomatous disease (for a review, see Conley, 1992; Royer-Pokora *et al.*, 1986). Chromosomal mapping assigned the IL-2Rγ gene to Xq13, which has been shown to be the locus for XSCID; furthermore, mutations were detected in the IL-2Rγ gene derived from three patients with XSCID (Noguchi *et al.*, 1993a). The same results were independently observed by several laboratories, confirming that all the patients with XSCID carry mutant genes of IL-2Rγ (Puck *et al.*, 1993; Ishii *et al.*, 1994b; DiSanto *et al.*, 1994a; Markiewicz *et al.*, 1994). Thus, IL-2Rγ mutations were demonstrated to be causatively related to XSCID, confirming that IL-2Rγ plays a pivotal role in early T cell development. The IL-2Rγ mutations so far detected in XSCID vary from patient to patient in both type and locus. The mutation loci spread over the IL-2Rγ molecule including the WS motif, the conserved cystein residues, and the SH2-like domain (Table II). Apart from base substitutions leading to missense and nonsense mutations, frameshift mutations by one and two base deletions as well as deletions of exons were seen, generating truncations in the extracellular and cytoplasmic domains of IL-2Rγ.

2. Impaired Function of IL-2Rγ Mutants Associated with XSCID

Mutants of IL-2Rγ in XSCID were presumed to lack the function necessary for early T cell development. We therefore studied how defective the mutants we identified were in terms of their abilities to form functional IL-2 receptors since IL-2Rγ was initially identified as an indispensable subunit of the functional IL-2 receptor (Ishii *et al.*, 1994b). The first patient lacked the second exon in IL-2Rγ mRNA, the second (AV mutant) showed one amino acid substitution in the extracellular domain, and the third (tSH mutant) had a two-base deletion causing a frameshift of the coding region in the SH2-like domain in the cytoplasmic region. As the first mutant yielded a short truncated product which could not be retained on the cell surface, further analysis of this mutant was not done. The second (AV) and third (tSH) mutants, and the wild-type of IL-2Rγ, were stably introduced into a unique human T cell line, ED40515(−). This cell line, originally established from PBL of a patient with adult T cell leukemia, was

TABLE II
SUMMARY OF IL-2Rγ MUTATIONS IN 20 PATIENTS WITH XSCID[a]

Patient	Mutation Type	Nucleotide Change	Amino Acid Change	Reference
1	Missense	G (216) → A	Glu (68) → Lys in exon 2	Markiewicz *et al.*, 1994
2	Missense	C (255) → T	Gln (61) → Phe in exon 2	Markiewicz *et al.*, 1994
3	Missense	G (355) → A	Gly (114) → Asp in exon 3	Puck *et al.*, 1993
4	Missense	G (358) → A	Cys (115) → Phe in exon 3	DiSanto *et al.*, 1994a
5	Missense	T (472) → A	Ile (153) → Asn in exon 4	Puck *et al.*, 1993
6	Missense	C (481) → T	Ala (156) → Val in exon 4	Ishii *et al.*, 1994b
7	Missense	C (690) →T	Arg (226) → Cys in exon 5	DiSanto *et al.*, 1994a
8	Missense	G (734) → C	Trp (240) → Cys in exon 5	DiSanto *et al.*, 1994a
9	Missense	C (737) → T	Ser (241) → Ile in exon 5	DiSanto *et al.*, 1994a
10	Nonsense	T (200) → A	Cys (62) → stop in exon 2	Puck *et al.*, 1993
11	Nonsense	A (369) → T	Lys (119) → stop in exon 3	Noguchi *et al.*, 1993a
12	Nonsense	C (444) → T	Gln (144) → stop in exon 3	Markiewicz *et al.*, 1994
13	Nonsense	C (879) → T	Arg (289) → stop in exon 7	Noguchi *et al.*, 1993a
14	Nonsense	C (937) → A	Ser (308) → stop in exon 7	Noguchi *et al.*, 1993a
15	Splice/missense	G (129) → A	Frameshift or Asp (39) → Asn in exon 1	DiSanto *et al.*, 1994b
16	Splice/deletion		Deletion of exon 2, frameshift	Ishii *et al.*, 1994b
17	Splice/deletion	G (468) → A	Disruption of splice donor site, frameshift in exon 3	Puck *et al.*, 1993
18	Deletion	A (711)	Ser (233) → frameshift in exon 5	Markiewicz *et al.*, 1994
19	Deletion	GATT (830-833)	Leu (272) → frameshift in exon 6	DiSanto *et al.*, 1994a
20	Deletion	GA (971-972)	Ser (320) → frameshift in exon 8	Ishii *et al.*, 1994b

[a] The exon localization of the mutations is from Noguchi *et al.* (1993c) and Puck *et al.* (1993).

adequate for examination of IL-2Rγ function because it expresses both IL-2Rα and IL-2Rβ but little or no IL-2Rγ (Arima *et al.*, 1992). The transfectant clones of the wild IL-2Rγ expressed high-affinity IL-2 receptors and proliferated in response to IL-2, while transfectant clones of the AV and tSH mutants could not respond to IL-2 at all (Ishii *et al.*, 1994b). The AV mutant clone showed no intermediate-affinity IL-2 binding after treatment with anti-IL-2Rα, demonstrating that the Ala residue at 156 in the extracellular domain is essential for interaction with IL-2. Furthermore, the AV mutant failed to form the functional receptor for IL-4 and IL-7 (our unpublished observations). These results suggest that the Ala 156 is critical for the solid structure of IL-2Rγ, which is required for interaction with ligands and/or coreceptor subunits. Indeed, the Ala 156 is located in the hinge region between two fibronectin type III-like domains, the N and C domains, of IL-2Rγ, as expected for the cytokine receptor superfamily (Bazan, 1990). Phenotypes associated with the tSH mutant are consistent with those of clones transfected with the deletion mutant of the SH2-like region (Asao *et al.*, 1994); that is, the intracellular signal transduction is not mediated by the tSH mutant even after binding IL-2. Similarly, other distinct mutants of IL-2Rγ originating from XSCID have been shown to have no ability to form the completely functional IL-2 receptor complex (DiSanto *et al.*, 1994a; Russel *et al.*, 1994).

3. *IL-2 Dysfunction Cannot Account for Mechanisms of Impaired T Cell Development in XSCID*

IL-2Rγ mutants derived from patients with XSCID were documented to have lost the function of a full-fledged IL-2 receptor. The question then posed is whether the impairment of T cell development in XSCID is primarily caused by the dysfunction of the IL-2 receptor. The following observations should help in providing part of the answers to this question; both IL-2-deficient SCID patients and mice could generate a normal number of T cells in the periphery (Pahwa *et al.*, 1989; Weinberg *et al.*, 1990; DiSanto *et al.*, 1990; Schorle *et al.*, 1991; Sadlack *et al.*, 1993; Kündig *et al.*, 1993). These observations indicated that the dysfunction of IL-2Rγ used as a subunit for the IL-2 receptor does not confer a defect of T cells in XSCID, suggesting the possibility that IL-2Rγ is a component of the functional receptor complexes for cytokines other than IL-2. In this regard, it is noteworthy to discover that IL-2Rγ is shared among functional receptors for at least IL-2, IL-4, IL-7, IL-9, and IL-15, as described below in detail. Consequently, one would plausibly assume that IL-2Rγ plays the exclu-

sive role in the development of T cells as a component of the receptor complex for IL-4, IL-7, IL-9, IL-15, or the other unknown cytokine(s). Since IL-4-deficient mice are also known to generate normal numbers of T cell subsets, IL-4 may not be necessary for early T cell development (Kuhn *et al.*, 1991; Kopf *et al.*, 1993). IL-15 has been shown to share IL-2Rβ as well as IL-2Rγ (Grabstein *et al.*, 1994; Giri *et al.*, 1994; Carson *et al.*, 1994), but double-negative ($CD4^-8^-$) thymocytes including the most immature T cells, though expressing IL-2Rγ, do not express the IL-2Rβ; thus, it is tempting to speculate that IL-15 may have no role in early T cell development (Kondo *et al.*, 1994a).

Possible involvement of IL-7 in early T cell development was examined by using the mouse thymic organ cultures (Hozumi *et al.*, 1994). When the most immature thymocytes, $Pgp1^+/c\text{-}kit^+$pro-T cells with $CD4^-8^-$, sorted from Day 15 fetal thymus, were cultured under high oxygen pressure in lobes of fetal thymus pretreated with deoxyguanosine to eliminate preexisting lymphocytes, $CD4^+$ and $CD8^+$ T cells were detected 7–10 days later. Simultaneous addition of blocking mAbs specific for the α-chain of IL-7 receptor (A7R34) and IL-2Rγ (TUGm2) into the culture completely inhibited development of the double-negative $Pgp1^+/c\text{-}kit^+$ pro-T cells into double-positive ($CD4^+8^+$) T cells, indicating that IL-7 plays a crucial role for early T cell development at least in the thymic organ culture system. The result suggests that the IL-2Rγ mutants in XSCID accompany the dysfunction of IL-7, resulting in the defect of T cell development. Indeed, the AV mutant of IL-2Rγ, described previously, has also been shown to have no ability to form the functional IL-7 receptor (our unpublished observations). Administration of anti-IL-7 antibodies into mice, however, induced a significant loss of B cells in addition to T cells, indicating that IL-7 also contributes to early B cell development in mice (Namen *et al.*, 1988a,b). The observation that IL-7 acts on the development of both T and B cells in mice seems to differ with that of XSCID in human with respect to the number of B cells in the periphery. XSCID exhibits a normal or increased number of B cells. There are currently three schools of thought to explain this inconsistency: (1) IL-7 may be required for T cell development but not B cell development in human; (2) there may be two distinct IL-7 receptors in the human system, one expressed on precursor T cells associates with IL-2Rγ, whereas the other expressed on precursor B cells does not recruit IL-2Rγ, (3) Another cytokine, such as IL-9, may be responsible for T cell development in human, because it is unlikely that the mouse thymic organ culture system reflects the *in vivo* development of T cells. These hypotheses, however, still remain to be resolved.

4. *Effects of IL-2Rγ Mutation on B Cell Development and Function*

Patients with XSCID show little or a very low amount of immunoglobulins in serum, even though surface IgM-positive B cells are present in the periphery in normal or elevated numbers. This result illustrates that B cell development, at least by the IgM-positive B cell stage, is independent of IL-2Rγ function. One would have presumed that B cells would be incapable of secreting immunoglobulin, because of lack of T cell help in the patients. Alternatively, the other possibility is that mutations in IL-2Rγ affect B cell function. The cooperation *in vitro* between B cells from the patients and T cells from normal individuals is able to lead the B cells to immunoglobulin production (Seeger *et al.*, 1976). On the other hand, the insight into obligatory carrier females who are normal in immunological properties demonstrated nonrandom inactivation of the X chromosome in mature B cells contrary to random activation in normal individuals (Conley *et al.*, 1988), illustrating that mature B cells inactivate the X chromosome of an allele carrying the mutant IL-2Rγ gene. This is also true in T cells and NK cells in carrier females (Puck *et al.*, 1987; Goodship *et al.*, 1988; Conley *et al.*, 1990). Immature B cells expressing mutant IL-2Rγ may suffer a disadvantage at the conversion to mature B cells. Moreover, the repertoire of usage of the immunoglobulin J_H segment was found to be biased in a patient with XSCID (Minegishi *et al.*, 1995). These observations may reflect the crucial function of IL-2Rγ in B cells as well as T cells.

5. *Atypical XSCID*

There are reports describing two atypical XSCID phenotypes with IL-2Rγ mutations (DiSanto *et al.*, 1994b; Russell *et al.*, 1994). One case showed a normal number of $CD4^+$ and $CD8^+$ T cells in the periphery (de Saint Basile *et al.*, 1992). Nevertheless, the peripheral T cells were poorly functional, because two different transcripts from the IL-2Rγ gene were present in the cells (DiSanto *et al.*, 1994b): one was nonfunctional due to aberrant RNA splicing, and the other was functional despite a mutation of Asp to Asn at 39. The former is abundant and the latter less abundant; molecular ratio is approximately 4:1. The result suggests that a small amount of IL-2Rγ is enough for T cell development but not for activation. The other case showed a mutation of Leu to Gln at 293 in the cytoplasmic region (Russell *et al.*, 1994) which exhibited less severe immunodeficiency with a reduced number of T cells in both $CD4^+$ and $CD8^+$ populations and normal

numbers of NK cells and B cells (Brooks *et al.*, 1990). T cells did not show the capacity to proliferate in response to PHA and an impaired production of immunoglobulins to specific antigen, diphtheria toxin, was seen.

Taken together with the fact that IL-2Rγ is a common component of other cytokine receptors, the functional implication of IL-2Rγ in T cell development could be different from that in T cell activation in immune responses. An intriguing observation was made with canine XSCID (Henthorn *et al.*, 1994; Somberg *et al.*, 1994). The canine IL-2Rγ gene is highly homologous to the human gene and was found to have, in affected animals, a four-base deletion in the first exon which generates a nonfunctional product. Despite the absence of the functional IL-2Rγ, presumably affecting development of $CD4^-$ $CD8^-$ double-negative thymocytes, the periphery contained normal numbers of T and B cells. T cells, however, failed to respond to PHA.

V. Signal Transduction Initiated by the Receptors Sharing the γc-Chain

A. IL-2/IL-2 Receptor System

1. Overview of IL-2-Induced Signal Transduction

Growth factor receptors, such as colony-stimulating factor-1 (CSF-1), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), contain large cytoplasmic domains with tyrosine kinases. Binding of these growth factors to their receptors induces activation of their tyrosine kinases, which triggers downstream signals to eventually evoke cell growth responses (Cantley *et al.*, 1991). Therefore, tyrosine phosphorylation of cellular proteins has been considered to be an indispensable event for cell growth signal transduction. There is accumulating evidence that the downstream signals initiated by the growth factors involve activations and cascade interactions of various signal-transducing effector molecules, such as PI3 kinase, PLCγ, Grb2, SOS, Ras, GAP, Raf-1, MAP kinase, and so on. IL-2, like most other cytokines, also exerts its function through interaction with the cell surface receptor, which generates similar biochemical events to those initiated by the growth factors. Both IL-2Rβ and IL-2Rγ, the essential components for IL-2-induced cell growth, belong to the cytokine receptor superfamily, of which the cytoplasmic domains, however, do not contain any consensus motifs of effector molecules for intracellular signal transduction, such as tyrosine kinases. It has therefore been the primary objective to identify signal-transducing effector molecules associated with the IL-2 recep-

tor complex. IL-2 rapidly induced serine/threonine phosphorylation and tyrosine phosphorylation of some cellular proteins, suggesting that serine/threonine kinase(s) and tyrosine kinase(s) are involved in the IL-2-mediated signal transduction (Ishii *et al.*, 1986; Gaulton and Eardley, 1986; Saltzman *et al.*, 1988; Ferris *et al.*, 1989; Piau *et al.*, 1989; Mills *et al.*, 1990). Molecular identification of IL-2Rβ enabled us to detect IL-2-induced tyrosine phosphorylation of IL-2Rβ, indicating a possible direct interaction of tyrosine kinase(s) with the IL-2 receptor complex (Asao *et al.*, 1990; Fung *et al.*, 1991). These studies led to the recent findings of the association of the Src family and the JAK family tyrosine kinases with the IL-2 receptor complex.

Biological activities of cytokines are known to show multifunctions characterized by pleiotropy and redundancy for their target cells. IL-2 functions as a growth factor for various lymphocytes, an enhancing factor for Ig production of B cells and for cytotoxicity of NK cells and macrophages, and a costimulating factor for induction of T cell anergy and apoptosis. On the other hand, not only IL-2 but also IL-4, IL-7, IL-9, and IL-15 induce growth of T cells, while at least IL-4 acts on B cells for Ig class switching. These pleiotropic and redundant functions of the cytokines may be due to receptor components and signal-transducing effector molecules involved in the signal-transducing pathways from the receptor complexes. The receptor complexes for IL-2, IL-4, IL-7, IL-9, and IL-15 contain the common subunit, IL-2Rγ, along with the receptor subunits specific for each receptor complex. Since both IL-2Rβ and IL-2Rγ are known to be responsible for IL-2-mediated signal transduction, it is of great interest to decipher the exact signal-transducing pathways present and what effector molecules participate in the IL-2/IL-2 receptor system.

2. *Critical Regions of the Cytoplasmic Domain of IL-2Rγ*

Reconstitution studies of IL-2 receptor complexes with IL-2Rγ and IL-2Rβ revealed the essential involvement of the cytoplasmic domains of both IL-2Rγ and IL-2Rβ in IL-2-mediated intracellular signal transduction. To define the critical regions of the cytoplasmic domain of IL-2Rγ for the signal transduction, various cytoplasmic deletion mutants of IL-2Rγ were prepared and used for reconstitution of IL-2 receptor complexes together with IL-2Rα and IL-2Rβ. The prepared IL-2Rγ mutants included γdC30, γdC68, and γdSH, which are deleted of the C-terminal 30 amino acids, the C-terminal 68 amino acids, and the SH2-like region, respectively. The wild-type or these mutant IL-2Rγ genes were introduced into a mouse fibroblastoid L929 subline expressing exogenous IL-2Rα and IL-2Rβ (Asao *et al.*, 1993). All the

transfectant cell lines expressed the full repertoire of the high-affinity IL-2 receptor complexes consisting of the transfected IL-2Rγ mutants and IL-2Rα and IL-2Rβ on their surfaces, indicating that a significant portion of the cytoplasmic domain of IL-2Rγ does not contribute to IL-2-binding affinity. The L929 transfectants were examined for their IL-2 responsiveness in terms of cell proliferation, IL-2 internalization, tyrosine phosphorylation of IL-2Rβ, and induction of protooncogenes such as c-*myc*, c-*fos*, and c-*jun*. The wild IL-2Rγ transfectants showed no IL-2-mediated growth promotion, but formed functional IL-2 receptor complexes for IL-2 internalization, tyrosine phosphorylation of IL-2Rβ, and induction of the three protooncogenes. On the other hand, the γdC30 transfectants showed IL-2 responsiveness similar to that of the wild-type transfectants except for induction of the protooncogenes. In the γdC30 transfectants, IL-2 induced c-*myc* gene induction but no induction of the c-*fos* and c-*jun* genes, indicating that there are at least two different signal-transducing pathways from the IL-2 receptor complex: one for c-*myc* gene induction and the other for induction of c-*fos* and c-*jun* genes (Fig. 5). Such different signaling pathways from the IL-2 receptor complex were previously observed with transfectants of various cytoplasmic deletion mutants of IL-2Rβ as described below. The γdC68 and γdSH mutants formed high-affinity receptors, but they were unable to transduce any signal except for IL-2 internalization, indicating that the SH2-like region of IL-2Rγ plays a crucial role in the IL-2-mediated intracellular signal transduction and that the large portion (at least C-terminal 68 amino acids) of the IL-2Rγ cytoplasmic domain is not required for IL-2 internalization. The ED40515(−) transfectants with these IL-2Rγ mutants also showed similar IL-2 responses to the L929 transfectants in tyrosine phosphorylation and induction of the protooncogenes. Furthermore, ED40515(−) transfectants with the wild-type and γdC30 mutants responded well to IL-2 for proliferation in a condition of serum starvation, whereas the L929 transfectants did not (Asao *et al.*, 1994). Thus, the functional IL-2 receptor complexes were apparently different in their abilities to induce IL-2-mediated growth signals between L929 and ED40515(−) transfectants. One possible mechanism underlying the above difference may be the differential expression of JAK3 tyrosine kinase as discussed under Section V,B,2.

3. *Critical Regions of the Cytoplasmic Domain of IL-2Rβ*

The cytoplasmic domain of IL-2Rβ contains at least three unique regions—the serine-rich, acidic, and proline-rich regions. To elucidate their significances in the IL-2-mediated signal transduction, the human

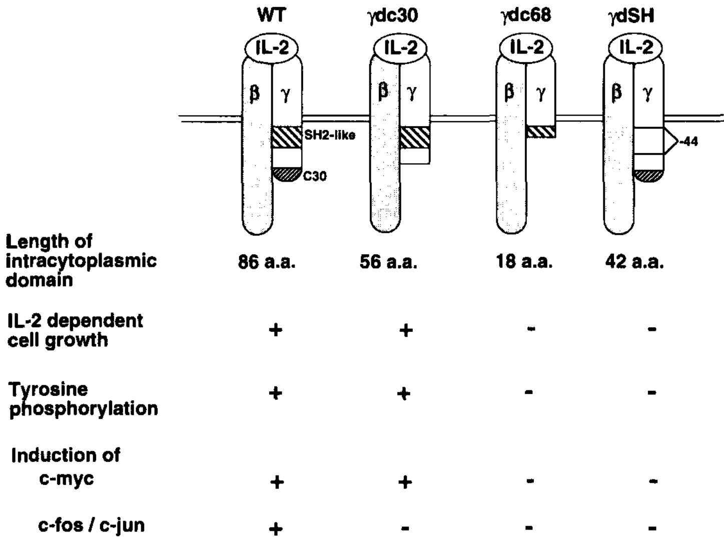

FIG. 5. Formation of functional IL-2 receptor complexes with cytoplasmic deletion mutants of IL-2Rγ. The wild-type γ gene (WT) and mutant γ genes with deletion of the C-terminal 30 amino acids (γdC30), deletion of the C-terminal 68 amino acids (γdC68), and deletion of the SH2-like region (γdSH) were stably introduced into the ED40515(−) cell line and the L929 cell line together with IL-2Rβ gene. The transfectant clones expressing each transgene were examined for their abilities to respond to IL-2 in terms of [^{3}H]thymidine uptake, tyrosine phosphorylation of cellular proteins, and induction of the protooncogenes such as c-*myc*, c-*fos*, and c-*jun*. Positive and negative results are indicated by + and −, respectively.

IL-2Rβ mutants deleted of the serine-rich region (S mutant), the acidic region (A mutant), and most of the cytoplasmic domain (ST mutant) were prepared and introduced into a mouse IL-3-dependent pro-B cell line, BAF-B03, expressing endogenous mouse IL-2Rα and IL-2Rγ (Hatakeyama *et al.*, 1989b), and a human T cell line, MOLT-4, expressing endogenous human IL-2Rγ (Tanaka *et al.*, 1994). The wild IL-2Rβ transfectants expressed the functional IL-2 receptor complex to induce IL-2-dependent cell growth. Similarly, the transfectants with A mutant showed IL-2-induced cell growth, whereas the transfectants with S mutant and ST mutant did not respond to IL-2 for their growth. IL-2-induced protooncogene expression was also examined with these transfectants; induction of c-*myc*, c-*fos*, and c-*jun* genes was detected in the wild transfectants and only c-*myc* gene induction was deleted in the A mutant transfectants. No significant induction of the three protooncogenes was seen in the transfectants with the S mutant (Hatakeyama *et al.*, 1992; Satoh *et al.*, 1992). These results suggest that the

serine-rich region of IL-2Rβ plays a crucial role in the IL-2-mediated signal transduction for cell growth and induction of at least the three protooncogenes (Fig. 6). The acidic region deletion mutant failed to induce expression of c-*fos* and c-*jun* genes but retained the ability to induce expression of the c-*myc* gene and cell growth. These observations provided a significant breakthrough in unraveling two distinct signaling pathways from the IL-2 receptor complex, one for the c-*myc* gene induction and the other for induction of c-*fos* and c-*jun* genes, which was later supported by the studies with the IL-2Rγ mutants as described previously. The acidic region of IL-2Rβ is essentially involved in the induction of c-*fos* and c-*jun* genes, and this region seems to be dispensable for induction of the cell growth signals. However, the transfectants with the A mutant showed a relatively lower response to IL-2 compared with those with the wild IL-2Rβ, suggesting an auxiliary effect of the acidic region in IL-2-mediated cell growth signalings (Hatakeyama *et al.*, 1989b; Tanaka *et al.*, 1994).

The cytoplasmic domain of IL-2Rβ also contains the so-called box1 and box2, which were originally defined in the cytoplasmic domain

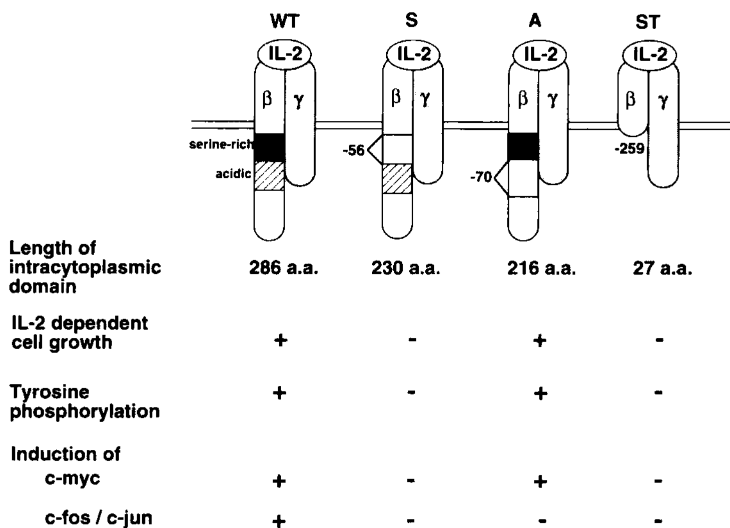

FIG. 6. Formation of functional IL-2 receptor complexes with cytoplasmic deletion mutants of IL-2Rβ. The wild-type β gene (WT) and mutant β genes with deletion of the serine-rich region (S), deletion of the acidic region (A), and a large cytoplasmic domain (ST) were stably introduced into the MOLT-4 and BAF-BO3 cell lines. The transfectant clones expressing each transgene were examined similar to the methods described in the legend to Fig. 5.

of gp130 and conserved among several cytokine receptors such as G-CSF receptor, Epo receptor, and the common β-chain of IL-3 receptor (Murakami *et al.*, 1991). Both box1 and box2 of IL-2Rβ are located proximally to the transmembrane domain, and box2 but not box1 is included in the serine-rich region of IL-2Rβ. The Epo receptor mutant in box2 was shown to be unresponsive to Epo (Miura *et al.*, 1993). Similarly, replacement of leucine 299 in box2 with proline resulted in the failure of IL-2Rβ to transduce IL-2-mediated cell growth signals (Mori *et al.*, 1991). Substitution of alanine for aspartic acid 258 in box 1 markedly compromised the receptor function for cell growth signaling, whereas substitution of glutamic acid restored the receptor function, indicating that the acidic character of the side chain of Asp 258 may be critical to its function (Goldsmith *et al.*, 1994). These findings documented that the membrane proximal domain including the box1 and serine-rich regions is indispensably involved in cell growth signaling.

4. *Chimeric Molecules of IL-2Rβ and IL-2Rγ*

The biological functions of the cytoplasmic domains of IL-2Rβ and IL-2Rγ were also investigated by using chimeric receptors containing their cytoplasmic domains. The chimeric receptors were constructed with the intracellular region of either IL-2Rβ or IL-2Rγ and the extracellular region of c-kit, a receptor for SCF (Nelson *et al.*, 1994). These chimeras were expressed on a pro-B cell line, BA/F3, and a T cell line, CTLL-2, and they were examined for their SCF responsiveness. Binding of SCF to the chimeric receptor consisting of the cytoplasmic βγ heterodimer-induced proliferation and tyrosine phosphorylation of some cellular proteins in both BA/F3 and CTLL-2 transfectants. Interestingly, the receptor consisting of the cytoplasmic β homodimer had the ability to respond to SCF stimulation in BA/F3 transfectants but not in CTLL-2 transfectants. These observations suggested that heterodimerization of the cytoplasmic domains of IL-2Rβ and IL-2Rγ is necessary and sufficient for signal transduction in T cells, whereas homodimerization of the cytoplasmic domain of IL-2Rβ seems to be adequate only in the pro-B cell line. Similar results confirming the requirement of the heterodimerization of the cytoplasmic domains of IL-2Rβ and IL-2Rγ were also obtained in 32D myeloid progenitor cells with other constructs in which the extracellular domains of IL-2Rβ and IL-2Rγ were replaced with that of IL-2Rα (Nakamura *et al.*, 1994). Homodimerization of the cytoplasmic domain of IL-2Rγ had no ability to induce growth of T, B, and myeloid cells (Nelson *et al.*,

1994; Nakamura *et al.*, 1994). However, it has been recently reported that cross-linking of IL-2Rγ with its specific mAb on the cell surface induces activation of JAK3 kinase and transduces signals for the prevention of T cell anergy similar to the anergy prevention by natural ligands such as IL-2, IL-4, and IL-7 (Boussiotis *et al.*, 1994).

B. Involvement of Tyrosine Kinases

1. The Src Family

Involvement of a tyrosine kinase in the IL-2-mediated signal transduction was originally suggested by the detection of IL-2-induced tyrosine phosphorylation of some cellular proteins (Saltzman *et al.*, 1988; Ferris *et al.*, 1989; Piau *et al.*, 1989). Subsequently, IL-2Rβ was shown to be phosphorylated on tyrosine residues within 1 min after IL-2 stimulation and well correlated with IL-2-induced cell growth (Asao *et al.*, 1990; Mills *et al.*, 1990). In fact, immune complexes containing IL-2 and IL-2Rβ contained tyrosine kinase activity, manifesting direct interaction of tyrosine kinase with IL-2Rβ (Fung *et al.*, 1991; Asao *et al.*, 1992). Accordingly, much effort was directed to identify the tyrosine kinase that is associated with the IL-2 receptor. In this context, one of the Src family tyrosine kinases, Lck, was initially noticed to be activated by IL-2 stimulation (Horak *et al.*, 1991). Thus, physical association of Lck with IL-2Rβ was verified by using the transfectants with IL-2Rβ mutants as described previously (Hatakeyama *et al.*, 1991). Lysates of COS-7 cells transfected with Lck ($p56^{lck}$) and the wild or mutants of IL-2Rβ were precipitated with Mik-β1, an anti-IL-2Rβ mAb, then immunoblotted with anti-Lck antibody. Lck was coprecipitated with the wild IL-2Rβ, but not with the A mutant IL-2Rβ, indicating that the acidic region of IL-2Rβ contains the interaction site to Lck. Similarly, other Src family tyrosine kinases, such as Fyn and Lyn, were found to be closely associated with the IL-2-mediated signal transduction; in particular, direct association of Fyn with the acidic region of IL-2Rβ was also detected (Kobayashi *et al.*, 1993). These findings predict that such Src family tyrosine kinases are involved in the signal-transducing pathway for induction of c-*fos* and c-*jun*, which is initiated by the acidic region of IL-2Rβ. Apart from IL-2-induced signaling, IL-7 was also found to activate Fyn and Lyn kinases in a human pre-B cell line, and Fyn was coimmunoprecipitated with IL-7Rα, suggesting a possible involvement of such Src family tyrosine kinases in the IL-7-induced signaling (Venkitaraman and Cowling, 1992; Seckinger and Fougereau, 1994).

2. *The JAK Family*

IL-2 induced tyrosine phosphorylation of IL-2Rγ as well as IL-2Rβ *in vivo* and *in vitro* (Asao *et al.*, 1992). Tyrosine phosphorylation of IL-2Rγ was a suitable indicator for the detection of tyrosine kinase activation in transfectants with the IL-2Rβ mutants. IL-2-induced tyrosine phosphorylation of IL-2Rγ or of some cellular proteins was observed in cells negative for Lck and transfectants with the A mutant of IL-2Rβ, implying that IL-2Rβ and IL-2Rγ may be associated with tyrosine kinase(s) other than the Src family tyrosine kinases such as Lck, Fyn, and Lyn (Asao *et al.*, 1992; our unpublished observation). Currently, novel tyrosine kinases belonging to the family of Janus kinases (JAKs) have been reportedly implicated in signal transduction initiated by several cytokines (Ihle *et al.*, 1994). JAK1, JAK2, and Tyk2 were activated by IFNα/β and IFNγ, and their activations essentially participated in signal transduction in response to IFNs (Velazquez *et al.*, 1992; Müller *et al.*, 1992; Watling *et al.*, 1993; Shuai *et al.*, 1993; Silvennoinen *et al.*, 1993a). Similarly, the JAK–TYK family tyrosine kinases was shown to be functionally associated with the LIF receptor β-chain (Stahl *et al.*, 1994), gp130 of IL-6 receptor (Lutticken *et al.*, 1994), and receptors for Epo (Witthuhn *et al.*, 1993), IL-3 (Silvennoinen *et al.*, 1993b), G-CSF (Nicholson *et al.*, 1994), and growth hormone (Argetsinger *et al.*, 1993). Thus, involvement of the JAK–TYK family tyrosine kinases in the IL-2-mediated signal transduction was assumed. In this context, it was demonstrated that IL-2 induced activation of JAK1 and JAK3, a new member of the JAK family which is detectable as a cross-reactive 114-kDa molecule with anti-JAK2 antibody, but not of JAK2 and Tyk2 (Tanaka *et al.*, 1994; Asao *et al.*, 1994; Johnston *et al.*, 1994; Witthuhn *et al.*, 1994; Kirken *et al.*, 1994; Beadling *et al.*, 1994). Furthermore, using transfectants with various cytoplasmic deletion mutants of IL-2Rβ and IL-2Rγ, their association sites with JAK1 and JAK3 were dissected; the serine-rich region of IL-2Rβ and the transmembrane proximal region containing the SH2-like region of IL-2Rγ participated in association with JAK1 and JAK3, respectively (Asao *et al.*, 1994; Miyazaki *et al.*, 1994; Russell *et al.*, 1994). It is noteworthy that a mouse fibroblastoid 3T3 subline expressing the exogenous IL-2Rα, IL-2Rβ, and IL-2Rγ responds to IL-2 for DNA synthesis only when it is transfected with the JAK3 gene (Miyazaki *et al.*, 1994). The reconstituted IL-2 receptor complex with the α-, β-, and γ-chains on fibroblastoid cells negative for JAK3 is known to exhibit IL-2 responsiveness in terms of tyrosine phosphorylation and induction of protooncogenes but hardly cell growth. Therefore, it

is suspected that JAK3 activation is one of the essential events required for the IL-2-induced cell growth signaling.

Recent studies have documented that the family of signal transducer and activator of transcription (STAT) proteins participate in the signal-transducing pathways from some cytokine receptors (for a review, see Ihle *et al.*, 1994). The STATs are thought to play an important role downstream of the JAK family tyrosine kinases; they form homo- and heterodimeric complexes via their SH2 domains upon tyrosine phosphorylation induced by JAKs, and their complexes interact with specific DNA sequences to effect gene expression (for a review, see Darnell *et al.*, 1994). STAT1 and STAT2 were originally characterized as components of IFNα-stimulated gene factor 3 (ISGF-3) (Fu *et al.*, 1992). STAT3 (APRF) was found to be involved in the signaling initiated by gp130 (Akira *et al.*, 1994). Novel member(s) of the STAT family have been suggested to be involved in the IL-2-mediated signal transduction, but their molecular characterization remains to be elucidated (Lamb *et al.*, 1994; Gilmour and Reich, 1994; Beadling *et al.*, 1994).

Another tyrosine kinase, Syk ($p72^{syk}$), a member of the Syk/ZAP-70 family, was also found to be activated in T cells within a few minutes after IL-2 stimulation (Qin *et al.*, 1994). Furthermore, it has recently been suggested that Syk is associated with the serine-rich region of IL-2Rβ, while IL-2-induced activation of Syk tyrosine kinase is possibly implicated in the c-*myc* gene induction (Minani *et al.*, 1995).

C. Other Biochemical Events Involved in Signal Transduction

1. *Ras*

A GTP-binding protein, Ras ($p21^{ras}$) has been known to play a key role in the signal-transducing pathways mediated by various extracellular stimuli for cell proliferation and transformation, of which activity is regulated by bound GDT/GTP (for a review, see McCormick, 1989). Involvement of Ras evidenced in signal transductions induced by some cytokines such as IL-2, IL-3, and GM-CSF (Satoh *et al.*, 1991; Graves *et al.*, 1992; Izquierdo *et al.*, 1992). IL-2 stimulation of T cells induced activation of Ras in terms of increased GTP-binding Ras, and both the serine-rich and the acidic regions of IL-2Rβ were essentially involved in its activation (Satoh *et al.*, 1992). The IL-2-induced activation of Ras was significantly inhibited by treatment of cells with herbimycin A, a specific inhibitor for tyrosine kinases, suggesting that a certain tyrosine kinase is coupled with the Ras activation pathway

(Izquierdo *et al.*, 1993). The serine-rich and acidic regions of IL-2Rβ are associated with JAK1 and Lck, respectively, and the A mutant has the ability to activate JAK1 kinase but not Lck kinase, which predicts that Lck may contribute to the Ras activation pathway. The involvement of tyrosine kinases in Ras activation may be due to tyrosine phosphorylation of Shc which operates upstream of Ras as described under Section V,C,2. In contrast to IL-2, IL-4 sharing IL-2Rγ showed no effect on Ras-bound GDT/GTP, indicating that Ras is not involved in the IL-4-mediated signaling (Satoh *et al.*, 1991; Welham *et al.*, 1994).

2. *Shc, Grb2, and SOS*

An SH2 domain-containing adapter protein, Shc ($p52^{shc}$), is reportedly known to act upstream of Ras (Rozakis-Adcock *et al.*, 1992). As Ras activation was detected in IL-2-stimulated cells, the possible involvement of Shc in the IL-2-mediated signal transduction was examined. As expected, IL-2 induced tyrosine phosphorylation of Shc within 5 min after treatment of T cells. Again, anti-Shc immunoprecipitates from IL-2-stimulated cells contained a tyrosine kinase activity (Burns *et al.*, 1993). Furthermore, it was demonstrated that Shc is associated with IL-2Rβ, tyrosine-phosphorylated Shc interacts with the SH2 domain of Grb2, another adapter protein, and Grb2 further binds to the Ras GDT/GTP exchange factor, SOS (Ravichandran and Burakoff, 1994; Zhu *et al.*, 1994). Thus, the cascade interaction of IL-2Rβ, Shc, Grb2, SOS, and Ras, together with the activation of tyrosine kinase, may be induced during IL-2 signaling.

3. *Raf-1 and Map Kinases*

A number of studies have demonstrated that serine/threonine kinases, Raf-1 and MAP kinases, present downstream of Ras, play key roles in signal transduction pathways for cell cycle progression initiated by growth factor receptors (for a review, see Nishida and Gotoh, 1993). In particular, the activation of Raf-1 and MAP kinases induced by IL-2 has also been observed. IL-2 induced rapid tyrosine/serine/threonine phosphorylation and activation of Raf-1 kinase in IL-2-dependent T cells (Turner *et al.*, 1991; Zmuidzinas *et al.*, 1991). Kinase activity of Raf-1 was regulated by its tyrosine phosphorylation because *in vitro* treatment of Raf-1 with CD45, a tyrosine phophatase, abolished the kinase activity of Raf-1 (Turner *et al.*, 1993). IL-2 also induced activation of MAPs ($p44^{erk\text{-}1}$ and $p42^{erk\text{-}2}$) in Con A-activated T cells (Watts *et al.*, 1993) and IL-3-dependent pro-B cells expressing reconstituted IL-2 receptors (Perkins *et al.*, 1993). Transfectants with the S mutant of IL-2Rβ, which has no ability to induce IL-2-mediated growth

signaling, failed to activate MAP ($p42^{erk-2}$) kinase; however, the MAP kinase activation appeared insufficient to induce IL-2-dependent cell proliferation because rapamycin, an immunosuppresant, showed suppression of IL-2-induced cell growth but could not inhibit activation of MAK kinase (Perkins *et al.*, 1993). On the other hand, IL-4 failed to activate $p44^{erk-1}$ and $p42^{erk-2}$ as well as Ras (Welham *et al.*, 1994). Thus, signal transduction pathways from IL-4 receptor may not include the Ras–Raf-1–MAP kinase pathway unlike those from IL-2 receptor.

4. *Phosphatidylinositol 3 Kinase (PI3-K)*

PI3-K was shown to be activated by, and associated with, receptors for several growth factors such as PDGF, EGF, CSF-1, insulin, and IGF-1 (for a review, see Parker and Waterfield, 1992). Although the physiologic role of PI3-K is still unknown, the receptor mutants lacking the association site of PI3-K failed to induce their cell growth signaling, indicating that PI3-K plays an important role in cell growth signal transduction (Coughlin *et al.*, 1989). Participation of PI3-K in signaling induced by IL-2 was suggested by the observation of IL-2-induced activation of PI3-K and the association of PI3-K with IL-2Rβ (Remillard *et al.*, 1991; Merida *et al.*, 1991; Augustine *et al.*, 1991). The SH2 domain of the p85 subunit of PI3-K was shown to recognize phosphorylated tyrosine 329 of IL-2Rβ (Truitt *et al.*, 1994). Furthermore, in CTLL-2 cells, Src family tyrosine kinases, Lck and Fyn, those associated with the acidic region of IL-2Rβ, were activated by IL-2 stimulation as described previously; furthermore, transfection with an activated form of mutant Lck induced the activation of PI3-K irrespective of IL-2 stimulation (Taichman *et al.*, 1993). These findings suggest that Lck or Fyn is responsible for activation of PI3-K in the IL-2 receptor complex, and activated PI3-K is implicated in the downstream signaling. However, PI3-K activation itself appeared insufficient for transduction of cell growth signals because when CTLL-2 was stably transfected with polyoma middle T antigen, both Fyn and PI3-K were activated, but they showed no IL-2-independent growth (Augustine *et al.*, 1991). Similarly, IL-4 and IL-7 induced activation of PI3-K, and at least immunoprecipitates of IL-4Rα were shown to contain activity of PI3-K (Wang *et al.*, 1992; Izuhara and Harada, 1993; Venkitaraman and Cowling, 1992). These results also suggest the possible involvement of PI3-K in the signal transduction pathways of these cytokines.

5. *Protein Kinase C (PKC)*

Activation of inositol phospholipid turnover is well known to be associated with transmembrane control mechanisms for signal trans-

duction induced by neurotransmitters, hormones, and growth factors (for a review, see Nishizuka, 1992). Two metabolites, diacylglycerols and inositol phosphates, have potent activities to activate protein kinase C (PKC) and calcium mobilization, respectively. IL-2-induced activation of PKC in terms of translocation of PKC from cytosol to membrane fractions was previously reported with IL-2-dependent T cells (Farrar and Anderson, 1985), but PKC-deficient or depleted cells showed appropriate responses to IL-2 for their proliferation, indicating that PKC is not essential for IL-2-mediated cell growth signaling (Mills *et al.*, 1988; Valge *et al.*, 1988). On the other hand, a recent study revealed that IL-2 apparently activates membrane-associated inactive PKC without its translocation from cytosol to membrane, and suggested the possibility that IL-2-induced activation of PKC may be implicated in the IL-2-dependent survival of T cells (Lu *et al.*, 1994). In this context, it is of interest that a TPA-dependent permanent cell line, TPA-Mat, has been established from an IL-2-dependent human T cell line (Takeshita *et al.*, 1988). TPA-Mat showed an absolute dependency on either IL-2 or TPA. IL-7 also stimulated inositol phospholipid turnover accompanied with tyrosine phosphorylation of some cellular proteins in immature B and T cells (Uckun *et al.*, 1991a,b).

6. *S6 Kinase*

Phosphorylation of ribosomal protein S6 is coupled with increased efficiency of protein synthesis and several steps of cell cycle progression (for a review, see Erikson, 1991). IL-2 was also found to stimulate S6 phosphorylation and protein synthesis (Evans and Farrar, 1987). S6 kinases consist of two families of 90- and 70-kDa kinases. IL-2 induced activation of the 70-kDa S6 kinase, and this activation was blocked by rapamycin, which inhibits IL-2-dependent proliferation (Calvo *et al.*, 1992; Kuo *et al.*, 1992). These findings suggest that the 70-kDa S6 kinase may participate in IL-2-mediated signal transduction for cell growth.

7. *GPI Hydrolysis*

Glycosylphosphatidylinositol (GPI) molecules have been shown to be involved in signal-transducing pathways initiated by insulin (Saltiel *et al.*, 1986; Mato *et al.*, 1987) and NGF (Chan *et al.*, 1989). Insulin stimulated rapid hydrolysis of GPI molecules through the activation of endogenous GPI phospholipase C, and the hydrolyzed products, inositol phophoglycan (IPG), mediated some of the effects of insulin on lipid and carbohydrate metabolism. Insulin-mediated hydrolysis of GPI molecules was detected in various cells including T cells

(Gaulton *et al.*, 1988). Therefore, its involvement in IL-2-mediated signaling was also investigated. IL-2 rapidly hydrolyzed GPI lipids in IL-2-dependent T cells, and the IPG was shown to have synergistic effects with IL-2 in T cell growth responses (Merida *et al.*, 1990). These findings suggest the possibility that GPI lipids contribute to IL-2-mediated signal transduction. On the other hand, in a murine lymphoma cell line, IL-4 antagonized the effect of IL-2 to induce rapid hydrolysis of GPI lipids, indicating that GPI hydrolysis is not involved in the IL-4-mediated signaling (Eardley and Koshland, 1991).

8. *Nuclear Events in IL-2 Signaling*

IL-2-induced signals are transduced from the receptor complex through the cytoplasm to nucleus, where transcription of various genes is activated. As described previously, c-*myc* and c-*fos*/c-*jun* inductions are regulated by different signaling pathways from the IL-2 receptor. In addition to these protooncogenes, c-*myb* was transiently induced within 3 hr of IL-2 stimulation during the G1 progression phase of the cell cycle (Stern and Smith, 1986), and similarly, a zinc-finger protein, NGFI-A, was also transiently induced within 1 hr of stimulation in G1 lymphoblasts (Perez-Castillo *et al.*, 1993). Expressions of other several immediate-early genes were also stimulated during this phase, although their molecular characteristics have not yet been elucidated (Sabath *et al.*, 1990; Beadling *et al.*, 1993). Cell cycle progression is exerted by activation of cyclin-dependent kinases (CDKs) that are negatively regulated by G1 cyclin–CDK inhibitors. In IL-2-induced G1 progression to S phase, IL-2 was found to inactivate the Cdk2 inhibitor, probably $p27^{Kip1}$, which is included in the G1 cyclin–Cdk2 complex (Firpo *et al.*, 1994).

IL-2-dependent cells generally fall into apoptosis upon IL-2 deprivation. A protooncogene, *bcl-2*, is one of the critical factors rescuing cells from apoptosis. In the culture of IL-2-dependent CTLL-2 cells, deprivation of IL-2 induced downregulation of *bcl-2* to undetectable levels, resulting in apoptosis, whereas addition of IL-2 led to the reexpression of *bcl-2* within 2 hr and protected the cells from apoptosis. Additionally, deregulated expression of *bcl-2* prolonged the survival of CTLL-2 cells in the absence of IL-2 (Deng and Podack, 1993). The interplay between IL-2 and the expression of this cellular oncogene, *bcl-2*, has important implications in the survival and growth of IL-2-dependent cells.

Apart from the tyrosine kinases associated with the IL-2 receptor complex, a novel tyrosine kinase, Itk, was found to be induced within 1 hr after IL-2 stimulation (Siliciano *et al.*, 1992; Tanaka *et al.*, 1993).

Itk is a T cell-specific tyrosine kinase that belongs to a new family of tyrosine kinases including Btk and Tec. Although the physiologic role of Itk in signal transduction has not been defined yet, it may possibly contribute to signaling in T cells. In addition, IL-2 induced gene expression of a tyrosine phosphatase, LC-PTP, within 1 hr after stimulation, which should regulate the tyrosine phosphorylation level in IL-2-dependent cell proliferation (Adachi *et al.*, 1994).

VI. Concluding Remarks

Molecular identification of IL-2Rγ has furnished us with incredible knowledge for understanding the structures and signal-transducing functions of various cytokine receptors, and particularly has made a great contribution toward elucidation of the molecular mechanisms of human XSCID occurrence. IL-2Rγ, so-called the γc-chain, is utilized as a common receptor subunit among multiple cytokines such as IL-2, IL-4, IL-7, IL-9, and IL-15. Mutations of IL-2Rγ in patients with XSCID cause dysfunction of these cytokines, resulting in impairment of early T cell development, a typical feature of XSCID. These cytokine receptors contain specific subunit(s) for each receptor along with the γc-chain. The γc-chain participates in increasing ligand-binding affinities, except for IL-9, and in intracellular signal transduction for all these cytokines. These cytokines have the ability to induce their pleotropic as well as redundant signals in their target cells. One may speculate that their pleiotropic and redundant signals are transduced from their specific subunits and from the γc-chain, respectively. In the IL-2/IL-2 receptor system, IL-2Rβ and the γc-chain have been demonstrated to be associated with JAK1 and JAK3, respectively; however, receptors for at least IL-4, IL-7, and IL-9 were also found to be associated with JAK1 and JAK3 (our unpublished observations). It is plausible that effector molecules associating with downstream elements of JAK1 and JAK3 may be different in cell types that differentially express these cytokine receptors, and that other distinct effector molecules are coupled with each receptor. Indeed, the JAK family kinases directly interact with adequate STAT molecules, which also form the STAT family, although it is still unknown which member(s) of the STAT family can be associated with JAK1 and JAK3. Furthermore, IL-2Rβ has been shown to interact directly with other effector molecules such as the Src family, Syk, and P13 kinases. Such effector molecules, together with the JAK family, may contribute to the transduction of common and IL-2-specific signals that regulate expression of various genes in the nucleus. With regard to gene expression, since

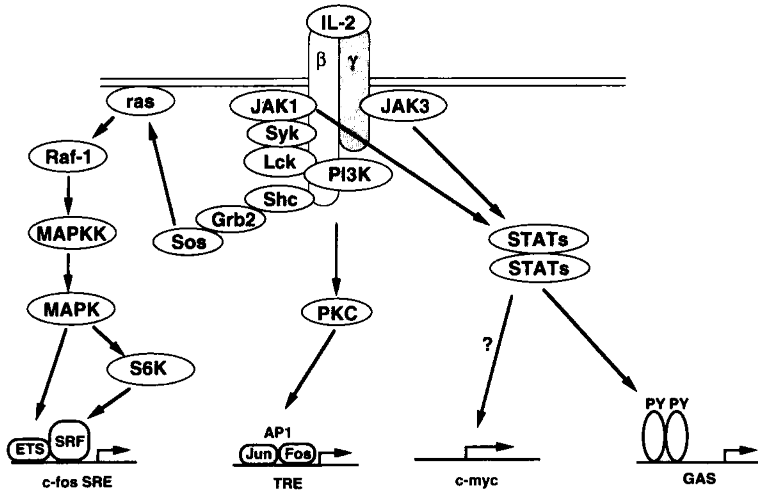

FIG. 7. Signal-transducing effector molecules possibly involved in signaling pathways from IL-2 receptor. JAK1 and JAK3 are associated with IL-2Rβ and IL-2Rγ, respectively. Syc, Lck, PI3K, and Shc are also associated with IL-2Rβ.

both cytoplasmic domains of IL-2Rβ and the γc-chain essentially participate in two discrete signal-transducing pathways, further investigation into the relationship between the two signaling pathways and the effector molecules associated with the IL-2 receptor complex is warranted (Fig. 7).

Human XSCID has been discovered to be caused by a deficiency of a single gene, the γc-chain. Although the precise mechanism of XSCID occurence still remains to be resolved, embarking upon gene therapy as well as the development of a definitive diagnosis for XSCID has now become feasible.

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This article was accepted for publication on 2 February 1995.

ADVANCES IN IMMUNOLOGY, VOL. 59

Self-Tolerance Checkpoints in B Lymphocyte Development

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I. Introduction

Our understanding of discrimination between self and non-self centers on the concept of a switch within each lymphocyte that either promotes clonal expansion and immunity or clonal elimination/ silencing and tolerance. This notion stems from Burnet (Burnet, 1959) and has been elaborated and expanded upon by successive waves of immunologists (Lederberg, 1959; Smith, 1961; Dresser and Mitchison, 1968; Bretscher and Cohn, 1970; Weigle, 1973; Lawton and Cooper, 1974; Lafferty and Cunningham, 1975; Metcalf *et al.*, 1979; Dintzis *et al;* 1983; Nossal, 1983; Schwartz, 1990). Throughout, the key issue has been what causes the switch to be thrown toward immunity or toward tolerance. Burnet's original concept involved developmental hard wiring, such that fetal lymphocytes would always respond to antigen by clonal elimination or at least inhibition from multiplication, whereas postnatal cells would always respond by multiplying and producing antibody (Burnet, 1959). Lederberg's modification, taking into account new production of lymphocytes well after birth, was that hard wiring of the switch would be pinned to the stage of each lymphocyte's development rather than development of the animal as a whole (Lederberg, 1959). Location and timing would thus ensure that self-antigens

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triggered a different cellular response from foreign, because self but not foreign would usually be encountered early in a cell's development within primary lymphoid organs (Nossal, 1994).

The notion of a developmentally hard-wired switch has received a great deal of support, beginning with the neonatal tolerance experiments of Billingham *et al.* (1953). These whole-animal level experiments were extended to the cellular level through *in vivo* and *in vitro* tolerance experiments with B cells (Lawton and Cooper, 1974; Nossal and Pike, 1975; Raff *et al.*, 1975; Sidman and Unanue, 1975; Metcalf and Klinman, 1976; Nossal, 1983; Scott, 1993) and T cells (Smith *et al.*, 1989), and culminated in the demonstration of intrathymic clonal deletion by Kappler *et al.* (1987) and the first wave of superantigen and transgenic mouse experiments demonstrating deletion of immature self-reactive T and B cells (Kappler *et al.*, 1988; MacDonald *et al.*, 1988; Kisielow *et al.*, 1988; Sha *et al.*, 1988; Nemazee and Bürki, 1989).

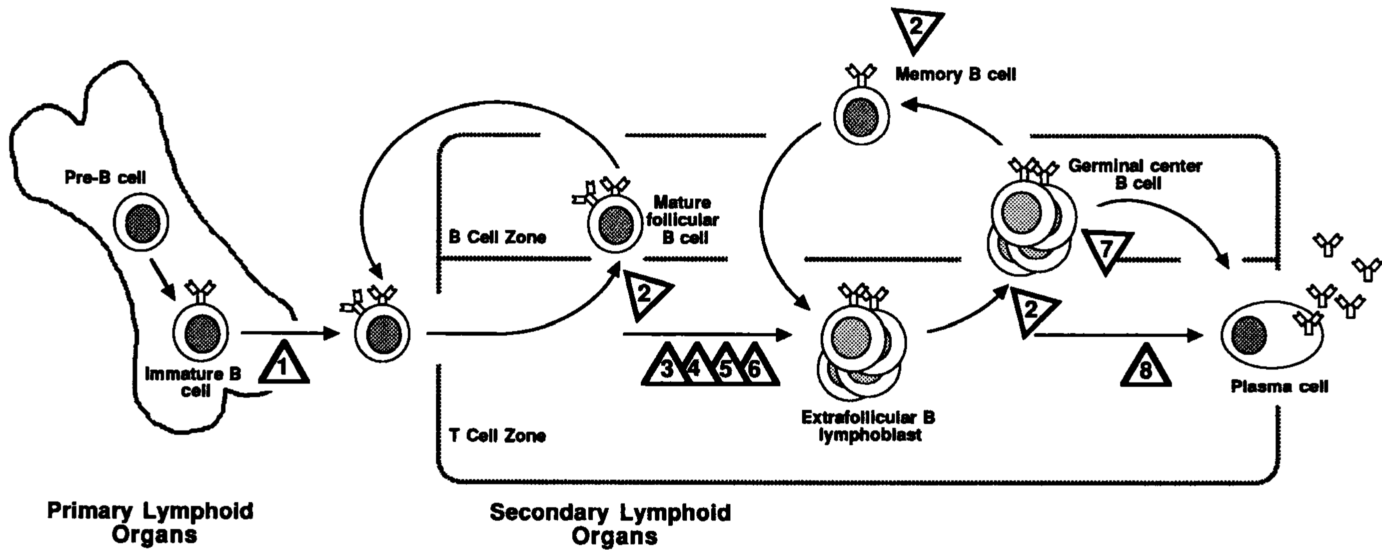
Two sets of observations have continually been at odds with the notion of a developmentally hard-wired switch. The first is the ability of infectious antigens, such as viruses, to trigger immunity rather than tolerance even when encountered very early in development (Burnet *et al.*, 1950; Nossal, 1957; Oldstone and Dixon, 1969). The second is the ability to induce tolerance in adult animals (Smith, 1961; Dresser and Mitchison, 1968; Weigle, 1973). One view of the latter is that they represent immune regulatory mechanisms unrelated to self–nonself discrimination (Burnet, 1976), such as immune deviation (Parish and Liew, 1972) or antibody feedback (Sinclair, 1990). An alternate view of both sets of observations is that the immunity-tolerance switch is decided not by developmental hard wiring of the response to antigen (signal one) but by input from a second, nonantigen costimulus associated with foreign antigens. In the absence of an appropriate costimulus, the response to antigen would be clonal silencing and tolerance, whereas the presence of the costimulus would trigger clonal expansion and immunity. This two-signal model of self–nonself discrimination began with Bretscher and Cohn (1970) with the concept of a costimulus developed by Lafferty and Cunningham (Lafferty and Cunningham, 1975; Lafferty *et al.*, 1983). Experimental support for such a model began with adult tolerance experiments *in vivo* (Claman, 1963; Golub and Weigle, 1967; Golan and Borel, 1971; Weigle, 1973a; Lafferty *et al.*, 1983) and *in vitro* (Metcalf *et al.*, 1979; Teale *et al.*, 1979; Jenkins and Schwartz, 1987), leading up to the recent identification of the role of CD28 (reviewed by Linsley and Ledbetter, 1993) among many other findings.

A third view of adult tolerance phenomena has been that immunity

or tolerance are determined not by location/timing or costimuli, but by the amount and form of antigen. In this model, optimal spacing of antigen-clustered receptors sets the switch to clonal expansion, but too much or too little receptor stimulation results in clonal elimination/silencing and tolerance (Dresser and Mitchison, 1968; Howard and Mitchison, 1975; Klaus *et al.*, 1976; Dintzis *et al.*, 1983,1989; Moskophidis *et al.*, 1993). Elegant experiments with haptenated polymers of T-independent antigens by Dintzis *et al.*, (1983,1989) provided a striking example in favor of this model. This quantitative view has been brought to the fore again recently by experimental evidence that positive or negative selection in thymocytes may be decided by the amount and affinity of antigen/MHC (Robey *et al.*, 1992; Ashton-Rickardt *et al.*, 1993; Hogquist *et al.*, 1993; Killeen and Littman, 1993).

None of the models described above are exclusive. From a general viewpoint it has therefore been useful to think of an amalgum of location/timing, costimuli, and antigen strength that sets the immunity/tolerance switch within each lymphocyte in one of four positions—proliferation, ignorance, anergy, or elimination. Little is known about the molecules that form the "wiring" of this hypothetical cellular switch, although it is often assumed that some balance between negative and positive signaling molecules determines the final outcome.

Instead of single hypothetical switch, the theme emphasized in this chapter is that it may be more accurate to view humoral self-tolerance as the cumulative action of a series of separate checkpoints placed along the B cell lineage (Fig. 1). This notion is particularly important as the field moves toward a genetic and molecular understanding of tolerance and autoimmunity, since each checkpoint may depend on distinct sets of genes and molecules. The strategy of employing multiple and distinct checkpoints presumably minimizes the risk that one or two inherited or somatically acquired mutations leads to uncontrolled autoantibody production, analogous to the role of checkpoints in the cell cycle that minimize neoplasia. From a genetic perspective, the use of broad terms such as "deletion" or "anergy" will lead to confusion in the long term because each of these labels can be used to describe several distinct checkpoint mechanisms that likely involve discrete molecular pathways. Effort is made in this chapter to define each censoring process more specifically, although it is not yet possible to identify each by a unique dependence on particular gene products. The review is divided into two sections, discussing self-tolerance checkpoints acting (1) during formation of the preimmune B cell repertoire and (2) during subsequent formation of an immune repertoire (Fig. 1).


Pre-B cell
Immature B cell
Mature follicular B cell
B Cell Zone
T Cell Zone
Memory B cell
Germinal center B cell
Extrafollicular B lymphoblast
Plasma cell
1
2
3
4
5
6
7
8
Primary Lymphoid Organs
Secondary Lymphoid Organs
Preimmune Repertoire
Immune Repertoire

II. Checkpoints during Formation of the Preimmune Repertoire

A. Developmental Stages, Locations, and Markers

There are some differences in the names and markers used to refer to particular stages in B cell development within the preimmune repertoire; those used here are defined below.

1. *Mature Follicular B Cells*

In the mouse, a large component of the preimmune repertoire consists of small, mature, recirculating B cells. Phenotypically, these cells are distinguished from other B cell stages (see Fig. 2) by high levels of surface IgD (sIgD; Blattner and Tucker, 1984), a range of levels of IgM (Hardy *et al.*, 1983), high levels of B220 (Forster *et al.*, 1989; Hardy *et al.*, 1991), low levels of heat stable antigen (HSA; Allman *et al.*, 1993), moderate levels of CD23 (Waldschmidt *et al.*, 1991), and moderate amounts of complement receptors 1 and 2 (CR1/2; Gelfand *et al.*, 1974; Kinoshita *et al.*, 1990; Hartley *et al.*, 1993). They correspond to fraction "F" in Hardy's nomenclature (Hardy *et al.*, 1991). There are approximately 2×10^8 of these cells in the mouse, at least half of which do not divide or turn over during a period of 4 weeks (Howard, 1972; Sprent and Miller, 1972; Sprent and Basten, 1973; Gray, 1988; Forster *et al.*, 1989; Forster and Rajewsky, 1990; Forster *et al.*, 1991; MacLennan *et al.*, 1989). These cells are continuously migrating between lymph, blood, and secondary lymphoid organs such

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FIG. 1. Checkpoints for censoring self-reactive B cells. B cells develop toward antibody-secreting cells in two phases. Prior to infection and exposure to new foreign antigens, a preimmune repertoire is established composed of newly formed B cells emigrating from the bone marrow and mature follicular B cells that continuously recirculate through secondary lymphoid organs. During this phase, self-reactive B cells are removed at two checkpoints: △1 cell-autonomous elimination in the bone marrow (see Sections II,C–II, E), and △2 elimination by competitive exclusion from primary follicles in the periphery (see Section, II,F). After infection or immunization, an immune repertoire is formed by clonal expansion of selected B cell clones in extrafollicular foci within T cell zones and in germinal centers in the B cell zone. The progeny of these reactions (a) become memory cells that reside in marginal zones, recirculate, or are recruited back into clonal expansion, or (b) terminally differentiate into antibody-secreting plasma cells. During this phase, self-reactive B cells can be impeded or removed by the following: △3 absence of T cell help (see Section III,A); △4 antigen receptor modulation (see Section III,C); △5 desensitization of antigen receptor signaling, reducing mitogenesis and costimulatory function (see Section III,D); △6 CD95 (Fas/Apo-1)-dependent killing by CD4⁺ T cells (see Section III,E); △7 cell death in germinal centers (see Section, III,F); △2 elimination by exclusion of memory cells from follicles and trapping in the T cell zone (see Section II,F and III,F); and △8 inhibition of terminal differentiation into plasma cells (see Section III,G).

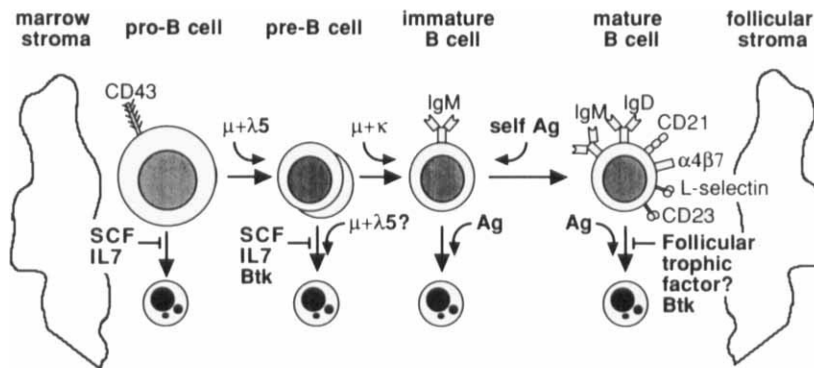

FIG. 2. Model outlining the control of B cell maturation and survival in the preimmune repertoire. As discussed in the text, B cell survival is apparently determined by interplay between signals from the antigen receptor and signals from stromal cells. Pro-B and pre-B cells express receptor for trophic factors, such as SCF and IL-7, that are produced by bone marrow stromal cells. At this stage, antigen receptor signals from the μ-chain/$\lambda5$ surrogate light-chain pre-B cell receptor trigger proliferation and maturation into small pre-B cells. The tendency of antigen receptor signaling to activate cell death may be counterbalanced in pre-B cells by survival signals such as IL-7. Ig-induced maturation causes the cells to lose receptors for stromal factors, however, and possibly to be displaced from the stromal cell niches. Thus, in immature B cells that bind self-antigens receptor signals from IgM are not likely to be counterbalanced by trophic factors from bone marrow stroma. The time lag between binding áutoantigen and activation of cell death may therefore depend on the decay rate of survival signals and their intracellular mediators, such as Bcl-2, and on the strength of antigen receptor signaling and its ability to overcome residual survival signals. During the period prior to cell death, antigen receptor signaling arrests further maturation preventing expression of homing receptors that allow migration of mature B cells to secondary lymphoid tissues. B cells that do not bind autoantigen with sufficient avidity to trigger developmental arrest become, mature B cells, acquire lymphoid homing receptors, and live long enough to migrate into primary follicles in spleen and lymph nodes. Follicular niches promote survival of mature B cells and counterbalance the tendency of autoantigen to activate cell death. By analogy with the bone marrow, this survival effect could be due to trophic factors produced by the follicular stroma. In mice, the Btk mutation, *xid*, appears to compromise survival of mature follicular B cells, while Btk mutations in humans compromise survival of pre-B cells in the bone marrow.

as lymph nodes, spleen, and Peyer's Patches (Gutman and Weissman, 1972; Sprent, 1973; Gutman and Weissman, 1973; Nieuwenhuis and Ford, 1976), and in the absence of infection they are the predominant B cell type in all these sites. In secondary lymphoid organs they account for the majority of cells in primary follicles and in the mantle zone of secondary follicles; for this reason, this subset will be referred to as follicular B cells herein.

2. *Marginal Zone B Cells*

Approximately 5–10% of spleen B cells in the mouse are resident in the marginal zones that lie between the follicles of the white pulp and the red pulp (reviewed by MacLennan *et al.*, 1990; Kraal, 1992). A less numerous but apparently equivalent subset appears to exist in an analogous location in the subcapsular sinus of lymph nodes, beneath the dome epithelium of Peyer's Patches, and beneath the crypt epithelium of tonsillar tissue (Stein *et al.*, 1980; Spencer *et al.*, 1985; van Krieken *et al.*, 1989; MacLennan *et al.*, 1990). These cells are predominantly long-lived, nondividing, nonrecirculating, are slightly larger, and have a distinct phenotype: IgD^{low}, $CD23^-$, and $CR1/2^{high}$ (Gray *et al.*, 1982,1984; MacLennan *et al.*, 1990; Waldschmidt *et al.*, 1991; Kraal, 1992). Some marginal zone B cells are memory B cells that are part of the immune repertoire, but others are formed in the absence of immunization and presumably must be considered part of the preimmune repertoire (Kumararatne *et al.*, 1981; Liu *et al.*, 1988; MacLennan *et al.*, 1990; Liu *et al.*, 1991). During ontogeny and following irradiation, marginal zone B cells appear several days after follicular B cells, but the immediate precursor of marginal zone B cells has yet to be determined (MacLennan and Gray, 1986).

3. *B1 B Cells*

Many B cells that can be flushed from the peritoneal and pleural cavities of unimmunized mice have a distinct phenotype of being large, IgD^-, IgM^{hi}, $B220^{low}$, $CD23^-$, and $Mac1^+$ (Kantor and Herzenberg, 1993). Because these cells are present in germ-free mice, they presumably also represent part of the preimmune repertoire. They nevertheless exhibit some characteristics that might be expected for memory B cells, such as the ability to self-renew and expand after adoptive transfer, production of much of the serum IgM and IgA in unimmunized mice, and recurrent use of particular heavy- and light-chain V-region combinations (Reininger *et al.*, 1987,1988; Kantor and Herzenberg, 1993; Arnold *et al.*, 1994).

4. *Immature B Cells*

Two predominant subsets of B cells bearing surface Ig are present in the bone marrow of adult mice: mature follicular-type B cells that are recirculating in the blood or may have reentered the bone marrow stroma, and immature B cells that have developed from pre-B cells within the previous day or two (Forster *et al.*, 1989; Hardy *et al.*, 1991). Immature B cells are mostly small cells with the characteristic phenotype of $sIgD^-$, a range of levels of sIgM, $B220^{low}$, HSA^{high}, $CD23^-$, and $CR1/2^-$, and very little to no staining with the S7 antibody

to CD43 (Forster *et al.*, 1989; Hardy *et al.*, 1991; Hartley *et al.*, 1993). They correspond to fraction "E" in Hardy's nomenclature. A transitional stage between immature B cells and mature follicular B cells can be identified as recent bone marrow emigrants in the spleen and blood of adult mice; these recent emigrants are only distinguished from immature B cells by their expression of low levels of IgD and low levels of CR1/2 (Allman *et al.*, 1993; Hartley *et al.*, 1993). The only marker at present distinguishing immature B cells from their immediate precursors, small pre-B cells, is surface IgM. Surface IgM can nevertheless be completely modulated from the cell surface by binding of some self-antigens, so that distinguishing $sIgM^-$ autoreactive immature B cells from pre-B cells is currently unreliable.

5. *Small Pre-B Cells*

These cells constitute a considerable fraction of bone marrow B lineage cells in adult mice, have a functionally rearranged heavy-chain (Hc) gene, have mostly dropped out of cell cyle, and are in the process of rearranging light-chain (Lc) genes (Coffman, 1982; Osmond, 1990; Hardy *et al.*, 1991). They are $sIgM^-$, $B220^{low}$, HSA^{high}, $CD43^{low\text{-}negative}$, and correspond to Hardy's fraction "D." Expression of a functional membrane IgM heavy chain is necessary for their development (Kitamura *et al.*, 1991; Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992; Reichman-Fried *et al.*, 1993).

6. *Large Pre-B and Pro-B Cells*

These cells are in the process of rearranging their heavy-chain genes or have just done so and are large, cycling cells (Coffman, 1982; Osmond, 1990; Hardy *et al.*, 1991). They are characterized as $sIgM^-$, $B220^{low}$, $HSA^{low\text{-}high}$, $CD43^+$, and correspond to Hardy's fractions A–C. Expression of functional heavy chains is not needed for the development of pro-B cells in fractions A–C (Ehlich *et al.*, 1993; Reichman-Fried *et al.*, 1993).

B. Diversity of the Preimmune Repertoire

Approximately 10^{11} different antibody V regions can be generated by combinatorial and junctional diversity following Hc and Lc gene rearrangement (Davis and Bjorkman, 1988). This number represents the potential size of the preimmune repertoire. Only a fraction of this potential is represented as sIg receptors on recirculating follicular B cells in the functional preimmune repertoire of mice, since there are only approximately 2×10^8 of these cells and most turnover with a half-life greater than 4 weeks (Sprent and Miller, 1972; Sprent and

Basten, 1973; Gray, 1988; MacLennan *et al.*, 1989; Forster *et al.*, 1989; Forster and Rajewsky, 1990; Forster *et al.*, 1991). A larger fraction of the potential repertoire may be represented as sIg on immature B cells in the bone marrow, which are produced at an estimated rate of approximately 1.7×10^7 new cells per day (Osmond, 1986,1990; Allman *et al.*, 1993). A considerable fraction of these new B cells appear to reach the secondary lymphoid tissue (primarily spleen) as recent emigrants from the bone marrow (Osmond, 1986; Bazin *et al.*, 1985; Lane *et al.*, 1986; Chan and MacLennan, 1993). The number of newly formed B cells reaching the spleen has been estimated at 1.5×10^6 new B cells per day (Allman *et al.*, 1993). This number is likely to be an underestimate due to the very short half-life of most of these cells, since reconstitution experiments indicate that enough B cells are exported to replenish the circulating pool within 4 days (Bazin *et al.*, 1985; Lane *et al.*, 1986). The antibody specificities that are represented in the recirculating preimmune repertoire appear not to be a random subset of the potential repertoire, based upon findings that V-region usage is skewed in follicular B cells compared with patterns seen in immature and pre-B cells (Klinman *et al.*, 1976; Yancopoulos *et al.*, 1984; Perlmutter *et al.*, 1985; Teale and Kearney, 1986; Riley *et al.*, 1986; Yancopoulos *et al.*, 1988; Denis *et al.*, 1989; Malynn *et al.*, 1990; Freitas *et al.*, 1990; Gu *et al.*, 1991; Decker *et al.*, 1991; Huetz, 1993). It is not known whether this skewing reflects positive or negative selection of particular V regions, although it occurs in a stereotypic manner between birth and maturity (Klinman *et al.*, 1976; Perlmutter, 1987; Yancopoulos *et al.*, 1988).

The diversity of the preimmune repertoire makes it difficult to identify a B cell with any one particular sIg receptor specificity, let alone track where and how that clone may be selected. Analysis of checkpoints that prevent particular antibody specificities from being represented in the preimmune repertoire has therefore been greatly helped by two experimental strategies. The first has been to expose developing B cells to antibodies that react with Ig-constant region determinants, which will affect all B cells. The second approach has been to artificially elevate the frequency with which particular antibody specificities are generated in the preimmune repertoire by introducing functionally rearranged antibody Hc and Lc genes (Storb, 1987; Goodnow, 1992). This strategy makes use of the fact that rearranged Hc and Lc transgenes suppress rearrangement of endogenous Hc and Lc genes, respectively, so that the preimmune repertoire is almost monoclonal in a Hc + Lc transgenic mouse. Suppression of endogenous gene arrangement is always leaky and, depending on the transgenic line and age of the mice, these endogenous receptor-

positive cells can vary from only a few percent of circulating B cells to greater than 90% (Goodnow, 1992).

C. Checkpoint 1: Elimination of Self-Reactive Cells in the Bone Marrow

Binding of self-antigens to sIgM receptors on immature B cells in the bone marrow can trigger a checkpoint that physically eliminates self-reactive clones. This checkpoint closely resembles the mechanism envisioned by Burnet (1959) and Lederberg (1959). Its existence was first indicated by Lawton and Cooper, who showed that mature follicular B cells failed to develop in birds and mice treated continuously from hatching or birth with antibodies that engaged IgM receptors on developing immature B cells (Lawton and Cooper, 1974). Further evidence came from *in vitro* experiments with antigens or antibodies to IgM showing that immature B cells and their transformed lymphoma counterparts were irreversibly inactivated or inhibited from developing when exposed to antigen receptor ligands (Raff *et al.*, 1975; Sidman and Unanue, 1975; Metcalf and Klinman, 1976; Teale *et al.*, 1979; Ralph, 1979; Boyd and Schrader, 1981; Nossal, 1983; Jakway *et al.*, 1986; Scott, 1993). Proof that self-reactive B cells were indeed physically eliminated at this checkpoint came from transgenic experiments of Nemazee and Burki (Nemazee and Bürki, 1989; Nemazee and Buerki, 1989). This work and subsequent transgenic studies by Hartley (Hartley *et al.*, 1991,1993), Okamoto (Okamoto *et al.*, 1992), and Weigert's group (Erikson *et al.*, 1991; Gay *et al.*, 1993; Radic *et al.*, 1993a), which collectively establish the generality of this checkpoint and some of its parameters, are summarized below.

1. *Elimination of H-2K-Reactive B Cells*

The strategy used by Nemazee and Bürki (Nemazee and Bürki, 1989; Nemazee and Buerki, 1989) to track the fate of self-reactive B cells was to generate transgenic mice carrying rearranged Hc and Lc genes encoding an IgM antibody (and later IgM and IgD, with similar results; Russell *et al.*, 1991) that bound to the allelic forms of class I major histocompatibility molecules, H-2K^k and H-2K^b. The antibody did not bind products of the H-2K^d allele (Ozato *et al.*, 1980), providing a control strain background where the antibody would not be autoreactive. The initial transgenics were produced in H-2^d strain mice and contained many splenic and lymph node B cells that carried transgene-encoded IgM and could be tracked with an anti-idiotype antibody.

To determine the fate of B cells developing in an animal in which their transgene-specified receptors reacted with self-H-2K antigens, the Ig transgenic mice were crossed with H-2K^k or H-2K^b strain mice.

Three key observations established that the autoreactive B cells were eliminated in the bone marrow (Nemazee and Bürki, 1989; Nemazee and Buerki, 1989; Nemazee *et al.*, 1991b): (1) B cells expressing the transgene-encoded idiotype were undetectable in the spleen or lymph nodes, but were present in the bone marrow where they had modulated much of the IgM from their cell surface; (2) the overall number of spleen and lymph node B cells was reduced one- to fourfold and the remaining cells expressed normal amounts of IgM that did not react with the anti-idiotype; (3) based on sampling by hybridoma panels from LPS blasts, most of these remaining cells carried endogenous κ or λ Lc gene rearrangments that presumably contributed non-H-2K-reactive light chains to their IgM receptors. The receptor editing process that allows these nonautoreactive cells to escape deletion is discussed under Section II,D,5.

2. *Elimination of mHEL-Reactive B Cells*

Hartley *et al.*, (Hartley *et al.*, 1991,1993; Hartley and Goodnow, 1994b) followed a slightly different strategy to demonstrate elimination of self-reactive B cells in the bone marrow; they used transgenic mice carrying rearranged Hc and Lc genes encoding IgM and IgD antibodies (or IgM alone) that bound to hen egg lysozyme (HEL; Goodnow *et al.*, 1988). Greater than 90% of the recirculating follicular B cells in spleen, lymph node, Peyer's Patches, and blood carried lysozyme-binding IgM and IgD as their sole antigen receptor and could be tracked by binding HEL or by anti-allotypic monoclonal antibodies (Goodnow *et al.*, 1988; Mason *et al.*, 1992). To determine how the fate of HEL-binding B cells was altered if they bound HEL during their development, the Ig transgenic mice were crossed with transgenic mice that expressed HEL on most cells in a membrane-bound form (mHEL) anchored by a membrane-spanning segment from H-2K. HEL-binding B cells were eliminated in the resulting mHEL/Hc + Lc double-transgenic animals, since the number of spleen or lymph node B cells was reduced approximately 10-fold. Of the few remaining B cells, many expressed normal cell surface densities of transgene-encoded IgM and IgD but did not bind HEL apparently due to loss of expression of the transgenic κ-light chain. A very small number of HEL-binding B cells were present in spleen and blood, and these expressed 20-fold lower amounts of cell surface IgM, lacked IgD, and were immature based on staining with a variety of developmental markers (Hartley *et al.*, 1993; Hartley and Goodnow, 1994b). Like the findings in H-2K-reactive Hc + Lc transgenic animals, immature HEL-binding B cells were present in normal numbers in the bone marrow but had modulated cell surface IgM, indicating they had encountered

mHEL as soon as they expressed IgM and were eliminated either in the bone marrow or shortly after emigration to the periphery.

3. *Elimination of Double-Stranded DNA-Reactive B Cells*

In contrast to the transgenic animals described previously that carried Hc + Lc transgenes cointegrated in one chromosomal site, Weigert and colleagues created separate Hc transgenic and Lc transgenic mice and then analyzed them on their own or crossed together (Erikson *et al.*, 1991; Gay *et al.*, 1993; Chen *et al.*, 1994). The Hc and Lc genes came from a panel of anti-DNA antibody-secreting hybridomas obtained from autoimmune MRL-*lpr* mice (Shlomchik *et al.*, 1990). Extensive structure/function analysis performed on the antibodies in this panel provided a strong background for predicting DNA-binding affinity and specificity (Radic *et al.*, 1993b; Radic and Weigert, 1994). The first transgenic mouse generated (Erikson *et al.*, 1991) carried a Hc transgene encoding μ-chains that generated double-stranded DNA (dsDNA)-binding antibodies when paired with light chains containing a number of common V_κ elements. Hybridoma panels derived from LPS-activated cells from the spleen of the Hc transgenic mice nevertheless lacked B cells making the dsDNA-reactive Hc/Lc combinations, implying that they had been deleted.

Gay *et al.* (1993) aimed to force B cells to express a dsDNA specificity by mating the Hc transgenic mouse with a transgenic mouse expressing the $V_\kappa4$ light chain from the original dsDNA-binding hybridoma. In the resulting Hc + Lc transgenic animals, the number of B cells was reduced in one neonatal mouse by twofold compared with a neonatal control animal carrying only the Hc transgene, but equivalent numbers accumulated in the spleen of both types of mice by adulthood. At either timepoint, however, none of the B cells bound an anti-idiotypic monoclonal antibody that recognized the transgenic V_H/V_L combination, despite the fact that they carried abundant surface IgM containing the transgenic Hc. This finding implied that the surface IgM displayed by the B cells contained other light chains, and this was confirmed by analyzing hybridomas derived from the Hc + Lc transgenic mice. Of 72 hybridomas that secreted IgM containing the transgenic Hc, none secreted antibody that was recognized by the V_H/V_L-specific antibody or bound dsDNA in the expected manner. All expressed mRNA encoding the transgenic κ-light chain, but they also expressed an endogenous non-DNA-binding κ-light chain that was preferentially assembled with the transgenic heavy chains. Thus, a fraction of autoreactive B cells escaped censoring by expressing a different receptor that did not bind dsDNA.

Autoreactive B cells escape censoring in the Hc + V_κ4-Lc mice more efficiently than in the H-2K or mHEL models described above, since relatively few B cells are present in H-2K or mHEL animals even at 12–15 weeks of age (Goodnow *et al.*, 1988; Nemazee and Buerki, 1989; Tiegs *et al.*, 1993). The rate at which B cells escape by expressing an endogenous light chain is likely to reflect how efficiently the transgenic light chain triggers allelic exclusion of the endogenous light-chain genes. In the HEL-reactive and H-2K-reactive transgenic mice, the κ-light chain transgenes included a strong κ 3′ enhancer which is necessary for efficient transgene expression and allelic exclusion (Storb, 1987; Meyer and Neuberger, 1989; Betz *et al.*, 1994). By contrast, the κ-light chain transgene used by Gay *et al.* lacked the 3′ enhancer and was an inefficient suppressor of endogenous light-chain rearrangement (Gay *et al.*, 1993).

Direct visualization of dsDNA-reactive B cell elimination in the bone marrow has recently been achieved by two additional maneuvres (Chen *et al.*, 1995). First, escape from censoring through coexpression of a non-DNA-binding endogenous light chain was minimized by using variant Hc transgenes bearing point mutations (56R or 76R) that introduced arginines in the heavy chain CDRs 2 and 3. The increased affinity for DNA conferred by these substitutions allowed dsDNA binding when paired with almost any light chain (Radic *et al.*, 1993b; Radic and Weigert, 1994). When expressed as Hc transgenes, the 56R and especially the 76R heavy chains caused many fewer mature B cells to be produced, presumably due to deletion of most Hc+Lc combinations. Of the B cells that did develop in 56R and 76R Hc transgenic mice, none expressed a dsDNA-binding receptor and most had lost expression of the transgenic heavy chain and gained expression of heavy chains from the mouse's endogenous Hc genes (Chen *et al.*, 1994,1995). In many of these B cells, loss of the transgenic heavy chain was due to chromosomal deletions spanning much of the transgene array (Chen *et al.*, 1994). An analogous somatic loss of transgenes occurs with a frequency of approximately 10^{-6} in hepatocytes that express a hepatotoxic plasminogen activator transgene, this frequency being sufficient to allow gradual regeneration of the liver (Sandgren *et al.*, 1991).

To block the option of regenerating a functional B cell repertoire through loss of the transgenic heavy chain, Chen *et al.* crossed the 56R and 76R Hc transgenic animals to mice carrying targeted disruptions of the J_H region of the endogenous Hc genes. Homozygous J_H-/- mice fail to develop pre-B or B cells because membrane μ-chains are essential for B cell development and survival beyond the pro-B cell stage

(Kitamura *et al.*, 1991). In the Hc transgenic J_H-/- mice, both the 56R and the 76R Hc transgene products were fully capable of rescuing maturation to the pre-B cell stage, indicating that the Hc transgenes could be expressed in a functional membrane-bound form. In contrast to the normal pool of pre-B cells in the Hc transgenic J_H-/- mice, however, few IgM^+ immature or mature B cells were present, particularly in the 76R mice with the potentially strongest DNA affinity. Thus, most dsDNA-binding B cells were eliminated in the bone marrow soon after they rearranged a light-chain gene and expressed a complete IgM receptor on their surface.

Two other anti-DNA transgenic models have also been investigated. Diamond and colleagues produced Hc-only transgenic mice expressing an IgG_{2b} heavy chain of a pathogenic anti-dsDNA antibody (Offen *et al.*, 1992). The overall number of splenic B cells was markedly reduced and only a fraction of the remaining B cells appeared to express IgG, while many expressed endogenous non-DNA-reactive IgM alone or in combination with IgG. The mice do not contain markedly elevated anti-DNA IgG2b in their serum, suggesting some form of censoring. Hybridomas derived from LPS-activated transgenic spleen cells revealed further evidence for censoring the transgenic B cells (Iliev *et al.*, 1994). Thus, only 5% of the hybridomas that secreted IgG2b antibody containing the transgenic heavy chain secreted a dsDNA-binding antibody. All of the DNA-binding hybridomas coexpressed an endogenous IgM heavy chain, whereas none of the non-DNA-binding IgG secretors coexpressed endogenous IgM. These findings are reminiscent of those made by Gay *et al.* (1993), and suggest that the only DNA-reactive B cells to mature were those that had decreased or lost cell surface expression of the transgenic heavy chain by coexpressing a non-DNA-binding μ-heavy chain.

Tsao and colleagues also made anti-DNA Ig transgenic mice carrying IgM Hc and Lc genes encoding a nephritogenic dsDNA-binding antibody (Tsao *et al.*, 1993). In this case, the anti-DNA B cells appeared not to be deleted in the bone marrow, because normal numbers of B cells expressing the transgenic Hc were present in the spleen and these were capable of secreting anti-DNA antibody when stimulated with LPS *in vitro*. Interestingly, the B cells could not be triggered to produce anti-DNA antibody *in vivo*, suggesting that they were anergic.

4. *Elimination of Erythrocyte-Reactive B Cells*

Okamoto *et al.* (1992) used a transgenic strategy comparable to that employed by Weigert's group (previously described) by generating separate Hc and Lc transgenic mice and then crossing them to produce

relatively monoclonal Hc+Lc transgenic offspring. The Hc and Lc genes used by Honjo's group also came from *bona fide* autoantibody-secreting B cells, like the genes used by Weigert and colleagues, although in this case the Ig genes were from a hybridoma originating in a different SLE-prone mouse strain, NZB (Shibata *et al.*, 1990). The autoantibody encoded by the Hc and Lc genes was characteristic of the antibodies accounting for autoimmune hemolytic anemia in NZB mice in that it recognized an unknown but abundant, nonpolymorphic antigen on erythrocytes with sufficient avidity to trigger red cell destruction *in vivo* (Shibata *et al.*, 1990).

Transgenic mice carrying either the Hc or Lc transgenes separately had normal numbers of mature B cells and no detectable anti-erythrocyte antibody in their circulation, consistent with the need for pairing of the two chains to generate the antierythrocyte specificity (Okamoto *et al.*, 1992). Moreover, transgene-encoded heavy chains were expressed as IgM at normal levels on most of the peripheral B cells in the Hc transgenic animals. In offspring that carried both Hc and Lc transgenes, by contrast, there were 10- to 100-fold fewer B cells in spleen or lymph nodes. Of the few peripheral B cells that were present, approximately half were mature but lacked expression of the transgenic light chain based upon staining with an anti-idiotype monoclonal antibody. The other half of peripheral B cells expressed both transgenic Hc and Lc products but the density of cell surface IgM was modulated 10-fold. Most of the cells expressed low levels of B220, suggesting they were either immature or had become B1-type cells. Immature B cells expressing the erythrocyte-reactive H and L chains were present in normal numbers in the bone marrow but had modulated surface IgM by 4-fold. Thus, as with the H-2K and mHEL models described previously, autoreactive B cells encountered autoantigen as soon as they expressed IgM and disappeared either in the bone marrow or shortly after emigration to the periphery.

One key difference between the antierythrocyte Hc+Lc transgenic mice and the H-2K, mHEL, or dsDNA models is that tolerance fails in many animals carrying the antierythrocyte transgenes. Thus, over half of the Hc+Lc mice develop spontaneous autoimmune hemolytic anemia accompanied by high amounts of transgene-encoded IgM bound to circulating erythrocytes (Okamoto *et al.*, 1992). By contrast, anti-HEL antibody-secreting cells or circulating antibody have never been detected in systemic mHEL-expressing mice on the same inbred C57BL/6 strain background (Hartley *et al.*, 1991,1993).

The basis for breakdown of tolerance in the antierythrocyte Hc+Lc transgenic mice is likely to lie in two unique features of this autoanti-

body. First, this Hc+Lc transgenic combination favors development and gradual expansion of cells with a B1 phenotype (Okamoto *et al.*, 1992; Murakami *et al.*, 1992). B1 cells are normally infrequent in secondary lymphoid organs or the circulation but predominate in the peritoneal cavity (Kantor and Herzenberg, 1993), and indeed large numbers of transgene-expressing B1 cells gradually accumulate between 2 and 8 weeks after birth in the antierythrocyte transgenics (Murakami *et al.*, 1992). Another V_H/V_L combination, $V_H12/V_\kappa4$, encoding antibodies that bind weakly to phosphatidylcholine, also favors formation and gradual expansion of B1 cells and precludes maturation into normal follicular-type B cells when expressed in Hc+Lc transgenic mice (Arnold *et al.*, 1994). The phosphatidylcholine-reactive antibody may represent an antibody to altered self rather than a true autoantibody, because (a) it only reacts with autologous erythrocytes after they have been protease treated, (b) does not cause hemolytic anemia, and (c) does not appear to trigger B cell elimination in the bone marrow. In contrast to these two receptor specificities, the V_H/V_L combination expressed in HEL-reactive IgM+D transgenic mice precludes formation of B1 cells in the presence or absence of HEL autoantigen, although it favors formation and accumulation of follicular-type B cells (Mason *et al.*, 1992; Cyster and Goodnow, 1995). It is not yet known how the expression of particular receptor specificities biases B cell maturation toward the B1 phenotype and promotes their accumulation in the peritoneum, although stimulation by environmental antigens of the T cell-independent type from the gut is a possibility (Bos *et al.*, 1989; Cong *et al.*, 1991).

The second important feature of the erythrocyte-specific autoantibody is the apparent lack of exposure to erythrocyte autoantigen in the peritoneum. Thus, in contrast to the extensive receptor modulation induced by autoantigen on transgene-expressing B cells in the bone marrow and secondary lymphoid tissues, B1 cells in the peritoneum of the erythrocyte-specific Hc+Lc transgenic mice express normal high densities of IgM (Murakami *et al.*, 1992). Moreover, when syngeneic erythrocytes are deliberately introduced into the peritoneal cavity, the transgene-expressing B cells undergo apoptotic cell death and disappear (Murakami *et al.*, 1992). The apparent protection of transgene-expressing B cells from autoantigen in the peritoneum combined with the unknown factors that selectively promote their expansion in this site provide a likely explanation for the high levels of autoantibody secreted in many of the animals. By contrast, the H-2K- or mHEL-reactive B cells fail to escape censoring in this way because (a) the autoantigens they recognize are present on all cells, and

(b) their receptor specificity is not permissive for expansion as B1 cells in the peritoneal cavity.

It remains to be determined how erythrocyte-specific B cells escape elimination in primary lymphoid organs to colonize and expand in the peritoneal cavity. On one hand, if censoring of immature B cells in primary lymphoid organs is less than 100% efficient, the peritoneal cells may be progeny of rare cells that evaded censoring by chance. Alternatively, the autoantigen recognized by this antibody may not be expressed on erythrocytes in fetal liver when many precursors for the B1 cell pool mature. Precedent for the latter possibility is set by the human I/i erythrocyte carbohydrate antigen system, which undergoes a fetal to adult shift in glycosylation pattern and interestingly is also a common target for autoantibodies in certain forms of autoimmune hemolytic anemia (Siegel and Silberstein, 1992).

D. Mechanism of Immature B Cell Elimination in the Bone Marrow

1. Developmental Arrest

Cell death does not immediately follow binding of mHEL, H-2K, or erythrocyte self-antigens by immature B cells in the bone marrow of Hc+Lc transgenic mice. The first evidence for this is the lack of any reduction in the steady-state pool of IgM^+ immature B cells in the bone marrow in the presence of self-antigen, despite the cells having already undergone self-antigen-induced receptor modulation (Nemazee and Burki, 1989; Hartley *et al.*, 1991; Okamoto *et al.*, 1992). In mHEL-bearing animals, Hartley *et al.* found a slight increase in the immature B cell pool composed mostly of typical small, noncycling immature B cells whose *in vivo* turnover was not shortened from the normal half-life of 18 hr (Hartley *et al.*, 1993).

What happens during the 18 hr between self-antigen encounter and B cell disappearance? Two approaches showed that antigen receptor engagement by mHEL triggered a reversible arrest of maturation in immature B cells prior to and independent of cell death (Hartley *et al.*, 1993; Fig. 2). First, immature autoreactive B cells sorted from $mHEL^+$ bone marrow matured into $B220^{high}$ IgD^+ cells in the absence of further exposure to mHEL-bearing cells in culture, but were blocked at the $B220^{low}$ IgD^- immature B cell stage if they continued to be exposed to mHEL antigen. When the arrested cells were stimulated with LPS, proliferation and differentiation into antibody-secreting cells was completely suppressed, but only if they remained in contact with mHEL autoantigen. By contrast, the viability of the cultured B

cells was unaffected by the presence or absence of mHEL autoantigen. Second, constitutive expression of a *Bcl-2* transgene that inhibits B cell death impeded elimination of mHEL-reactive B cells *in vivo* by a factor of 5- to 10-fold, but the accumulating autoreactive cells in bone marrow, spleen, blood, and lymph node remained blocked at the immature stage of B cell development. *In vivo* transfer studies showed that the life span of arrested mHEL-binding B cells was markedly extended by constitutive Bcl-2 expression and was longer than the time taken for maturation in the absence of further mHEL exposure (J.G. Cyster and C.C.G., unpublished results).

2. *Cell Death*

The inhibitory effect of Bcl-2 on the elimination of immature mHEL-reactive B cells implies that the rapid turnover and disappearance of autoreactive cells in the absence of Bcl-2 was due to cell death by apoptosis (Hartley *et al.*, 1993). It has nevertheless been impossible to demonstrate dying cells in the bone marrow due to a high natural background of apoptotic bodies within bone marrow stromal cells that presumably results from cell turnover in many hemopoietic lineages (Hartley and Goodnow, 1994b). Small numbers of immature autoreactive B cells are present in the blood and spleen in the mHEL, H-2K^b, and antierythrocyte models (Hartley *et al.*, 1991,1993; Nemazee *et al.*, 1991b; Okamoto *et al.*, 1992), indicating that a fraction may die after emigration from the bone marrow. Intriguingly, the same *Bcl-2* transgene that had a marked effect in the mHEL model had no effect on B cell elimination in the antierythrocyte model (Nisitani *et al.*, 1993), pointing to qualitative or quantitative differences in the way B cells are eliminated in these two cases.

What triggers the death of autoreactive immature B cells? Cell death in a variety of cell types can be triggered in two ways. First, signals connected to mitogenesis can directly activate the cell death program, as occurs in fibroblasts when the cellular *myc* or adenovirus E1a gene products are constitutively expressed (Evan *et al.*, 1992; Rao *et al.*, 1992). In these two well-studied fibroblast models, the decision between mitosis and apoptosis is also determined by extracellular survival signals, such as insulin-like growth factor 1 (Harrington *et al.*, 1994), or potentially downstream intracellular signals such as Bcl-2 (Fanidi *et al.*, 1992) or its viral homologue, E1B p17 (Rao *et al.*, 1992). These survival signals indirectly promote mitosis by suppressing the tendency of *myc* and E1a to activate cell death. Thus, cell death may be determined as much by withdrawing or inhibiting the activity of survival signals as by direct inducers of the death program. For exam-

ple, growth factor-dependent cells die rapidly when they are removed from factor unless they constitutively express Bcl-2 (Vaux *et al.*, 1988; Rodriguez-Tarduchy *et al.*, 1990; Williams *et al.*, 1990).

Like c-*myc* or E1a in the fibroblast models previously described, Ig receptor signaling activates either cell death or mitogenesis, depending on a variety of conditions, in B cells exposed to anti-Ig antibodies in culture (Sell and Gell, 1965; Parker, 1980; Sieckmann, 1980; DeFranco *et al.*, 1982; Howard and Paul, 1983; Melchers and Andersson, 1984; Paul and Ohara, 1987; DeFranco, 1987; Cambier and Ransom, 1987; Chang *et al.*, 1991; Brown *et al.*, 1992; Parry *et al.*, 1994a,b). In contrast to mature IgD^+ B cells, the mitogenic response to anti-Ig receptor cross-linking is difficult to reveal in immature B cells (Raff *et al.*, 1975; Sidman and Unanue, 1975; Sieckmann, 1980; Pike *et al.*, 1982; Yellen *et al.*, 1991; Yellen-Shaw and Monroe, 1992; Scott, 1993), whereas the apoptotic response to Ig cross-linking remains intact (Brown *et al.*, 1992). Moreover, certain B lymphoma cell lines that may represent transformed counterparts of immature B cells undergo cell cycle arrest and apoptosis when their Ig receptors are cross-linked (Ralph, 1979; Boyd and Schrader, 1981; Jakway *et al.*, 1986; Benhamou *et al.*, 1990; Hasbold and Klaus, 1990; Ishigami *et al.*, 1992; Scott, 1993). Ig-induced apoptosis is blocked if B cells or B lymphoma cells are concurrently stimulated by LPS (Jakway *et al.*, 1986) or through CD40 (Valentine and Licciardi, 1992; Tsubata *et al.*, 1993; Santos-Argumedo *et al.*, 1994; Lederman *et al.*, 1994; Parry *et al.*, 1994a). Collectively, these findings are most simply explained by direct activation of the cell death program by Ig signaling, although it is also possible that Ig signaling triggers death in these models by interfering with the reception of survival signals from factors in fetal calf serum.

Ig signaling can also bring about cell death indirectly by modulating reception of extracellular survival signals. In large pre-B cells, signals triggered by expression of membrane μ-chains paired with surrogate light chains, in combination with stimulation by stem cell factor (SCF) and IL-7, initially promote proliferation and/or survival and maturation into small pre-B cells (Fig. 2). Elimination of either the μ-chain, $\gamma5$ surrogate light chain, or IL7 causes a drastic loss of small pre-B cells (Kitamura *et al.*, 1991,1992; Ehlich *et al.*, 1993; Grabstein *et al.*, 1993; Reichman-Fried *et al.*, 1993). By promoting maturation, however, the same pre-B cell receptor signaling event shuts off expression of receptors for SCF and IL-7 in the resulting small pre-B cells and immature B cells, thus setting a limit to the numbers and life span of maturing B cells (Lee *et al.*, 1989; Era *et al.*, 1991; Sudo *et al.*, 1993; Reichman-Fried *et al.*, 1993; Rolink and Melchers, 1993). Extinguished reception

of extracellular survival signals may in turn account for the drop in intracellular Bcl-2 protein that occurs during maturation of pro-B cells into small pre-B cells and immature B cells (Li *et al.*, 1993; Merino *et al.*, 1994).

3. *A Two-Signal Model for B Cell Survival in the Preimmune Repertoire*

Interplay between Ig-mediated activation of cell death, concurrent stimulation by survival factors, and the effects of chronic Ig signaling on reception of these survival factors may collectively explain elimination of immature self-reactive B cells in the bone marrow (Figs. 2 and 3). In pro-B cells that have productively rearranged a H chain gene, μ-chain/surrogate light-chain Ig receptors may be cross-linked by a ligand on stromal cells (Rolink and Melchers, 1993). The resulting Ig signaling comes at a time and place when the pro-B cell is also strongly stimulated by growth and survival factors from stromal cells such as IL-7 and SCF. The latter may account for the high level of Bcl-2 expressed in pro-B cells (Li *et al.*, 1993; Merino *et al.*, 1994) and, thus, oppose the tendency for Ig signaling to activate the cell death program, allowing large pre-B cells to undergo mitosis and then survive as small pre-B cells. By contrast, when self-antigens cross-link μ-chain/light-chain IgM receptors on immature B cells, concurrent stimulation by SCF and IL-7 will be reduced or absent because the maturing B cells are losing receptors for these factors and may no longer be in intimate contact with stromal cells. At a certain point, the residual intracellular Bcl-2 and other mediators of survival fall below the amount needed to suppress Ig-mediated activation of the death program, and the B cell dies. This point will be reached earlier if self-antigen-induced Ig clustering and signaling is high, and will be reached later or not at all if weaker signaling is elicited. As discussed under Section II,F, survival of mature B cells may also depend on signals from stromal cells; in this case, in primary follicles of secondary lymphoid tissue. By arresting development of immature B cells, self-antigen-induced Ig signaling may thus also contribute to death by preventing expression of receptors needed for access to the follicular stroma in secondary lymphoid tissues (Hartley *et al.*, 1993; Hartley and Goodnow, 1994b).

Three experimental observations could be explained by the model outlined previously and in Figs. 2 and 3. First, the model explains how Ig signaling promotes growth of pro-B cells but death of immature B cells without needing to invoke a change in the intracellular signal-transduction apparatus associated with membrane μ-chains. Second, the model accounts for the ability of the Bcl-2.22 transgene to markedly

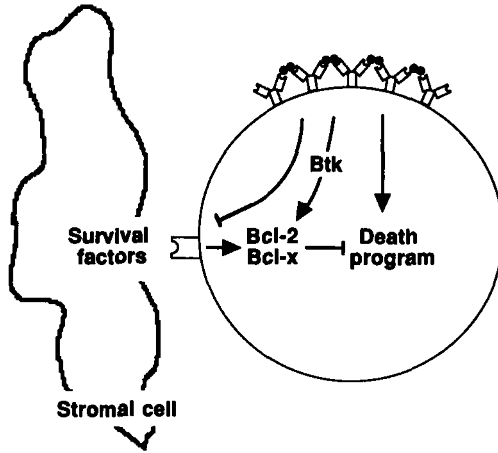

FIG. 3. Simplified model illustrating the interplay between antigen receptor signaling and trophic factors from stromal cells. Undefined signaling pathways triggered by antigen receptor clustering tend to activate the cell death program (as well as promote mitosis). Death is repressed, however, by concurrent stimulation by extracellular survival factors from neighboring stromal cells and by intracellular survival mediators, such as Bcl-2 or Bcl-X, which may be induced by the former. Thus, death may only occur if antigen receptor signaling exceeds a certain threshold. Alternatively, lower levels of signaling from the antigen receptor can trigger death indirectly by interfering with reception of survival signals. In the bone marrow, interference is brought about indirectly by the loss of receptors for stromal factors when pro-B cells mature in response to pre-B cell receptor signaling. In secondary lymphoid organs, interference with reception of survival signals is brought about by Ig signaling through its antagonistic effect on migration into follicles. As discussed in the text, survival is also compromised by the *xid* mutation in Btk, whose effect is (a) additive with the effects of chronic antigen receptor signaling, and (b) can be entirely corrected by constitutive expression of Bcl-2. Antigen receptor signaling through Btk may therefore transmit a signal that facilitates the induction or action of Bcl-2.

inhibit elimination of autoreactive B cells in response to mHEL autoantigen (Hartley *et al.*, 1993) but not in response to an erythrocyte autoantigen (Nisitani *et al.*, 1993). The erythrocyte autoantigen may be more abundant and thus promote greater receptor clustering and signaling, exceeding the capacity of transgene-derived Bcl-2 to inhibit the cell death program. Third, in contrast to the lack of any reduction in the pool of immature B cells induced by mHEL normally (Hartley *et al.*, 1993), a marked decrease is induced by mHEL when the B cells carry a mutation in the cytosolic protein tyrosine phosphatase, PTP1C (Cyster and Goodnow, 1995). PTP1C normally inhibits intracellular signaling by the Ig antigen receptor and exaggerated responses to a

given amount of antigen occur in its absence (Cyster and Goodnow, 1995). Death may be triggered by mHEL more rapidly in PTP1C-deficient immature B cells because the exaggerated Ig signaling quickly exceeds the capacity of survival signals to suppress death.

4. *Molecules Responsible for Developmental Arrest and Death*

While great progress has been made in recent years demonstrating that the B cell antigen receptor triggers a series of divergent signaling pathways (Gold and DeFranco, 1994; Cambier *et al.*, 1994), it is not known which of these is responsible for triggering developmental arrest or cell death in immature B cells. Understanding how Ig signaling and survival signals, such as Bcl-2, are integrated awaits identification of the active machinery for apoptosis and elucidation of how these two kinds of signals connect to the death machinery.

Another pathway for triggering cell death involves the CD95 (Fas/Apo-1) cell surface receptor (Trauth *et al.*, 1989; Itoh *et al.*, 1991; Oehm *et al.*, 1992). While loss of function mutations in CD95, such as *lpr*, are associated with autoantibody production, and CD95 deficiency in B cells is essential for autoantibody production in *lpr* mice (Watanabe-Fukunaga *et al.*, 1992; Sobel *et al.*, 1991; Cohen and Eisenberg, 1991), CD95 nevertheless appears not to be required for censoring immature autoreactive B cells in the bone marrow. Thus, mHEL triggers exactly identical arrest and elimination of immature HEL-reactive B cells whether or not they carry the *lpr* mutation (Rathmell and Goodnow, 1994). Because a small amount of cell surface Fas expression may remain in *lpr* animals (Adachi *et al.*, 1993; Chu *et al.*, 1993; Wu *et al.*, 1993a), it cannot be excluded that these residual receptors are still sufficient for normal censoring of bone marrow B cells in *lpr* animals. As described under Section III,E, the *lpr* mutation disrupts a B cell-censoring step in the periphery that is more likely to contribute to autoantibody production in mice with this mutation.

5. *Receptor Editing*

As an alternative to cell death, self-reactive B cells that become developmentally arrested in the bone marrow may instead "edit" their receptor to a nonautoreactive form and thus escape developmental arrest and subsequent death. The first evidence for this alternative was in H-2K^k-reactive Hc+Lc transgenic mice, where Tiegs *et al.* (1993) noted that B cells expressing non-H-2K^k-binding λ-light chains became more frequent in transgenic mice bearing the H-2K^k autoantigen. It was suggested that more λ-bearing cells were produced in the presence of the deleting antigen because the VDJ recombinase-

activator genes (RAG) were reinduced by antigen receptor engagement in immature B cells. Consistent with this notion, more RAG1 and RAG2 mRNA were present in the bone marrow of the H-$2K^k$ Ig transgenic mice. Moreover, λ-bearing cells were not elevated in metallothionein–H-$2K^b$ transgenic mice in which deletion is initiated at a later stage, arguing against the possibility that λ-bearing cells simply expanded to fill the space in the preimmune repertoire left after deletion of H-2K-reactive cells.

As discussed under Section II,C,3, Gay *et al.* (1993) also found many B cell clones that escaped elimination in anti-dsDNA Hc + Lc transgenic mice by receptor editing. The dsDNA-reactive B cells escaped elimination by rearranging an endogeneous light-chain gene whose product outcompeted the transgenic light chain for pairing with heavy chains. Chen *et al.*, found B cell clones in anti-dsDNA Hc transgenic mice that had escaped censoring by deleting most or all of the Hc transgene copies (Chen *et al.*, 1994).

Few B cells escape elimination by the editing mechanisms described above in Hc + Lc transgenic mice, as the number of peripheral B cells often remains low even at several months of age (Nemazee and Buerki, 1989; Hartley *et al.*, 1991,1993; Tiegs *et al.*, 1993) except when the transgenic light chain lacks an efficient enhancer (Gay *et al.*, 1993). In normal circumstances, however, the organization of the κ-light chain locus allows secondary rearrangements of upstream V_κ and downstream J_κ elements that would simultaneously delete an autoreactive VJ_κ rearrangement and replace it with a new rearrangement that may not be autoreactive (Tiegs *et al.*, 1993; Radic *et al.*, 1993a). Evidence for light-chain replacement by secondary rearrangements has been obtained in mice carrying only an anti-dsDNA Hc chain transgene, where light chains are derived from the endogenous light-chain locus rather than a transgene locus on another chromosome (Radic *et al.*, 1993a). In these studies, B cells that expressed the transgenic Hc but were not dsDNA reactive often expressed light-chain genes with rearrangements to $J_\kappa5$, rather than the usual preference for $J_\kappa1$ seen in nontransgenic mice (Nishi *et al.*, 1985).

The relative roles of receptor editing versus cell death for censoring immature autoreactive B cells remain to be determined. The tendency for most B cells to have rearrangments to $J_\kappa1$ (Nishi *et al.*, 1985) indicates that the majority of preimmune B cells do not arise through editing. On the other hand, particular heavy-chain V regions that have a high predisposition to autoreactivity may depend on editing to increase the chance that they are paired with nonautoreactive light chains. A possible example may be carbohydrate antigen-reactive H

chains, where particular light chains can change the fine specificity (Siegel and Silberstein, 1992) and thus may prevent reactivity with autologous carbohydrates while retaining reactivity with closely related bacterial carbohydrates (Galili *et al.*, 1987).

E. Thresholds for Triggering B cell Elimination in the Bone Marrow

B cell censoring in the bone marrow must have a stringency limit to avoid eliminating too great a fraction of the preimmune repertoire (Nossal, 1994). Dose–response experiments using antigens or antiimmunoglobulins both *in vivo* and *in vitro* have repeatedly emphasized the need for a critical concentration of antigen and minimal receptor affinity to induce B cell tolerance (Dresser and Mitchison, 1968; Siskind and Benacerraf, 1969; Weigle, 1973a; Metcalf *et al.*, 1979; Nossal, 1983). With the ability to visualize B cell censoring in the bone marrow of Ig transgenic mice, a high threshold of antigen receptor engagement has been firmly established as a key feature of this first tolerance checkpoint (Fig. 4).

1. *Antigen Valency*

Consistent with the notion of a censoring threshold, not all self-antigens trigger developmental arrest and elimination when they are bound by immature B cells in the bone marrow of Hc + Lc transgenic mice. The clearest example of this is seen in crosses between anti-HEL Hc + Lc transgenic mice and mice expressing soluble HEL (sHEL). By contrast with the developmental arrest that occurs in the presence of mHEL (Hartley *et al.*, 1991), no developmental arrest or elimination is triggered by sHEL (Goodnow *et al.*, 1988) even when the concentration in the extracellular fluid of the bone marrow is sufficient to saturate the IgM receptors on the developing immature B cells (Goodnow *et al.*, 1989a; Mason *et al.*, 1992). Binding of sHEL nevertheless triggers an intracellular signal in the immature B cells, since (a) IgM receptors are modulated 3- to 5-fold *in vivo* (compared with 15- to 20-fold for mHEL) (Mason *et al.*, 1992; Hartley and Goodnow, 1994b; Cyster and Goodnow, 1995), (b) an elevation of intracellular calcium occurs in Indo-1-loaded immature B cells exposed to sHEL *in vitro* (Cyster and Goodnow, 1995), and (c) binding of sHEL renders the immature cells anergic (Mason *et al.*, 1992). The signals elicited in immature B cells by binding sHEL are therefore either of insufficient quantity or of the wrong type to activate this first B cell censoring checkpoint. Since both mHEL and sHEL forms of the antigen are bound with the same intrinsic affinity and both are present at receptor-saturating concentra-

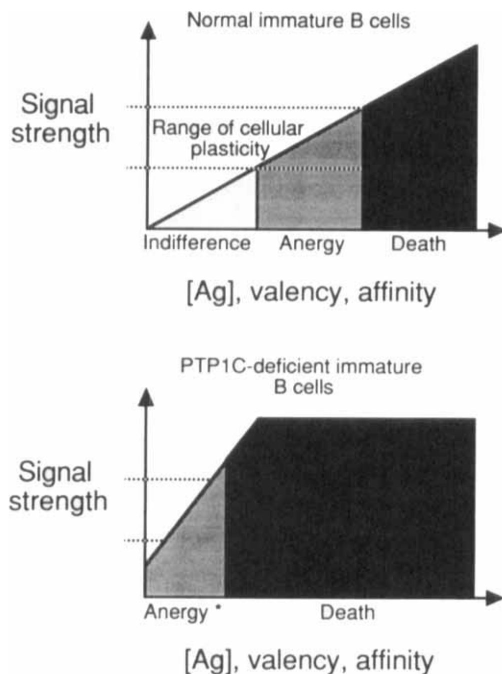

FIG. 4. Signaling threshold model to explain the differential induction of various censoring mechanisms in immature B cells. In normal circumstances (top), immature B cells have only been found to be eliminated in the bone marrow if they bind self-antigens that are abundant and highly multivalent, such as ubiquitous cell surface molecules or dsDNA. Less multivalent autoantigens, such as soluble HEL or ssDNA, or possibly weaker binding of antigens such as dsDNA, trigger weaker signaling and bring about anergy but not elimination, provided they are present in the bone marrow in sufficient concentration and bound with adequate affinity. When autoantigen concentration, valency, and affinity are too low to generate adequate intracellular signaling, neither censoring mechanism is activated in the bone marrow. On the other hand, when the signal-quenching effects of PTP1C are deficient within immature B cells (bottom) weak receptor clustering becomes sufficient to trigger death and elimination. Moreover, in PTP1C-deficient cells, enough Ig signaling occurs in the absence of appreciable autoantigen binding to trigger one of the features of anergy, namely IgM receptor modulation. Desensitization of the remaining receptors does not occur, however, suggesting that PTP1C may mediate the signaling block in anergic B cells.

tions, it is likely that the selective ability of mHEL to trigger developmental arrest lies in its valency and thus greater potential to cluster large numbers of antigen receptors together (Hartley *et al.*, 1991).

An analogous result occurs when membrane-bound H-$2K^b$ molecules are compared with a soluble form of the same antigen. Transgenic

mice expressing a soluble-secreted form of H-2K^b were produced (Arnold *et al.*, 1988) and crossed to anti-H-2K Hc + Lc transgenic mice (Nemazee *et al.*, 1991b). In contrast to the deletion and extensive IgM modulation induced in immature B cells by membrane-bound H-2K^b, the soluble antigen had no detectable effect on developing H-2K-specific B cells. This result again emphasizes a need for a minimum self-antigen valency to trigger B cell censoring in the bone marrow, although the concentration of the soluble antigen may have been insufficient to occupy a comparable proportion of receptors to that achieved by membrane H-2K^b.

A third example that may favor the importance of antigen valency for triggering the bone marrow censoring checkpoint comes from anti-DNA Hc + Lc transgenic mice. Whereas a number of transgenic Hc + Lc combinations encoding dsDNA-reactive receptors are censored in the bone marrow (Erikson *et al.*, 1991; Gay *et al.*, 1993; Radic *et al.*, 1993a; Chen *et al.*, 1994), a ssDNA-reactive receptor composed of the same heavy-chain transgene paired with a different light-chain transgene did not trigger B cell censoring in the bone marrow (Erikson *et al.*, 1991). Instead, the ssDNA-reactive B cells matured and populated the preimmune repertoire, but were rendered anergic in a manner comparable to that previously seen with sHEL (Nguyen *et al.*, 1994). Autoreactive B cells may encounter ssDNA in a less multimeric form than dsDNA, since ssDNA polymers are much more sensitive to cleavage and breakdown by tissue endonucleases. Nuclear antigens, such as DNA, are also displayed in potentially multivalent form on the surface of dying cells (Casciola-Rosen *et al.*, 1994); it is currently unclear whether dsDNA and ssDNA might differ in their relative presentation in this manner. By contrast, Tsao and colleagues found that B cells expressing a pathogenic anti-dsDNA antibody were not eliminated in the bone marrow of IgM Hc + Lc transgenic mice (Tsao *et al.*, 1993). The variable fate of nucleic acid-reactive B cells may instead be determined by receptor affinity or cross-reactions with other polyanionic self-antigens.

A fourth example of an oligovalent self-antigen that should be encountered by immature B cells in the bone marrow yet fails to trigger censoring at this checkpoint has been described in a model developed by Schlomchik *et al.* (1993). They produced Hc + Lc transgenic mice carrying transgenes encoding a rheumatoid-factor autoantibody that bound preferentially to IgG_{2a} of the a-allotype. B cells expressing this receptor specificity developed normally in mice bearing serum IgG_{2aa} compared with mice lacking this serum allotype. Like the experiments with soluble H-2K^b, it is not yet known whether the affinity of the

receptor and the concentration of serum IgG_{2a} antigen are sufficient to achieve a level of autoantigen binding and receptor occupancy in the bone marrow that compares with membrane antigens.

2. *Receptor Affinity*

The results above described in the preceding section collectively emphasize the importance of multivalent or cell surface antigens to trigger autoreactive B cell censoring in the bone marrow. Most V_H/V_L combinations borne by immature B cells in the bone marrow, having not been subjected to hypermutation and affinity maturation, bind cell surface antigens such as ABO carbohydrates with low intrinsic affinity (Landsteiner, 1945; Siskind and Benacerraf, 1969; Karush, 1978). When secreted as pentameric IgM molecules, however, this low intrinsic affinity can translate into high-avidity binding to blood-exposed multivalent antigens (Karush, 1978), as illustrated by the devastating lysis of transfused ABO-incompatible erythrocytes or the hyperacute ischemic necrosis after reconnecting the blood supply to ABO-incompatible kidney transplants (Starzl *et al.*, 1987). By contrast, each of the transgenic models of bone marrow censoring described above have studied the fate of B cells bearing hypermutated antibodies with high intrinsic affinity for their ligands. To explore the relevance of bone marrow B cell censoring for preventing low intrinsic affinity IgM autoantibodies, the intrinsic binding affinity of autoreactive B cells has been varied in transgenic models by two approaches. In one approach, Hartley and Goodnow (1994a) compared the fate of B cells whose receptors differed in their intrinsic affinity for HEL. To do this, they used a transgenic line carrying only the antilysozyme Hc gene, in which each different B cell expressed the trangene-encoded heavy chain paired with a different light chain. Approximately 5% of the B cells bound HEL in a saturable manner, but these fell into a series of discrete and reproducible subpopulations which required different concentrations of monomeric sHEL to saturate their receptors. A considerable proportion of the cells carried very low-affinity V_H/ V_L combinations that required 1–10 μM sHEL to achieve saturation, yet these low-affinity cells were still eliminated efficiently in animals that expressed mHEL as a systemic cell surface antigen.

As an alternative approach to vary the intrisic affinity, Lang and Nemazee (1994) made use of the large panel of bm-series mice, which bear spontaneous mutations in the H-2K^b molecule affecting the binding of K^b to the transgenic Ig receptor. Elimination of self-reactive B cells was triggered efficiently even when the anti-K^b Hc + Lc transgenic mice were crossed to the bm3 strain, which bear an altered

H-2K molecule that binds with an estimated intrinsic affinity in the submicromolar range.

The two experiments above provide persuasive evidence that this first B cell censoring checkpoint eliminates low-affinity B cells efficiently if they recognize abundant cell surface antigens on blood cells or other cells in the bone marrow. It is thus highly likely that this mechanism accounts for the absence of natural autoantibodies to abundant blood cell antigens such as ABO or RhD. Indeed, B cells capable of making specific anti-A or anti-B antibodies are undetectable in the circulation of A-positive or B-positive individuals (Rieben *et al.*, 1992; Conger *et al.*, 1993). It remains to be determined, however, whether low-affinity B cells are equally well censored at this stage if they recognize antigens that are less abundant on the surface of blood cells or are attached less firmly. Variation in antigen valency and receptor affinity could allow certain clones to escape censoring and help explain the occurence of autoantibodies to blood cell surface autoantigens in the autoimmune hemolytic anemias or in autoimmune thrombocytopenia. As mentioned under Section II,E,1, the failure of DNA-reactive B cells to be eliminated in the bone marrow in the ssDNA model of Erikson *et al.* (1991) or the dsDNA model of Tsao *et al.* (1993) may also be due to insufficient affinity.

3. *Antigen Concentration*

Triggering B cell elimination in the bone marrow clearly requires that the autoantigen be present in the bone marrow and encountered by B cells. For cell surface antigens, a third potentially important variable is therefore the number of cells bearing the autoantigen. In the H-2K, mHEL, and erythrocyte models, a large fraction of bone marrow hemopoietic and/or stromal cells in the bone marrow bear the target autoantigen. At the other extreme, when cell surface expression of mHEL is restricted to a peripheral organ, such as the thyroid epithelium or pancreatic islet β cells, no censoring of B cells can be detected in the bone marrow or at subsequent steps (S. Akkaraju and C. Goodnow, unpublished data). In the case of cell surface autoantigens that lie between these two extremes by being displayed abundantly only on rare blood cell types, two sets of experiments suggest that bone marrow censoring may not be efficient.

Hartley and Goodnow explored this issue of target cell frequency by studying the fate of lysozyme-binding B cells in Hc + Lc transgenic mice that contained progressively lower frequencies of mHEL-bearing hemopoietic cells (S. Hartley and C. Goodnow, unpublished data). To vary the frequency of mHEL-bearing cells, radiation bone marrow

chimeras were constructed by reconstituting nontransgenic mice with mixtures of Hc + Lc transgenic bone marrow and marrow that also carried the H-2K promoter-driven mHEL transgene. When only 1% of the hemopoietic cells carried the mHEL transgene, no detectable B cell censoring occured. When 10% of the hemopoietic cells carried mHEL, large numbers of lysozyme-binding B cells matured and emigrated from the bone marrow without evidence of encountering mHEL since their IgM receptors were not modulated in the bone marrow or in the blood. On reaching the spleen and to a much lesser extent the lymph nodes, the autoreactive B cells encountered mHEL as evidenced by receptor modulation at this site and were eliminated at the IgD^+ stage.

A striking example of failure to eliminate autoreactive B cells in the bone marrow to an abundant but T cell-specific surface antigen has been provided by Eibel *et al.* (1994). They constructed IgM Hc + Lc transgenic mice carrying transgenes encoding a high-affinity antibody to an allelic epitope, CD8.2, that results from a single amino acid polymorphism in the α-chain of CD8. Maturation of B cells expressing the transgene was followed by flow cytometry using the ability of transgenic B cells to bind a biotinylated soluble fusion protein of CD8α linked to C_K. Large numbers of mature follicular-type B cells expressing the transgenic receptor were present in the spleen of mice that expressed only the CD8.1 form of the antigen, which is not recognized by the transgenic antibody. By contrast, few mature follicular-type B cells expressing the transgenic receptor specificity were present in the spleen of mice bearing the reactive CD8.2 autoantigen. Immature B cells in the bone marrow and immature recent bone marrow emigrants were nevertheless present in normal numbers and were suggested not to have encountered the CD8 antigen at this stage since only 1% of bone marrow cells are $CD8^+$ T cells.

Both these models indicate that bone marrow censoring is inefficient when a large number of autoreactive B cells must be censored to surface antigens that are unique to small subsets of blood cells. In addition, they indicate that elimination can take place after developing B cells have migrated from the bone marrow to secondary lymphoid organs. The B cells were not yet mature at the time elimination was triggered, but had an IgD^{low} HSA^{high} phenotype typical of recent bone marrow emigrants or neonatal spleen B cells (Allman *et al.*, 1992, 1993). Consequently, it is unclear whether censoring occurs by the same mechanism as bone marrow censoring or by a separate process.

Along similar lines, Russell *et al.* (1991) found that B cell developmental arrest and editing was not triggered in the bone marrow of

anti-H-2K Hc + Lc transgenic mice bearing an H-$2k^b$ transgene controlled by the sheep metallothionein (MT) promoter. The MT-K^b construct was expressed predominantly in hepatocytes and kidney cells, leading to the conclusion that elimination was triggered during migration through these peripheral organs after emigration from the bone marrow. Some modulation of IgM appeared to occur on maturing Ig-D^{low} B cells in the bone marrow, however, raising the alternative possibility that low expression of the MT-K^b transgene on cells in the bone marrow may have triggered elimination at the IgD^{low} HSA^{high} transitional stage.

4. Signal Strength Determines Thresholds for Censoring Immature B Cells

Receptor affinity, autoantigen valency, and antigen concentration may collectively determine whether or not the censoring threshold is reached in immature self-reactive B cells by influencing intracellular signal strength (Fig. 4; Goodnow, 1992). Striking evidence for signal strength as the common integrator comes from the effect of mutations at the *motheaten* locus (Cyster and Goodnow, 1995). Two recessive alleles, *motheaten* (*me*) and *motheaten-viable* (*me^v*) have arisen as spontaneous mutations in C57BL/6 mice (Green and Shultz, 1975; Shultz *et al.*, 1984). Both mutations have similar pleiotropic effects on many hemopoietic cell lineages (Shultz, 1988), and the gene affected (Shultz *et al.*, 1993; Tsui *et al.*, 1993) encodes a cytosolic protein tyrosine phosphatase, PTP1C (also HCP and SHP), that is widely expressed in hemopoietic cells (Shen *et al.*, 1991; Matthews *et al.*, 1992; Plutzky *et al.*, 1992; Yi *et al.*, 1992; Pei *et al.*, 1993). PTP1C contains two *src*-homology 2 (SH2) domains and is thus likely to be recruited to intracellular sites of tyrosine kinase activation, as has been shown to occur following activation of the receptors for SCF or IL-3 (Yi *et al.*, 1993; Yi and Ihle, 1993). PTP1C protein expression is abolished in *me* homozygotes, while enzyme activity is greatly reduced in *me^v* (Shultz *et al.*, 1993; Kozlowski *et al.*, 1993). Four key B cell abnormalities occur in *me* or *me^v* homozygotes (reviewed by Shultz, 1988; Shultz, 1991): (1) bone marrow B lymphopoiesis is depressed (Greiner *et al.*, 1986; Medlock *et al.*, 1987; Hayashi *et al.*, 1988); (2) no mature IgD^{high} follicular-type B cells can be detected in spleen, lymph nodes, or blood, and antibody responses following immunization are greatly depressed (Shultz and Green, 1976; Sidman *et al.*, 1978a,b; Davidson *et al.*, 1979; Sidman *et al.*, 1986); (3) B1-type B cells are still present and appear highly activated and enlarged (Sidman *et*

al., 1986); and (4) serum IgM is greatly elevated and includes a range of autoantibody specificities (Shultz and Green, 1976; Painter *et al.*, 1988; Katsuri *et al.*, 1990). The B cell phenotype thus features both immunodeficiency and autoimmunity.

To study how deficiency of intracellular PTP1C affects B cell development and selection, Cyster and Goodnow crossed C57BL/6 antilysozyme Hc + Lc transgenic mice with the C57BL/6-me^v strain to generate me^v homozygotes with a monoclonal HEL-specific B cell repertoire (Cyster and Goodnow, 1995). Some of the B cell abnormalities in me^v mice, such as depression of B lymphopoiesis, are due to effects of the mutation in *trans* due to effects of dysregulated myeloid cell activity for example (Medlock *et al.*, 1987; Hayashi *et al.*, 1988). To minimize such *trans* effects and identify defects resulting from PTP1C deficiency in the B cells themselves, radiation bone marrow chimeras were constructed in which me^v Hc + Lc transgenic bone marrow was diluted with an excess of $Ly5^a$-marked Hc + Lc transgenic marrow that carried functional PTP1C genes. By comparing side-by-side matched lysozyme-specific cells that either carried or lacked normal levels of PTP1C, the first key observation made was that PTP1C-deficient cells showed a much greater and more rapid elevation of intracellular calcium after binding HEL antigen, indicating that the enzyme negatively regulates Ig receptor signaling. Second, when immature B cells with this exaggerated signaling trait encountered soluble HEL antigen during their development in the bone marrow, the response triggered was identical to that triggered by mHEL in wild-type B cells. Thus, binding of sHEL to PTP1C-deficient immature B cells triggered 15- to 20-fold modulation of surface IgM, developmental arrest, and elimination. PTP1C is therefore essential to dampen Ig signaling and ensure that bone marrow censoring is triggered only when large numbers of receptors are stably clustered, as would occur in cells that recognize abundant cell surface autoantigens (Fig. 4).

The increased stringency of bone marrow censoring brought about by PTP1C deficiency may account for the immunodeficiency and absence of mature IgD^{high} B cells in nontransgenic me^v animals. Many germline V_H/ V_L combinations appear to have low affinity for a variety of self-antigens (Haspel *et al.*, 1983; Ternynck and Avrameas, 1986; Holmberg *et al.*, 1986; Naparstek *et al.*, 1986; Casali and Notkins, 1989; Striebich *et al.*, 1990; Kearney *et al.*, 1992; Radic and Weigert, 1994) and this may be sufficient to trigger developmental arrest and elimination when PTP1C is unable to set the censoring threshold at an appropriate threshold. The biochemical targets of PTP1C and the basis for dysregulation of B1 cells in me^v mice remain to be elucidated.

F. Checkpoint 2: Competitive Elimination of Autoreactive B Cells by Follicular Exclusion

1. *Pros and Cons of Studying Tolerance in Monoclonal Repertoires*

As mentioned previously, binding of sHEL to immature B cells in the bone marrow of Hc + Lc transgenic mice does not trigger developmental arrest even at concentrations sufficient to saturate the antigen receptors (Goodnow *et al.*, 1988,1989a; Hartley *et al.*, 1991; Mason *et al.*, 1992). In these monoclonal animals, the autoreactive B cells mature, live for at least 1 or 2 weeks, and dominate the primary follicles and recirculating preimmune repertoire in numbers that are at least 75% of those in Hc + Lc transgenic littermates that lack sHEL autoantigen (Goodnow *et al.*, 1988; Mason *et al.*, 1992; Cyster *et al.*, 1994; J. Cyster and C. Goodnow, manuscript in preparation). A comparable outcome occurs in Hc + Lc transgenic mice expressing a ssDNA-binding autoantibody (Erikson *et al.*, 1991; Nguyen *et al.*, 1994) and may also occur in IgM Hc + Lc transgenic mice expressing a pathogenic dsDNA-binding autoantibody (Tsao *et al.*, 1993). In such animals, the primary bulwark against further maturation of autoreactive cells into antibody-secreting cells are biochemical changes that render the cells anergic: (a) a selective and long-lasting block in transport of new IgM receptors out of the endoplasmic reticulum toward the cell surface resulting in a 10- to 50-fold reduction in cell surface expression of this class of receptor (Bell and Goodnow, 1994); and (b) a biochemical block in the tyrosine kinase signaling cascade initiated by IgM and IgD receptors, inhibiting accumulation of phosphotyrosine on the receptor-associated CD79α and -β chains and on the collaborating *syk* tyrosine kinase (Cooke *et al.*, 1994). These biochemical changes efficiently prevent the triggering of cell multiplication and antibody secretion by T cell-dependent or T cell-independent antigens (Goodnow *et al.*, 1988; Adams *et al.*, 1990; Goodnow *et al.*, 1991; Cooke *et al.*, 1994). Because these biochemical changes have no effect on the maturation and survival of autoreactive cells in the preimmune repertoire, however, they are discussed with relevance to checkpoints in the immune repertoire under Sections III,B–D.

While monoclonal Hc + Lc transgenic animals of this type provide an essential tool to visualize and study the biochemistry of anergy in isolation from other B cell censoring processes, the absence of repertoire diversity obscures an important second checkpoint during formation of the preimmune repertoire. As discussed under Section II,B, the finite pool of long-lived recirculating preimmune B cells in normal mice contains only a small fraction of the B cells and potential antibody

diversity generated in the bone marrow. A large number of new B cells arrive daily in the spleen from the bone marrow and when the repertoire is saturated most disappear within 1–3 days, after reaching the T cell zones but without entering B cell-rich primary follicles (Brahim and Osmond, 1970; Lortran *et al.*, 1987; Chan and MacLennan, 1993; Allman *et al.*, 1993). Given the large attrition of B cells at this point, together with the skewing of antibody V region usage that occurs between immature bone marrow B cells or neonatal B cells and mature cells in the circulating repertoire of adults, it has been speculated that only B cells with particular specificities are retained in the long-lived circulating repertoire (Yancopoulos *et al.*, 1984,1988; Perlmutter *et al.*, 1985; MacLennan and Gray, 1986; Perlmutter, 1987; Riley *et al.*, 1986; Denis *et al.*, 1989; Malynn *et al.*, 1990; Freitas *et al.*, 1990; Decker *et al.*, 1991; Gu *et al.*, 1991; Huetz *et al.*, 1993).

2. *A Novel Censoring Step Revealed by Approaches to Study Clones in a Diverse Preimmune Repertoire*

Because preferential retention of particular clones could not be studied in the uniform repertoire of Hc + Lc transgenic mice, Cyster *et al.* (1994) developed three approaches to add repertoire diversity back yet preserve a small population of lysozyme-specific B cells that could be tracked by flow cytometry. The first approach employed the antilysozyme Hc-only transgenic mice described under Section II,E,2, in which approximately 1% of the circulating B cells bind HEL with high affinity. The second method was to construct radiation chimeras with mixtures of Hc + Lc transgenic bone marrow and nontransgenic bone marrow in ratios that resulted in a small fraction of uniform HEL-specific B cells and an excess of nontransgenic cells with a diverse repertoire of specificities. The third way of tracking retention of particular clones in a diverse repertoire was to follow a uniform population of lysozyme-specific B cells after they were taken from the spleens of Hc + Lc transgenic mice and transferred by intravenous injection into unirradiated recipients that contained a saturated, diverse preimmune repertoire.

In each of the approaches described, lysozyme-specific B cells competed efficiently with an excess of other B cells for survival in the circulating repertoire when no HEL autoantigen was present. In the presence of serum sHEL autoantigen, however, the lysozyme-specific cells still matured and emigrated from the bone marrow normally but were outcompeted by other B cells for survival in spleen, lymph nodes, and blood (Cyster *et al.*, 1994). Competitive exclusion of autoreactive B cells, either after emigration from the bone marrow or after recircula-

tion to spleen and lymph nodes following transfer, occurred after the B cells had entered the T cell zones at the point where they would normally migrate into B cell-rich primary follicles. Thus, whereas sHEL-binding autoreactive B cells migrated into follicles with a normal tempo of 3–6 hr when the only competing B cells were also autoreactive, they accumulated in the T cell zone and were unable to enter follicles when faced with competition by an excess of other B cells. Exclusion of autoreactive cells from the follicles was accompanied by premature cell death, with a half-life of 18 hr. B cell death was independent of and subsequent to exclusion from follicles because death could be inhibited by a constitutively expressed *Bcl-2* transgene but this had no effect on follicular exclusion (Cyster *et al.*, 1994).

Because the site and characteristics of autoreactive B cell elimination in these polyclonal transgenic models closely resembles peripheral attrition of B cells in normal mice (described previously), this competitive exclusion mechanism appears likely to represent a major checkpoint for censoring autoreactive B cells from the preimmune repertoire. Moreover, the striking correlation between entry into primary follicles and B cell survival implies that the follicular microenvironments function as limiting niches controlling the overall number and types of B cells in the circulating pool. This type of "social control" of cell number and type is well known in the developing nervous system (Cowan *et al.*, 1984; Raff, 1992).

3. *Basis for Competitive Entry into Primary Follicles*

It has been known for decades that B and T cells reside in two distinct zones in secondary lymphoid tissue, and that they selectively partition into these sites after entering the outer T cell zone along a common route (Ford, 1975; Nieuwenhuis and Ford, 1976). A great deal has been learned recently about the events that direct B and T cell migration along their common route from blood through high endothelium to the outer T cell zone (Gallatin *et al.*, 1983; Picker and Butcher, 1992; Springer, 1994). By contrast, nothing is known at present about the subsequent steps that direct divergent migration of B cells and T cells into the primary follicles and deeper into the T cell zone, respectively.

Layering of cells into discrete bands occurs during formation of many tissues and is thought to be brought about in one of three general ways. First, quantitiative differences in homotypic adhesion molecules between two cell types can cause the more adherent cells to form a discrete medulla and the less adherent cells to form a cortical layer

surrounding them (Steinberg, 1963; Steinberg and Takeichi, 1994). Second, cells can actively migrate toward a target along a chemotactic gradient such as that set by chemokines for a variety of leukocytes (Murphy, 1994; Springer, 1994) or by the netrins for neuronal growth cones (Kennedy *et al.*, 1994; Goodman, 1994). Third, migrating cells can be repelled from particular regions by chemoinhibitory gradients, as illustrated by the role of the collapsins in neuronal patterning (Lui *et al.*, 1993; Goodman, 1994). Which of these mechanisms accounts for differential movement of B cells into follicles is unknown. The fact that autoreactive B cells migrate into follicles normally in the absence of competing cells implies that their failure to compete for entry reflects a subtle quantitative difference in responsiveness to positioning cues rather than an absolute defect.

Based on the paradigms above, one could speculate that autoreactive B cells have slightly greater expression of an adhesion molecule than nonautoreactive B cells, causing them to layer between the T cells and the nonautoreactive B cells present in follicles. Alternatively, autoreactive B cells may express fewer receptors for a chemokine that attracts B cells toward follicles. In this case, follicular occupancy by nonautoreactive B cells bearing greater numbers of receptors might reduce the concentration of chemokine reaching the T cell zone: the reduced chemotactic ligand concentration together with fewer receptors on the autoreactive cells might then explain their reduced attraction for the B cell zones (Fig. 5).

Regardless of the molecular basis for competitive exclusion of autoreactive B cells from follicles, two key observations make it clear that this process is independent of the biochemical changes in antigen receptor expression and signaling that underly anergy. First, when anergic B cells from monoclonal Hc + Lc transgenic animals containing sHEL autoantigen are transferred to polyclonal nontransgenic recipients that lack sHEL autoantigen, the anergic B cells compete efficiently with the recipient's B cells, enter follicles normally, and survive (J. Cyster and C. Goodnow, manuscript in preparation). Thus, sHEL autoantigen is continuosuly required to bring about follicular exclusion, whereas the biochemial changes underlying anergy are not reversed under the same conditions of *in vivo* transfer to an antigen-free environment (Goodnow *et al.*, 1991; Cooke *et al.*, 1994). Second, when naive lysozyme-specific B cells from Hc + Lc transgenic animals, which are not anergic, are transferred to polyclonal recipients expressing sHEL autoantigen the HEL-specific B cells are immediately excluded from follicular entry and, in the absence of T cell help,

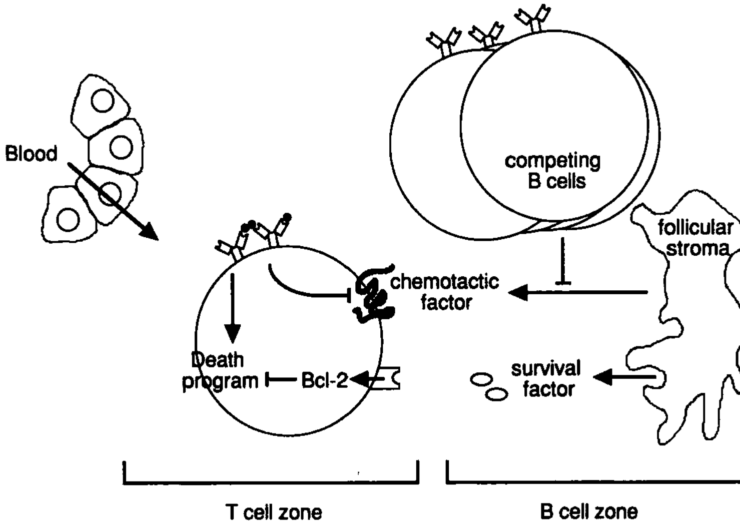

FIG. 5. A hypothesis to explain competitive elimination of self-reactive B cells by follicular exclusion. B and T cells enter secondary lymphoid tissue along a common route, via the high-endothelial venules in the case of lymph nodes and Peyer's patches or via the marginal sinus and terminal arteriolar sheaths in the spleen. After migrating for approximately 3 hr through the outer T cell zone, B cells selectively move into the B cell zones of primary follicles (and the equivalent mantle zones of secondary follicles). The model speculates that B cells move away from the T cell zone in response to a chemotactic gradient originating in the follicular stroma or in the marginal zone/ subcapsular sinus. The chemotactic gradient is reduced when follicles are saturated with competing naive B cells, either because abundant receptors on these cells consume the factor or because the cells reduce its production. Sufficient latitude in chemotactic responsiveness exists, however, so that the change in gradient brought about by follicular crowding has no effect on follicular entry of naive B cells. When B cells bind antigen, however, a signaling pathway is activated that depresses responsiveness to the chemotactic gradient, for example, by decreasing the numbers of receptors. The combination of decreased chemotactic ligand due to follicular crowding and depressed responsiveness together becomes enough to selectively stop antigen-binding cells in the T cell zone. In the case of self-reactive B cells, death follows within 1 or 2 days because (a) the B cells are unable to come in contact with follicular stromal cells, which may produce survival factors for mature B cells; (b) continued antigen receptor signaling due to autoantigen binding tends to activate cell death, which is now unopposed by follicular survival factors; and (c) the trapped B cells are unable to form productive interactions with helper T cells due to checkpoints 3–6.

die within 1 or 2 days in the T cell zone (J. Cyster and C. Goodnow, manuscript in preparation). Moreover, as described under Section III,F,2, antigen-primed germinal center B cells and memory B cells

also move rapidly to the T cell zone when they are exposed to sHEL antigen (Shokat and Goodnow, 1995). Similarly, hapten-primed memory B cells in the marginal zone move rapidly to the T cell zone when they are restimulated with the relevant hapten (Liu *et al.*, 1988,1991). Collectively, these findings imply that antigen receptor engagement and signaling on B cells transiently changes their tropism, increasing the attraction for the T cell zone and/or decreasing attraction for the B cell zones. The change in tropism clearly helps solve the problem of how antigen-specific B and T cells find one another during responses to foreign immunogens. The Ig signaling pathway that brings about this change in cell tropism and how it is triggered despite depression of the tyrosine kinase cascade in anergic B cells are important issues for the future.

4. *Relationship between Follicular Entry and Survival*

In the absence of competition in monoclonal Hc + Lc transgenic animals, autoreactive B cells that are continually exposed to sHEL antigen enter follicles and survive in a manner indistinguishable from that of nonautoreactive B cells over the course of 5 days (Mason *et al.*, 1992; Cyster *et al.*, 1994). Similarly, BrdU-labeling studies in monoclonal Hc + Lc transgenic animals show that at least 40% of autoreactive B cells live for at least 2 weeks; this figure is a minor reduction from the average life span of nonautoreactive cells of 3 or 4 weeks (J. Cyster and C. Goodnow, manuscript in preparation). When competing B cells are deliberately added by constructing mixed chimeras, however, the autoreactive B cells are excluded from follicles and BrdU-labeling reveals that their life span is shortened to less than 3 days (Cyster *et al.*, 1994; J. Cyster and C. Goodnow, manuscript in preparation).

Competition for follicular entry is likely to explain the short life span and dramatic reduction in autoreactive B cell numbers observed by Fulcher and Basten (1994). They studied 6–9 month old Hc + Lc trangenic mice of an F1 hybrid strain background instead of the 2–4 month old C57BL/6 strain Hc + Lc transgenic mice used in our earlier studies (Goodnow *et al.*, 1988; Mason *et al.*, 1992; Cyster *et al.*, 1994). As mentioned under Section II,B, suppression of endogenous Ig gene rearrangement is never complete in Hc + Lc transgenic mice, and endogenous receptor-bearing B cells which do not bind lysozyme preferentially accumulate to large numbers and outcompete autoreactive HEL-binding B cells in old mice (Mason *et al.*, 1992; C. Goodnow, unpublished data). Accumulation of nonautoreactive endogenous

receptor-bearing B cells is also greatly accelerated in F1 hybrid strain backgrounds such as that used by Fulcher and Basten (1994; C. Goodnow, unpublished data).

The striking correlation between follicular entry and B cell survival, both in the transgenic experiments (Cyster *et al.*, 1994; J. Cyster and C. Goodnow, manuscript in preparation) and in studies of B cell attrition in normal repertoires (Lortran *et al.*, 1987; Chan and MacLennan, 1993), indicates that these niches may be essential for maintaining the long life span of circulating preimmune B cells. B cells in primary follicles are in intimate contact with a stroma formed by dendritic projections of follicular dendritic cells (FDC; Nossal and Ada, 1971; Veerman and van Ewijk, 1975; van Ewijk and Brons, 1976; Mandel *et al.*, 1980). By analogy with the importance of intimate contacts between pre-B cells and bone marrow stromal cells (Dorshkind and Witte, 1987; Hayashi *et al.*, 1990; Kincade, 1991), it is therefore tempting to speculate that follicular dendritic cells also produce trophic factors that are essential for maintaining B cell survival. Several other observations are also consistent with this hypothesis: (1) when B cells are removed from primary follicles and placed in standard suspension cultures (which usually lack FDC because of their fragility) most die by apoptosis within 24–48 hr, despite their average life span of 4 weeks *in vivo* (Illera *et al.*, 1993); (2) the death of follicular B cells in suspension culture is dramatically suppressed if Bcl-2 protein is constitutively produced from a transgene controlled by an Ig enhancer (McDonnell *et al.*, 1989; Strasser *et al.*, 1991); (3) the endogenous bcl-2 protein is highly expressed in follicular B cells (Pezzella *et al.*, 1990; Merino *et al.*, 1994) and is important for the longevity of these cells *in vivo* (Veis *et al.*, 1993; Nakayama *et al.*, 1993).

Collectively, these findings are consistent with the hypothesis that trophic factors from FDC are bound by preimmune B cells during the period in each recirculation cycle when they reside in primary follicles. Binding induces sufficient expression of bcl-2 to maintain the B cell's survival for the next cycle of migration through lymph and blood. When placed in suspension culture or trapped in the T cell zone by competition, failure to bind trophic factor and reinduce bcl-2 leads to apoptosis within 24–48 hr. In each case, the need for extrinsic trophic factor can be supplanted to varying extents by constitutive bcl-2 expression (McDonnell *et al.*, 1989; Strasser *et al.*, 1991; Cyster *et al.*, 1994).

In addition to death by "default" due to withdrawal from follicular trophic factors, death of excluded autoreactive B cells may also be actively promoted by Ig signaling. For example, when B cells are removed from follicles and placed in suspension culture, the rapid

default cell death that ensues, presumably due to trophic factor withdrawal, can be further accelerated by cross-linking receptors on the B cells with anti-Ig antibody (Parker, 1980; Brown *et al.*, 1992; Parry *et al.*, 1994b). Exposure of tolerant Hc + Lc transgenic cells to sHEL in suspension culture has a similar effect (C. Goodnow, unpublished data). By contrast, *in vivo* exposure of follicular B cells to anti-Ig antibodies triggers little death of the cells during 1–3 days (Goroff *et al.*, 1986), yet a similar maneuvre triggers very rapid apoptosis in B cells that are not in follicles but reside in the peritoneal cavity (Murakami *et al.*, 1992). Thus, a similar situation to that described for bone marrow B cells may exist for B cells in secondary lymphoid tissues, where the ability of antigen receptor engagement to trigger B cell death and elimination will depend on the interplay between the strength of Ig signaling promoting death and survival signals from the microenvironment that opposes death (Figs. 2 and 3).

While the existence and nature of follicular survival signals remain to be determined, an intracellular molecule that appears to assist B cell survival is the cytosolic Bruton's tyrosine kinase (Btk). This enzyme is the target of mutations leading to severe B cell deficiency in humans with Bruton's-type X-linked aggammaglobulinemia (XLA; Tsukada *et al.*, 1993; Vetrie *et al.*, 1993) and a milder deficiency of circulating B cells in mice with X-linked immunodeficiency (*xid;* Rawlings *et al.*, 1993; Thomas *et al.*, 1993). In XLA, most B cells appear not to survive beyond the large pre-B cell stage of development (Tedder *et al.*, 1985; Conley, 1985; Compana *et al.*, 1990). While the precise stage at which attrition occurs requires further study, it appears to coincide with the stage of B cell development when engagement of the pre-B cell receptor in concert with stromal survival signals normally triggers proliferation and survival (see Section II,D,3 and Fig. 2). In murine *xid*, production and export of B cells from the bone marrow appear normal, but the number of IgD^{high} cells is markedly reduced and the remaining cells carry higher levels of cell surface IgM equivalent to those found normally only on a minority of follicular-type IgD^{high} B cells (Scher *et al.*, 1980; Scher, 1982; Scher *et al.*, 1983; Hardy *et al.*, 1983). Interestingly, B cells carrying the *xid* defect are dramatically outcompeted from the circulating repertoire by otherwise identical B cells carrying wild-type btk (Sprent and Bruce, 1984).

Cooke *et al.* have explored the effect of the *xid* mutation on development and peripheral retention of lysozyme-specific B cells in Hc + Lc transgenic mice (M. Cooke *et al.*, manuscript in preparation). The numbers of immature B cells in the bone marow and immature recent bone marrow emigrants in the spleen were unchanged in Hc +

Lc transgenic mice carrying the *xid* mutation. Lysozyme-binding follicular-type cells and marginal zone B cells were also produced, but their numbers were reduced approximately 5- to 10-fold. Moreover, when the *xid* defect was present in autoreactive cells that were exposed to sHEL autoantigen but lacked competition, very few follicular-type B cells remained. In the presence or absence of sHEL autoantigen, the deficiency in follicular B cell survival caused by *xid* could be entirely corrected by constitutive expression of a Bcl-2 transgene. Since autoreactive B cells in the monoclonal mice normally appear to have a subtle reduction in their numbers and life span, the added defect in peripheral B cell survival contributed by the *xid* mutation in btk appears to completely abolish survival. These findings suggest that autoantigen binding and *xid* interfere with peripheral B cell survival at independent, additive points in a trophic pathway that may be upstream of bcl-2 (Figs. 2 and 3).

5. *Ramifications of Censoring Self-Reactive Cells by Competitive Exclusion*

A key distinction between clone censoring by follicular exclusion and censoring by elimination in the bone marrow or by induction of anergy is that the latter are cell autonomous processes. For example, B cell elimination in the bone marrow depends on a threshold of intracellular signaling determined by the binding characteristics of the cell's receptor and is unaffected by the binding characteristics of other neighboring B cells or the number of B cells produced. By contrast, the efficiency of B cell censoring by follicular exclusion depends on the presence of an excess of competing B cells relative to the number of follicular niches. Selection pressure at checkpoint 2 will be relaxed if production of new B cells in the bone marrow is less than the peripheral capacity, as occurs in neonates, after irradiation, due to inherited immunodeficiencies such as XLA or XSCID (Ochs and Wedgwood, 1989), as a result of stress and glucocorticoid elevation (Garvy *et al.*, 1993; Merino *et al.*, 1994), or during pregnancy (Medina *et al.*, 1993). Children with XLA or XSCID-associated deficiencies in B cell production have a paradoxically high frequency of B-cell-mediated autoantibody diseases such as autoimmune hemolytic anemia (Ochs and Wedgwood, 1989). Failure to purge autoreactive B cells from the circulating preimmune repertoire by competitive exclusion may account for the high rate of autoimmunity in these individuals. Similarly, SCID mice grafted with B cell precursors fail to normalize their B cell repertoire to minimize the use of J-proximal V_H regions, which may tend to be autoreactive, if the total number of circulating

B cells remains low (Denis *et al.*, 1989; Malynn *et al.*, 1990). By contrast, when grafting is done in a way that normal numbers of peripheral B cells are produced, the J-proximal V regions appear to be excluded in favor of 5′ V elements (Denis *et al.*, 1989). Similar connections between inherited or induced T cell deficiency and T-cell-mediated autoimmunity have been found repeatedly (Penhale *et al.*, 1975,1976; Kojima *et al.*, 1976; Rose *et al.*, 1981; Greiner *et al.*, 1987; Fowell *et al.*, 1991; Fowell and Mason, 1993; Jacob *et al.*, 1992; Alderuccio *et al.*, 1993), suggesting that T cell homeostasis may also depend on competition for some type of limiting niche.

III. Checkpoints During Formation Of The Immune Repertoire

Section II summarized data identifying two key steps that markedly reduce the frequency of autoreactive B cells in the preimmune repertoire recirculating among secondary lymphoid organs. Because antibody-secreting clones are most likely to be drawn from this pool following infection, prior censoring of autoreactive cells at these steps is likely to make a major contribution to preventing autoantibody production. Elimination of B cells in the bone marrow nevertheless appears to censor only the most dangerous clones, namely those that have an appreciable affinity for abundant antigens on the surface of hemopoietic cells and other cell types exposed to the blood. Many of the remaining clones that recognize systemic extracellular autoantigens may be preened from the preimmune repertoire by follicular exclusion, at least in young healthy individuals in which a large excess of B cells competes for niches in the peripheral repertoire. Censoring by follicular exclusion is nevertheless vulnerable to breakdown if B cells are produced in numbers insufficient to create stringent competition (discussed previously). Moreover, autoreactive B cells in the process of being eliminated by follicular exclusion are concentrated in the T cell zone, increasing the risk that they interact with T cells and become triggered into multiplication and autoantibody production. The following sections review evidence that an additional series of checkpoints acts during the formation of an immune repertoire to further minimize the risk of autoantibody production.

A. Checkpoint 3: Lack of T Cell Help

Since antibody responses to many antigens depend on the interaction between B cells and helper T cells, elimination or inactivation of autoantigen-specific helper T cells is likely to be a major bulwark to prevent activation of autoreactive B cells. Autoreactive T cells are censored during the immature $CD4^+CD8^+$ double-positive stage of

development in the thymus in a cell autonomous manner resembling the elimination of autoreactive B cells in the bone marrow (Kappler *et al.*, 1987,1988; MacDonald *et al.*, 1988; Sha *et al.*, 1988; Kisielow *et al.*, 1988; von Boehmer, 1992). For $CD4^+$ helper T cells, much of the evidence for thymic censoring of autoreactive cells comes from studies of self-superantigens encoded by vertically transmitted endogenous mouse mammary tumor proviruses (Kappler *et al.*, 1987,1988; MacDonald *et al.*, 1988). With the use of transgenic mice carrying rearranged TCRα and -β chain genes, comparable elimination of $CD4^+$ T cells has also been shown to occur for T cells that recognize self-I-A^s alloantigens (Vasquez *et al.*, 1992), an Ig light-chain idiotype (Bogen *et al.*, 1993), sHEL, or mHEL (S. Akkaraju, B. Ho, M. Davis, and C. Goodnow, manuscript in preparation), and C5a complement protein (Stockinger and Zal, 1994). In the case of sHEL and C5a, thymic censoring appears incomplete, since cells with somewhat reduced levels of the transgenic TCRα chain are still exported to the periphery in sHEL transgenic mice and small numbers of C5a-reactive T cells can be detected in the periphery of C5a-bearing animals. Injection of large quantities of soluble foreign antigen or peptide into $CD4^+$ TCR transgenic mice triggers a wave of apoptosis in thymic $CD4^+8^+$ cells which may reproduce a normal censoring step for autoantigen-reactive T cells (Murphy *et al.*, 1990; Singer and Abbas, 1994). Since this maneuvre also activates a huge number of mature peripheral T cells, it has yet to be established that the death of cortical thymocytes under these conditions is not secondary to glucocorticoid release.

On the other hand, a considerable number of TCR transgenic experiments have revealed no thymic censoring of $CD4^+$ autoreactive T cells, including cells that recognized a pancreatic islet antigen (Katz *et al.*, 1993), myelin basic protein (MBP; Goverman *et al.*, 1993; Lafaille *et al.*, 1994), immunoglobulin IgG_{2a} (P. Ricciardi-Castignoli, personal communication), and influenza hemagglutinin under the control of the rat insulin promoter (RIP–HA; Scott *et al.*, 1994). In the latter three examples, lack of thymic censoring may not be explained simply by lack of antigen in the thymus, since MBP is transcribed in the thymus (Mathiesen *et al.*, 1993; Pribyl *et al.*, 1993), the RIP–HA transgene is expressed sufficiently in the thymus to trigger deletion of $CD8^+$ T cells (R. Liblau and L. Sherman, personal communication), and IgG_{2a} is a serum protein that would be expected to have access to the thymus. These findings, together with the limited censoring for sHEL and C5a, may indicate that the threshold for $CD4^+$ T cell censoring in the thymus is set so that only a fraction of the most strongly autoreactive cells are eliminated at this step, much as occurs for B

cells in the bone marrow (see Sections II,C–II,E). It is thus unclear at this stage whether thymic censoring of helper T cells plays the dominant role in maintaining tolerance or whether peripheral regulatory phenomena such as anergy (Schwartz, 1990; Schonrich *et al.*, 1991; Arnold *et al.*, 1993), antagonism by positively selecting ligands (Jameson *et al.*, 1994), some form of suppression (Powrie and Mason, 1990; Fowell *et al.*, 1991; Fowell and Mason, 1993), or possibly competitive exclusion (Cyster *et al.*, 1994) might control a larger fraction of autoreactive helper T cells.

It has been argued that tolerance in the helper T cell compartment should be entirely sufficient to prevent autoantibody production, rendering superfluous any censoring of the B cell repertoire (Mitchison, 1992). Three observations are inconsistent with this notion. (1) The same line of reasoning also leads to the conclusion that censoring of the CD8 T cell compartment should be equally superfluous, since antibody and cytotoxic responses both depend to a considerable extent on T cell help. Paradoxically, most of the evidence for stringent repertoire censoring (discussed previously) comes from $CD8^+$ cells rather than $CD4^+$ cells. (2) Antibody and cytotoxic cell responses to pathogens show varying degrees of dependence on specific cognate interactions with helper T cells. Many protective antibacterial antibody responses are helper T cell-independent as are certain antiviral T cell responses. (3) Cross-reactions or complexes between self- and foreign antigens circumvent helper T cell tolerance and can direct antiforeign T cell help to autoreactive B cells (Weigle, 1973b,1980; Goodnow *et al.*, 1989b). Given the current uncertainty about the cellular basis and extent of helper T cell tolerance, one must remain circumspect at present about the relative importance of censoring regulatory helper cells or effectors such as B cells and cytotoxic T cells. The least error-prone strategy would seem to be one that censored or regulated cells at many levels.

B. Checkpoints 4 and 5: Long-Lasting Biochemical Changes in B Cell Responsiveness to Antigen (Anergy)

In monoclonal Hc + Lc transgenic mice, autoreactive B cells that recognize sHEL (Goodnow *et al.*, 1988; Cyster *et al.*, 1994), ssDNA (Erikson *et al.*, 1991), or dsDNA in one case (Tsao *et al.*, 1993) are not eliminated from the circulating preimmune repertoire by cell autonomous developmental arrest and death in the bone marrow nor by competitive exclusion in the periphery. Autoantibody secretion by the circulating autoreactive B cells is nevertheless efficiently prevented in each of these models. Cessation of antibody secretion in the pres-

ence of sHEL autoantigen is not due to the absence of T cell help, because (a) IgM antibody-secreting plasma cells are always present in the spleen of Hc + Lc littermate controls lacking sHEL antigen (Goodnow *et al.*, 1988), and (b) the latter are formed by a T cell-independent route that still occurs in Hc + Lc transgenic mice carrying a targeted disruption of the RAG2 genes (F. Young, F. Alt, and C. Goodnow, unpublished data). Moreover, when T cell help is provided in the form of carrier-specific T cells (Goodnow *et al.*, 1988; Adams *et al.*, 1990), alloreactive T cells (Cooke *et al.*, 1994), or HEL-specific T cells (Rathmell *et al.*, 1995) the autoreactive B cells fail to mount an antibody response in mice that contain sHEL autoantigen. A weak response can be elicited after the tolerant B cells are transferred and stimulated in a mouse that lacks sHEL autoantigen (Goodnow *et al.*, 1988; Adams *et al.*, 1990), and once stimulated in the new nonautoreactive environment they can reacquire responsiveness (Goodnow *et al.*, 1991). The profound resistance of autoreactive B cells recognizing sHEL, ssDNA, or dsDNA to be triggered into multiplication and antibody secretion is consistent with the earlier evidence for B cell anergy from Nossal's group (Nossal and Pike, 1980; Pike *et al.*, 1982,1983). Several molecular pathways are altered in anergic B cells and these are considered separately in Sections III, C,D.

C. Checkpoint 4: Antigen Receptor Modulation

One of the obvious changes that accompanies nondeletional tolerance in the sHEL and ssDNA models is a marked decrease in the number of cell surface IgM antigen receptors (Goodnow *et al.*, 1988; Nguyen *et al.*, 1994). If the Ig transgenes encode only the IgM class of receptors, autoreactive B cells expressing these transgenes carry 5–10% of the normal numbers of antigen receptors (Brink *et al.*, 1992). Receptor deficiency persists for at least 5 days after the cells are removed from further stimulation by antigen (Goodnow *et al.*, 1991) and may thus contribute to poor triggering of autoreactive B cells by T cell-dependent or T independent antigens.

Curiously, when the Hc transgene also contains downstream sequences that allow IgD to be coexpressed by the normal process of differential RNA splicing, IgM is still modulated by 10 to 50-fold on autoreactive B cells but cell surface expression of IgD is relatively unaffected (Goodnow *et al.*, 1988). Because IgM and IgD contain the same V regions and bind HEL with identical affinity (Brink *et al.*, 1992), continued expression of IgD on autoreactive B cells maintains the total number of HEL-binding receptors at 30% of the numbers on

control Hc + Lc transgenic B cells that developed in the absence of sHEL autoantigen. It is unlikely that this small reduction in receptor numbers contributes markedly to the unresponsiveness of autoreactive cells because (a) similar numbers of receptors are present on many B cells in the normal repertoire (Hardy *et al.,* 1983; Goodnow *et al.,* 1989c), and (b) unresponsiveness is not seen in Hc + Lc transgenic B cells that have not been exposed to sHEL but have a similar pattern of IgM and IgD expression, due either to crossing an IgD-only Hc construct with an IgM-only construct (R. Brink, C. Goodnow, and A. Basten, manuscript in preparation) or to deficiency of PTP1C (Cyster and Goodnow, 1995).

Loss of IgM expression on tolerant B cells results from an interesting block in the intracellular transport of nascent receptor complexes (Bell and Goodnow, 1994). Thus, μ- and κ-chains are still synthesized in the endoplasmic reticulum (ER) of autoreactive B cells and assemble with the CD79α and -β chains (Igα, -β; or mb-1 and B29) which play an important role in the transport of IgM to the cell surface (Reth, 1992). CD79α and -β chains are synthesized in normal amounts in autoreactive B cells, but no transport of the assembled IgM receptor complexes to the medial Golgi can be detected and the receptor complex appears to be retained and degraded in the endoplasmic reticulum. Intracellular transport of MHC class II molecules nevertheless occurs normally in the autoreactive B cells, indicating that the IgM transport block is selective. IgD was also assembled with CD79α and -β chains in tolerant B cells, but transport of this receptor class to the Golgi and on to the cell surface still occurred, although at a slower rate than that in nonautoreactive HEL-specific cells.

It is not known how intracellular transport of IgM receptor complexes is selectively blocked out of all the cell surface proteins synthesized by autoreactive B cells. One hypothesis relates to the observation that ionomycin-sensitive stores of intracellular calcium are markedly depleted in autoreactive B cells from the sHEL/Hc + Lc transgenic animals (M. Cooke, R. Dolmetsch, R. Lewis, and C. Goodnow, unpublished data). Much of this intracellular calcium store appears normally to reside in the endoplasmic reticulum, possibly in the form a calcium/protein gel with the many resident chaperone proteins (reviewed by Sambrook, 1990). Calcium concentrations regulate chaperone activity *in vitro,* and retention or transport of different secretory proteins from the endoplasmic reticulum is altered by loss of function mutations in the calcium pump that concentrates calcium in this organelle (Rudolph *et al.,* 1989). Because proteins differ in their dependence on chaperone function and rate of transport from the endoplasmic reticulum, a subtle

quantitative decrease in chaperone activity can leave transport of many proteins unaffected, while completely preventing transport of particular proteins (Sambrook, 1990). IgM receptors may represent a protein complex that is particularly sensitive to changes in chaperone function, such that its transport is completely blocked when ER calcium is lowered by chronic receptor stimulation at the cell surface.

Differential expression of IgD and IgM is not unique to transgenic B cells, since most normal follicular B cells express uniform high amounts of IgD but vary from cell to cell by greater than 50-fold in their expression of cell surface IgM (Hardy *et al.*, 1983). It is possible that these clonal differences in IgM receptor expression reflect differences in the degree to which each cell is continuously binding self-antigen. The ability of IgD to be selectively expressed under these conditions highlights important differences in the cell biology of this receptor class that may be central to its function. Because no differences in the ability of IgM or IgD to activate downstream signaling pathways have been identified to date, the differences in cell responses after stimulation through IgM or IgD that have been detected in some instances (Scott, 1993; Carsetti *et al.*, 1993) might instead be connected to the striking differences in intracellular trafficking of the two receptor subtypes.

D. Checkpoint 5: Desensitization of Antigen Receptor Signaling

Despite the continued expression of IgD as a complex with CD79α and β on autoreactive B cells in Hc + Lc transgenic mice, receptor signaling is greatly depressed. Thus, when the autoreactive B cells are exposed to sHEL antigen *in vitro* no tyrosine phosphorylation of the CD79α and -β chains or *syk* kinase can be detected, and little or no increase in intracellular calcium follows (Cooke *et al.*, 1994). Some increase in tyrosine phosphorylation of *lyn* kinase may occur, suggesting that signaling is interrupted at the level of CD79 phosphorylation and recruitment of *syk*. Activation of the tyrosine kinase cascade appears depressed rather than absolutely blocked because moderate phosphorylation of CD79, *syk*, and other cellular proteins occurs if the IgD antigen receptors are clustered extensively using polyclonal anti-IgD antisera (Cooke *et al.*, 1994). Even in these circumstances the level of receptor activation appears somewhat lower, and the elevation of intracellular calcium that is triggered is short-lived compared to that of nontolerant B cells that express the same density of cell surface IgD. Importantly, receptor signaling remains depressed even

after the autoreactive cells have been parked for 36 hr in nontransgenic mice that lack sHEL antigen (Cooke *et al.*, 1994).

The biochemical basis for receptor desensitization on these cells is not currently known. Because PTP1C acts as a negative regulator of proximal Ig signaling in B cells (Cyster and Goodnow, 1995), one attractive hypothesis is that the activity of this tyrosine phosphatase is somehow increased in the vicinity of the antigen receptors on tolerant cells, allowing it to oppose the actions of receptor-associated tyrosine kinases and prevent full activation of the cascade. Interestingly, the receptors on tolerant cells remain fully competent to activate the MAP kinase/RSK signaling pathway to the nucleus (J. Healy and C. Goodnow, manuscript in preparation). This finding indicates that continuous partial activation of the receptors is occurring in autoreactive B cells, sufficient to activate only a subset of the normal spectrum of nuclear events. How such partial activation may contribute to induction or maintainence of depressed B cell responsiveness will be an important issue for future study.

1. Depression of Costimulatory Function

One of the early downstream consequences of antigen binding and Ig signaling in nontolerant B cells is the appearance of CD86 (B70/B7.2) on the cell surface within 4 hr (Azuma *et al.*, 1993; Freeman *et al.*, 1993a,b,c; Hathcock *et al.*, 1993; Lenschow *et al.*, 1993; Boussiotis *et al.*, 1993; Razi-Wolf *et al.*, 1993; Wu *et al.*, 1993b). This costimulatory molecule is one of two ligands for CD28 on resting T cells, delivering a signal into T cells that strongly synergizes with TCR signaling to induce lymphokine synthesis (reviewed by Linsley and Ledbetter, 1993), and expression of CD40 ligand (Klaus *et al.*, 1994). By contrast, as a result of the proximal block in Ig signaling in tolerant B cells little CD86 is induced by binding sHEL antigen *in vitro*, and none can be detected despite chronic sHEL exposure *in vivo* (Cooke *et al.*, 1994; M. Cooke, J. Cyster, and C. Goodnow, unpublished data). The poor induction of CD86 costimulatory molecules after binding antigen, together with the poor induction of other costimulatory molecules, such as ICAM-1 (Cooke *et al.*, 1994) and others that may yet be identified, renders tolerant B cells unable to trigger lymphokine secretion by HEL-specific resting T cells from TCR transgenic mice (Ho *et al.*, 1994). Thus, presentation of HEL peptides by the tolerant B cells occurs normally and elicits TCR-dependent responses that do not require CD28 costimulation (Ho *et al.*, 1994; Kanost and McCluskey, 1994; Eris *et al.*, 1994). Presentation of HEL autoantigen is sufficient

to trigger lymphokine production from resting HEL-specific T cells if the lack of costimulation is corrected by addition of anti-CD28 antibodies (Ho *et al.*, 1994). Like the proximal desensitization of Ig signaling, depressed induction of CD86 is not an absolute defect because extensive clustering of Ig receptors by anti-IgD antibodies or membrane HEL induces normal expression of CD86 on tolerant B cells (Cooke *et al.*, 1994).

The poor induction of costimulatory function in tolerant autoreactive B cells may play an important part in their inability to be triggered by T cell-dependent antigens (Cooke *et al.*, 1994). Blocking CD86 and the related CD80 (B7.1) molecule with soluble CTLA-4 Ig fusion protein or loss of CD28 receptor due to targeted mutation in both cases interferes with primary antibody responses to T-dependent antigens *in vivo* (Damle *et al.*, 1991; Linsley *et al.*, 1991,1992; Shahinian *et al.*, 1993; Ronchese *et al.*, 1994; Lane *et al.*, 1994). Collectively, these findings are consistent with a model for antigen-specific T cell–B cell interactions that proposes that delivery of T cell help requires recognition not only of peptide/MHC complexes presented by the B cell but also of costimulatory molecules induced by antigen binding to the B cells antigen receptor (Cooke *et al.*, 1994). Presentation of self-peptides by tolerant B cells, in the absence of CD86 costimulatory molecules, may potentially buttress tolerance in the helper T cell repertoire by inactivating autoreactive T cells (Eynon and Parker, 1992; Fuchs and Matzinger, 1992; Gilbert and Weigle, 1992; Eynon and Parker, 1993; Morris *et al.*, 1994).

2. *Block in Mitogenic Signaling by Ig*

In addition to inducing CD86, a later effect of B cell antigen receptor cross-linking is the delivery of intracellular signals promoting B cell transition from G_0 to S-phase of cell cycle (Sell and Gell, 1965; Sieckmann, 1980; Parker, 1980; DeFranco *et al.*, 1982; Howard and Paul, 1983; Melchers and Andersson, 1984; DeFranco, 1987; Cambier and Ransom, 1987). Mitogenic responses to Ig signaling frequently depend on additional complementary signals that can be provided by interleukin 4, which is not itself mitogenic for G_0 B cells (Howard and Paul, 1983; Paul and Ohara, 1987), by submitogenic concentrations of lipopolysaccharide (DeFranco *et al.*, 1982; DeFranco, 1987), or by suboptimal stimulation through the CD40 molecule (Banchereau *et al.*, 1994). Certain polymeric antigens with a critical number and spacing of antigenic epitopes, such as pneumococcal polysaccharide (Howard and Mitchison, 1975), haptenated dextran or acrylamide polymers (Dintzis *et al.*, 1983,1989), or anti-Ig antibodies coupled to polymeric dextran (Brunswick *et al.*, 1988), are potently mitogenic for B cells that specifi-

cally bind them in the relative absence of LPS or T cell-derived signals. Mitogenesis by these T cell-independent type-2 antigens is thought to result from their ability to stably cluster 20 or more antigen receptors, creating a signaling unit with sufficient potency or duration to drive entry into S-phase on its own (Dintzis *et al.*, 1983). The potency of these antigens may or may not also be assisted by their ability to cocluster other receptors on the B cells, for example, by fixing complement and becoming decorated with C3d (Carter *et al.*, 1988; Hebell *et al.*, 1991). The intracellular pathways that mediate the mitogenic effects of Ig receptor clustering are not yet clearly understood (Cambier *et al.*, 1994; Gold and DeFranco, 1994).

Mitogenic responses cannot be elicited by any degree of antigen receptor cross-linking in anergic B cells from sHEL-expressing Hc + Lc transgenic mice (Cooke *et al.*, 1994) and in ssDNA-reactive B cells (Nguyen *et al.*, 1994). The complete block in mitogenic signaling differs from proximal signaling events and induction of CD86 since the latter are depressed but can be elicited when antigen receptors are extensively clustered by polyclonal anti-IgD antisera (Cooke *et al.*, 1994; see above). For example, anti-IgD antibodies coupled to polymeric dextran trigger no proliferation in tolerant Hc + Lc B cells from mice expressing sHEL, despite being extraordinarily mitogenic for nontolerant B cells from Hc + Lc transgenic control animals (M. Cooke and C. Goodnow, manuscript in preparation). The poor mitogenesis cannot be explained by differences in receptor density between the two cell types since tolerant and nontolerant B cells express identical levels of cell surface IgD (Goodnow *et al.*, 1988; Bell and Goodnow, 1994). The tolerant B cells nevertheless proliferate normally when stimulated by LPS, through CD40, or by the combination of phorbol esters and ionomycin, indicating that mitogenic signaling by Ig receptors is selectively blocked (Cooke *et al.*, 1994). Furthermore, the normal response to stimulation by phorbol esters and ionomycin implies that the mitogenic signaling block associated with the antigen receptor may be proximal to activation of the *ras*/*raf*/MAP kinase pathway and elevation of intracellular calcium, which are directly triggered by these two compounds. Consistent with a proximal block, when intracellular calcium is elevated by extensive antigen receptor clustering on tolerant B cells, the calcium response remains transient compared to that in nontolerant cells (Cooke *et al.*, 1994). The duration of intracellular calcium elevation appears important for antigen receptor-induced mitogenesis in B cells (Yamada *et al.*, 1993). Thus, the complete absence of a mitogenic response to Ig signaling in tolerant cells may simply be a more stringent indicator of the one proximal signaling block. A

more distal block in the mitogenic signaling pathways from Ig nevertheless cannot be excluded at present.

What is the significance of blocking mitogenic signaling by Ig receptors on autoreactive B cells? Two pathways to antibody production that depend on mitogenic signaling through Ig are B cell responses to T cell-independent antigens and to lymphokines in noncognate interactions with T cells (Howard and Paul, 1983; Melchers and Andersson, 1984; Cambier and Ransom, 1987; DeFranco, 1987). Responses to T cell-dependent antigens, by contrast, may depend markedly on Ig-induced CD86 (Damle *et al.*, 1991; Linsley *et al.*, 1991, 1992; Shahinian *et al.*, 1993; Cooke *et al.*, 1994; Ho *et al.*, 1994; Ronchese *et al.*, 1994; Lane *et al.*, 1994) and other Ig-induced costimulatory molecules, but are thought to be independent of mitogenic signaling by Ig. To test the role of mitogenic signaling, Cooke *et al.* (M. Cooke, W. Ho, M. Davis, and C. Goodnow, manuscript in preparation) made use of the *xid* mutation in the Btk kinase which selectively abolishes the mitogenic response to Ig clustering (Scher, 1982; Thomas *et al.*, 1993; Rawlings *et al.*, 1993). Mature IgD$^+$ lysozyme-specific B cells from Hc + Lc transgenic mice carrying the *xid* defect exhibit normal sHEL antigen-induced proximal signaling and normal induction of CD86. Despite this, the HEL-specific *xid* B cells proliferated 20-fold less efficiently in an *in vivo* cell transfer assay in the presence of sHEL antigen and an equal number of naive HEL-specific T cells from a TCR transgenic mouse. Similarly, when nontransgenic *xid* mice are immunized with a limiting amount of T cell-dependent antigen, while secondary antibody responses are generally normal, the primary antibody response is depressed or absent (Scher, 1982). These findings indicate that Ig receptor-induced mitogenic signaling plays an important although not essential role in T cell-driven antibody responses. Synergism between Ig signals and IL-4 (Howard and Paul, 1983) or suboptimal CD40 stimulation (Banchereau *et al.*, 1994) could explain the role of Ig-dependent mitogenic signaling at this stage of the immune response. The significance of blocking Ig mitogenic signaling in autoreactive B cells is stressed by the ability of the *xid* defect to suppress autoantibody production in the systemic lupus-prone NZB/W and BXSB mouse strains (Theofilopoulos and Dixon, 1985).

E. Checkpoint 6: CD95 (Fas/Apo-1)-Dependent Killing of Autoreactive B Cells by CD4$^+$ T Cells

As mentioned under Section III,B, when T cell help is provided to tolerant follicular B cells from sHEL/Hc + Lc double-transgenic mice, they mount poor antibody responses *in vivo* and undergo little or no blastogenesis or antibody production *in vitro* (Goodnow *et al.*,

1988; Adams *et al.*, 1990; Cooke *et al.*, 1994; Ho *et al.*, 1994). To determine if any signal is transmitted to tolerant autoreactive B cells when they interact with HEL-specific T cells, Rathmell *et al.* tracked their fate after transfer with an equal number of TCR transgenic $CD4^+$ cells *in vivo* (Rathmell *et al.*, 1995). HEL-specific $CD4^+$ T cells triggered B cell proliferation and antibody production when they recognized HEL presented by nontolerant lysozyme-specific B cell controls acutely exposed to sHEL antigen at the time of transfer. By contrast, HEL-specific $CD4^+$ T cells not only failed to promote proliferation or antibody production when they interacted with tolerant B cells presenting HEL antigen, but instead they eliminated the autoreactive B cells. Helper cell-mediated killing was HEL-specific and depended on expression of CD95 (Fas/Apo-1) on the tolerant B cells because tolerant B cells carrying the *lpr* mutation in CD95 were protected from killing. When killing was disrupted by the *lpr* mutation, the $CD4^+$ T cells now activated the tolerant B cells and promoted their proliferation. T cell-driven proliferation and differentiation of nontolerant B cells, on the other hand, was reduced when the B cells were CD95 deficient, indicating that in the context of acutely activated B cells CD95 promoted proliferation instead of death.

These observations identify CD95 as a key switch regulating the outcome of interactions between antigen-specific B and T cells. Studies with $CD4^+$ cells in mixed lymphocyte cultures or with Th1 T cell clones have also recently provoked the hypothesis that Fas-dependent cytotoxicity by $CD4^+$ T cells might be important for regulating B cells (Ju *et al.*, 1994; Vignaux and Golstein, 1994). A model to explain how CD95 mediates death in tolerant B cells but assists proliferation in nontolerant B cells is outlined in Fig. 6. In mature recirculating B cells that have been acutely exposed to antigen, signaling by the Ig receptor is robust and triggers expression of costimulatory molecules on the B cell as well as signals that promote mitosis and perhaps suppress the death program. Expression of costimulatory molecules on the B cell ensures high-level production of CD40L, IL-4, and other cytokines by interacting T cells, and these molecules also promote mitosis and suppress death in the B cell. Thus, in the context of an acutely antigen-activated B cell, the tendency of CD95 to trigger cell death may be fully counterbalanced by concurrent signals that suppress death and promote mitosis. By contrast, in tolerant B cells that have been chronically exposed to self-antigen, Ig signaling is depressed and provides little stimulus to promote mitosis or suppress death, leaving the cell vulnerable to censoring by CD95-based cytotoxicity.

The importance of CD95 for regulating autoreactive B cells has

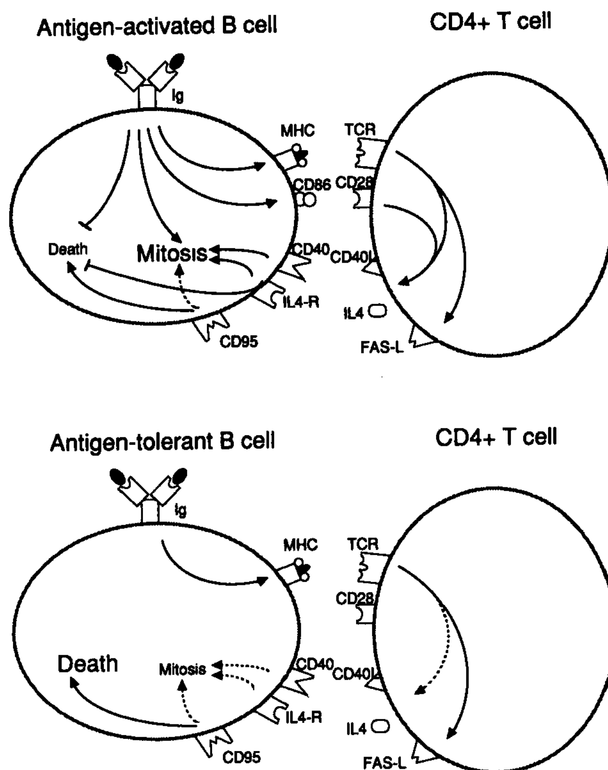

FIG. 6. Model to explain how autoreactive B cells are prevented from receiving T cell help in secondary lymphoid tissues. Mature B cells that suddenly encounter antigen (top) receive an acute activating stimulus from Ig receptor signaling, in addition to internalizing antigen for loading into MHC class II molecules and presentation to the T cell receptor (TCR). Acute Ig signaling in the B cell triggers expression of CD86 (B70/B7.2) as well as a number of other potential costimulatory molecules on the cell surface, and triggers internal changes that promote mitosis and may oppose apoptosis. Costimulation of the T cell through the TCR and CD28 triggers synthesis of CD40 ligand (CD40L) and IL-4 synergistically; these in turn promote B cell mitogenesis through CD40 and IL-4 receptor, in concert with mitogenic signals from Ig. IL-4R signaling, and possibly signaling by CD40, also suppress apoptosis. TCR signaling also triggers expression of CD95 (Fas/Apo-1) ligand. In the context of acutely activated B cells interacting with T cells, where the balance of signaling favors mitosis and opposes apoptosis, CD95 signaling does not trigger death but weakly augments mitosis. In tolerant B cells that have been chronically stimulated with autoantigen (bottom) desensitization of antigen receptor signaling depresses induction of CD86 and blocks transmission of mitogenic signals from Ig receptors. Peptides from self-antigens, or from foreign antigens that cross-react or form complexes with self, are still presented to T cells. In the absence of costimulation, however, CD40L and IL-4 are poorly induced. Combined with the block in Ig signaling, the net effect is little promotion of mitosis and little suppression of apoptosis. T cell production of CD95 ligand still occurs, and in the absence of signals to suppress apoptosis CD95 signaling in turn triggers death. Disruption of CD95 expression on the B cell prevents delivery of the apoptotic signal and allows residual mitogenic signals to activate autoreactive B cells and break anergy.

already been well established in nontransgenic *lpr* mice (Cohen and Eisenberg, 1991; Watanabe-Fukunaga *et al.*, 1992). Three spontaneous mouse mutations affect CD95 function: *lpr*, which reduces CD95 protein expression by at least 90% (Watanabe-Fukunaga *et al.*, 1992; Adachi *et al.*, 1993; Drappa *et al.*, 1993; Wu *et al.*, 1993a); lpr^{cg}, a separate allele of CD95 that disrupts signaling by CD95 (Matsuzawa *et al.*, 1990; Watanabe-Fukunaga *et al.*, 1992); and *gld*, a point mutation in the CD95 ligand that renders it unable to bind and trigger CD95 (Suda *et al.*, 1993; Takahashi *et al.*, 1994; Lynch *et al.*, 1994; Ramsdell *et al.*, 1994b). Each of these mutations results in gradual development of a systemic lupus-like syndrome of autoantibodies to systemic autoantigens, such as DNA, although the onset, severity, and spectrum of autoantibodies varies depending on modifier genes at other loci (Theofilopoulos and Dixon, 1985; Cohen and Eisenberg, 1991; Watson *et al.*, 1992). Autoantibody production in mixed *lpr*/wild-type chimeras only occurs from B cells that lack CD95 (Sobel *et al.*, 1991; Nemazee *et al.*, 1991a; Katagiri *et al.*, 1991), consistent with the necessity for CD95 in censoring autoreactive B cells identified by Rathmell *et al.* (1995). Importantly, the *lpr* mutation in CD95 perturbs censoring of autoreactive B cells only by this unique T cell-dependent process because *lpr* has no effect on autoreactive B cell elimination by developmental arrest in the bone marrow, by follicular exclusion in the periphery, or on the induction of anergy (Rathmell and Goodnow, 1994; J. Rathmell, J. Cyster, C. Goodnow, unpublished data).

Autoantibody production in *lpr* mice also depends on $CD4^+$ MHC class II-restricted T cells (Steinberg *et al.*, 1980; Wofsy *et al.*, 1982, 1985; Hang *et al.*, 1984; Mosbach-Ozmen *et al.*, 1986; Santoro *et al.*, 1988; Gilkeson *et al.*, 1992; Jabs *et al.*, 1992; Jevnikar *et al.*, 1994), and the T cells also need to carry the *lpr* gene defect (Sobel *et al.*, 1993). Thymic censoring of autoreactive $CD8^+$ T cells and self-superantigen-reactive $CD4^+$ T cells nevertheless appears relatively normal in *lpr* animals (Kotzin *et al.*, 1988; Singer *et al.*, 1989; Sidman *et al.*, 1992), except in some cases (Matsumoto *et al.*, 1991; Zhou *et al.*, 1992). The effect of CD95 deficiency has yet to be examined for censoring $CD4^+$ T cells that recognize self-peptides in the context of MHC class II molecules. It is important to note that some functional CD95 apparently remains in *lpr* thymocytes (Ju *et al.*, 1994), and this may be sufficient to trigger elimination of some autoreactive clones but not others. When high doses of cytochrome c antigen are injected into *lpr*-strain TCR transgenic mice expressing a cytochrome c-specific class II-restricted receptor, thymic elimination occurs normally (Singer and Abbas, 1994), consistent with the lack of an absolute defect in thymic

censoring. In the same experiments, however, dissappearance of peripheral T cells after initial activation and expansion is blocked by the *lpr* mutation (Singer and Abbas, 1994), consistent with earlier *in vitro* evidence that CD95 plays a role in the death of overstimulated mature T cells (Russell *et al.*, 1993; Bossu *et al.*, 1993; Gillette-Ferguson and Sidman, 1994; Ramsdell *et al.*, 1994a).

Taken together, these findings suggest that autoantibody production in *lpr* mice results from errors at two checkpoints. First, some autoreactive $CD4^+$ T cells escape censoring either in the thymus or after reaching the periphery because they lack sufficient CD95. Second, these unchecked autoreactive helper T cells encounter autoreactive B cells presenting autoantigens in secondary lymphoid tissues, most likely B cells that have been concentrated in the T cell zones by follicular exclusion. In normal circumstances, the escape of a few autoreactive T cells would simply reinforce tolerance through CD95-dependent killing when they recognized autoantigens presented by the B cells. When CD95 is lacking on the B cell, however, this checkpoint fails and an inappropriate interaction occurs that triggers the B cell into multiplication. The considerable time lag before autoantibody production ensues in *lpr* mice may reflect the fact that other checkpoints are unaffected by CD95 deficiency, requiring other inherited and/or somatic mutations in rare clones before their full autoaggressive potential can be realized.

F. Checkpoint 7: B Cell Censoring within Germinal Centers and in Memory Cells

Following infection or immunization, clonal expansion of B cells within germinal centers is central to affinity maturation and the development of an anamnestic secondary response (Wakefield and Thorbecke, 1968; Nieuwenhuis and Keuning, 1974; Klaus and Humphrey, 1977; Coico *et al.*, 1983; Kraal *et al.*, 1988; MacLennan, 1994). Germinal centers represent the site at which B cell clones that have successfully negotiated all of the tolerance checkpoints discussed previously undergo somatic hypermutation of the V-region elements of their Ig heavy- and light-chain genes (Jacob *et al.*, 1991b; Berek *et al.*, 1991; Jacob *et al.*, 1993; McHeyzer-Williams *et al.*, 1993). Selection of variant progeny with higher affinity for the immunizing foreign antigen, which underlies affinity maturation of T cell-dependent humoral responses (Siskind and Benacerraf, 1969; Berek and Milstein, 1988; Kocks and Rajewsky, 1989), is also likely to occur within the germinal center (MacLennan, 1994). Positive selection of higher-affinity B cells is thought to be driven by depots of foreign antigen held for long periods

as immune complexes on the follicular dendritic cell meshwork in the apical light zone of the germinal center (Nossal and Ada, 1971; Klaus and Humphrey, 1977; Mandel *et al.*, 1980; Tew *et al.*, 1993). Cells that bind these antigen depots the best or the fastest (Foote and Milstein, 1991) may outcompete other B cells for receiving a survival signal that could be transmitted by (a) the resulting engagement of Ig receptors on the B cell or (b) following subsequent presentation of these antigens to T cells (Liu *et al.*, 1989; MacLennan, 1994). $CD4^+$ memory T cells are present in the apical zone of the germinal center (Gutman and Weissman, 1972; Stein *et al.*, 1980; Berman *et al.*, 1981; Poppema *et al.*, 1981; Stein *et al.*, 1982) and contain a preformed pool of CD40 ligand that can be displayed on their surface within several minutes of their TCR being engaged (MacLennan, 1994). By preferentially capturing antigens from the FDC, high-affinity B cell centrocytes emerging from the proliferating pool of basal dark zone centroblasts in the germinal center may selectively interact with these T cells and receive survival signals via CD40 and perhaps other molecules (Liu *et al.*, 1989; MacLennan, 1994; Gray *et al.*, 1994; Fig. 7).

In addition to promoting affinity maturation to foreign antigens, hypermutation of Ig genes within germinal center B cells can also generate clones with a new reactivity or higher affinity for self-antigens. The first clear example of this risk was illustrated by a single nucleotide change in the Ig genes of the S107 myeloma, converting the antibody from its original specificity for bacterial phosphorylcholine into an autoantibody that bound dsDNA (Diamond and Scharff, 1984). Subsequent analysis of clonally related hybridomas isolated from individual autoimmune mice of MRL–*lpr* or NZB/W strains revealed that ongoing mutation and clonal selection could indeed select progressively higher affinity and more pathogenic anti-dsDNA autoantibodies when allowed to proceed unchecked (Shlomchik *et al.*, 1987a,b,1990; Tillman *et al.*, 1992; Diamond *et al.*, 1992; Radic *et al.*, 1993b; Radic and Weigert, 1994). These observations, therefore, make a strong case for the existence of censoring steps that normally remove self-reactive variants arising after an immune response is under way in germinal centers (Goodnow *et al.*, 1990).

1. *Indirect Evidence for B Cell Censoring after Immunization*

Several experimental observations point to the possibility of B cell censoring during germinal center responses. Linton *et al.* (1991) adoptively transferred T cells primed to hemocyanin antigen together with unprimed B cells into irradiated mice and stimulated them with the hapten DNP coupled to hemocyanin. The conditions used favored

formation of hapten-specific memory B cells and somatically mutated progeny within 11 days *in vivo* (Linton *et al.*, 1989). To model the fate of B cells that mutated to recognize self-antigen, for which T help should be lacking, the responding T and B cells were exposed to DNP hapten on an irrelevant carrier in small fragments of spleen in culture. When the cultured splenic fragments were exposed to hapten on the irrelevant carrier after first being stimulated for 2 days with the immunogenic DNP–hemocyanin, the frequency of cultures producing anti-DNP antibody was reduced three- to fivefold. These findings led to the interpretation that, after activation by T cells, DNP-specific B cells became particularly sensitive to some type of censoring mechanism when their receptors were engaged by hapten in the absence of T cell help. These experiments could not identify whether this "second window" of tolerance corresponded to the extrafollicular or intrafollicular phases of B cell proliferation or to the subsequent formation of plasma cells. It will be important to establish whether or not germinal centers can form in cultured fragments of irradiated spleen, as this system would offer great potential for dissecting germinal center selection steps.

Nossal and colleagues have also explored B cell censoring after an immune response is under way (Karvelas and Nossal, 1991; Nossal *et al.*, 1993). Their approach was to initiate a primary antibody response in unmanipulated mice by immunizing with the hapten NP coupled to HSA in adjuvant. At different times after initiating the response, a high dose of deaggregated NP–HSA or NP on an irrelevant carrier was injected intraperitoneally. When the high dose of antigen was administered as late as 6 days after the immune response was under way, it reduced by 20-fold the frequency of high-affinity NP-specific B cells that could be enumerated in limiting dilution cultures on Day 14 of the response (Nossal *et al.*, 1993). This tolerizing maneuver appeared to block B cell multiplication and affinity maturation in part through effects on the T cells, since a 5- to 10-fold reduction in the production of high-affinity B cells could be achieved with HSA carrier lacking the NP hapten. It was not possible to ascertain whether high-affinity germinal center B cells are censored after they are formed in these experiments, however, because injection of deaggregated NP–HSA after Day 7 of the response when germinal center formation begins led to boosting rather than tolerance.

Dintzis and Dintzis have also generated evidence for some type of efficient B cell censoring after an immune response is well under way (Dintzis and Dintzis, 1992). They initiated a T cell-dependent antibody response to the hapten, FITC, by immunizing with FITC conjugated

to ovalbumin in adjuvant, and boosting with the same material in adjuvant 20 days later. Fifteen days after boosting, when germinal centers and memory B cells would be well established, the animals received FITC coupled to short, nonimmunogenic polymers of dextran by intraperitoneal injection. This form of FITC antigen would be unable to mobilize or recall T cell help because of the chemical nature of the dextran carrier, and the dextran polymers were not long enough to trigger B cells into multiplication by T cell-independent mechanisms (Dintzis *et al.*, 1983,1989). Exposure to this nonimmunogenic FITC conjugate ablated the anti-FITC IgM and IgG antibody response, such that 10- to 30-fold fewer anti-FITC antibody-secreting cells were present in the spleen after continued boosting and treatment with FITC–dextran. While these data provide striking evidence for B cell censoring within the immune repertoire, the stage and cellular mechanism by which antibody-forming cells were ablated could not be determined.

2. *Direct Demonstration of B Cell Elimination in Germinal Centers and Postgerminal Center Stages*

The rarity and heterogeneity of antigen-specific B cells in normal mice creates great difficulties for studying how B cells may be censored in germinal centers or at other stages of B cell clonal expansion and memory formation. To circumvent this problem, Shokat *et al.*, developed a system to track the fate of monoclonal lysozyme-specific B cells from Hc + Lc transgenic mice after seeding them into developing germinal centers in nontransgenic mice immunized with duck egg lysozyme (DEL) (Shokat and Goodnow, 1995). The seeded B cells could be readily enumerated by FACS analysis for a unique $Ly5^a$ marker or visualized histochemically and multiplied remarkably over the following 5 days to account for most of the Ig-bearing centrocytes in the germinal centers of the recipients' spleen.

To model the fate of self-reactive B cell variants that arise in germinal centers, the recipient mice received a large dose of hen egg lysozyme by intravenous injection at the height of the germinal center reaction (Shokat and Goodnow, 1995). HEL did not cross-react appreciably with DEL at the helper T cell level in the recipient B6 mice, but nevertheless bound to Ig receptors on the transferred B cells at least 1000-fold better than DEL. When HEL was given to the mice it therefore shifted the germinal center B cell population to binding an antigen that (a) lacked T cell help and (b) was free in the circulation rather than being displayed on follicular dendritic cells. This sudden change in binding had two effects on lysozyme-specific germinal center B

cells. First, large numbers of apoptotic cells appeared in the germinal center within 4 hr. Second, lysozyme-binding B cells disappeared from the apical zone of the germinal center and from the mantle and marginal zones within 4 hr and became concentrated in the outer T cell zones of the spleen. The relocated cells persisted in the T cell zone until 12 hr when a wave of apoptosis began in this region accompanied by their disappearance. Constitutive expression of a *Bcl*-2 transgene blocked the death and disappearance of lysozyme-specific B cells that had relocated to the T cell zone, but had little effect on the more rapid phase of cell death that took place within the germinal center itself.

Pulendran *et al.*, have made strikingly similar observations of antigen-induced B cell death in germinal centers (Pulendran *et al.*, 1995). They initiated a germinal center immune response to the hapten NP coupled to HSA. To model the binding of self-antigen by variant B cells arising in germinal centers, they injected soluble NP coupled to different carriers at the height of the germinal center response when previous work had established that NP-reactive cells are abundant in these sites. A wave of apoptosis in the germinal centers was triggered by NP coupled to a self-protein carrier, mouse serum albumin, or to the immunizing HSA carrier. Apoptotic cells were detectable within 1 hr and, like the data of Shokat and Goodnow (1995), peak numbers ocurred 4 hr after giving soluble antigen. Interestingly, expression of the *Bcl*-2.22 transgene did impede apoptosis in this model, in contrast to the HEL antigen system (Shokat and Goodnow, 1995). Because HEL antigen bound to receptors on germinal center cells with much higher affinity than NP, it is possible that Bcl-2 was simply insufficient to block HEL-induced death.

These observations suggest two distinct censoring steps for B cells arising in germinal centers (Fig. 7). The wave of cell death triggered by HEL or NP antigens in the germinal center within 4 hr may reflect B cells that were in transition from centroblasts in the basal dark zone into centrocytes in the apical light zone of the germinal center. Centrocytes are constantly and rapidly being replaced with a half-life of 8 hr by progeny of proliferating centroblasts in the dark zone (reviewed by MacLennan, 1994). Short-term survival of these cells appears dependent on antigen receptor cross-linking because when human germinal center cells are placed in culture they rapidly enter apoptosis unless their surface Ig receptors are extensively cross-linked with anti-Ig antibodies immobilized on sheep erythrocytes (Liu *et al.*, 1989). Interestingly, soluble anti-Ig was unable to mediate this effect (Liu *et al.*, 1989). The survival signal transmitted to germinal center

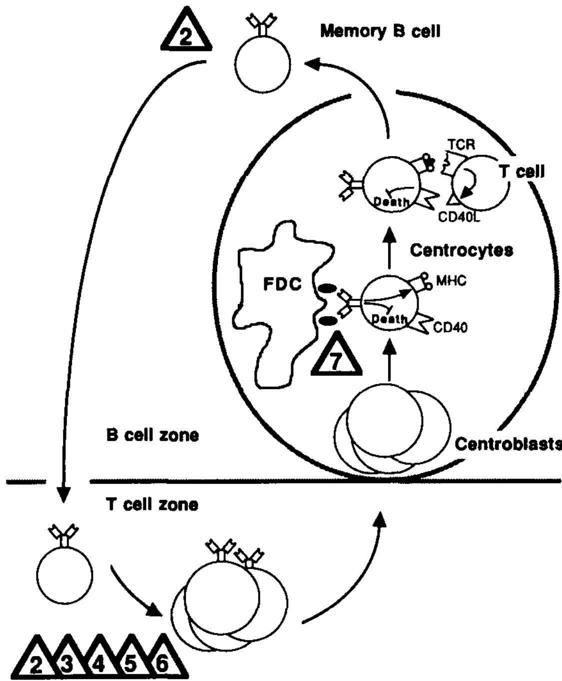

FIG. 7. Model to explain B cell censoring in germinal centers. As discussed in the text, proliferating Ig-negative B centroblasts in the basal dark zone of the germinal center generate centrocyte progeny that reexpress Ig antigen receptors and move into the apical light zone. Immobilized antigen on follicular dendritic cells engages Ig receptors on young centrocytes, possibly transmitting a short-term survival signal. Processing and subsequent presentation of the antigen to T cells in the apical zone triggers CD40L expression on the T cells, which may transmit a longer-term survival signal to the B centrocyte and allow emigration either as memory cells or as plasma cells. If antigen receptors on young centrocytes bind preferentially to self-antigens that are in the germinal center but not on the FDC meshwork, self-antigen binding will compete with binding of foreign antigen on the FDCs and interfere with delivery of short-term survival signals resulting in rapid cell death ⚠7. If a centrocyte binds a self-antigen that is immobilized on the FDCs, the specificity of germinal center T cells for foreign antigens will prevent delivery of long term survival signals and thus also trigger cell death. Memory B cells that cross-react with systemic autoantigens but fail to encounter them within the germinal center may potentially receive survival signals but will subsequently be shifted out of the B cell zones when they bind these autoantigens outside the germinal center due to the effect of Ig signaling on B cell tropism ⚠2. Continued binding of self-antigen traps the cells in the T cell zone, reducing their life span to 1 or 2 days. Rescue of these cells through extrafollicular or germinal center proliferation may be prevented by the absence of T cell help ⚠3, loss of antigen receptors on the autoreactive B cells ⚠4, desensitization of receptor signaling due to chronic receptor binding ⚠5, and CD95-dependent killing ⚠6.

B cells by extensive antigen receptor cross-linking *in vitro* appeared short lived, and survival for 48 hr could only be achieved by clustering CD40 molecules with antibodies (Liu *et al.*, 1989). These observations suggest the model (MacLennan, 1994; Fig. 7) that germinal center B cell survival depends first on binding immobilized antigen on FDC, conferring a transient reprieve from death. Subsequent presentation of internalized antigen to T cells in the apical zone of the germinal center may then deliver a longer-term survival signal via CD40L–CD40 interactions. The rapid germinal center cell death triggered by soluble HEL or NP antigens may thus reflect their ability to occupy antigen receptors on early centrocytes and prevent extensive cross-linking by immobilized antigen on the FDC. In the case of antigen receptor occupancy with the high-affinity HEL antigen (Shokat and Goodnow, 1995), this would also prevent internalization of DEL antigen and subsequent presentation to DEL-specific T cells, thus preventing delivery of longer-term survival signals via CD40. A similar case may apply when NP hapten is delivered on an irrelevant carrier (Pulendran *et al.*, 1995). On the other hand, since germinal center cell death was also induced by NP coupled to the immunizing carrier; the key trigger for censoring B cells at this step may be interference with Ig binding to immobilized antigen on the FDC.

By the same reasoning, the delayed wave of cell death in lysozyme-specific B cells that had relocated from the germinal centers to the T cell zone (Shokat and Goodnow, 1995) may represent older centrocytes that had already received a longer-term survival signal from DEL-specific T cells in the apical light zone before encountering circulating HEL antigen. Rapid movement of antigen-specific B cells to the T cell zone has previously been shown to occur when hapten-primed memory B cells in the marginal zone are restimulated with the same hapten (Liu *et al.*, 1988,1991). Similarly, as discussed with relation to the phenomenon of follicular exclusion under Section II,F, acute antigen receptor engagement appears to trap naive lysozyme-specific B cells in the T cell zone, and cessation of receptor engagement relieves autoreactive B cells from being trapped in this region (Cyster *et al.*, 1994; J. Cyster and C. Goodnow, manuscript in preparation). These findings collectively imply a change in the tropism of B cells brought about by antigen receptor signaling. Thus, when their antigen receptors bind antigen, individual B cells favor the T cell zone rather than the follicular and marginal zones that are favored in the absence of antigen. As discussed under Section II,F, nothing is known about the molecules that determine tropism of T and B cells for different zones in secondary lymphoid tissue.

The change in tropism toward the T cell zone that occurs after antigen binding in centrocytes, memory B cells, and naive B cells obviously helps solve the problem of how antigen-specific T and B cells find one another during responses to foreign antigens. Indeed, when hapten-primed memory B cells are relocated to the T cell zone by injecting a hapten-carrier complex to which T cells have been primed, the relocated B cells rapidly form proliferating foci with CD40L-bearing T cells in the T cell zone (Liu *et al.*, 1988,1991; Van den Eertwegh *et al.*, 1993). Moreover, provision of T cell help to naive lysozyme-specific B cells that have been trapped in the T cell zone rescues them from death and triggers their proliferation and differentiation (J. Cyster and C. Goodnow, manuscript in preparation). The progeny of these restimulated foci then appear either to form plasma cells that migrate to the splenic red pulp or may reenter developing germinal centers (Liu *et al.*, 1988,1991; Van den Eertwegh *et al.*, 1993; Jacob *et al.*, 1991a; Jacob and Kelsoe, 1992; J. Cyster and C. Goodnow, manuscript in preparation). By contrast, the lysozyme-specific centrocytes and memory cells that were relocated to the T cell zone within 4 hr after HEL injection do not proliferate, make antibody, or reenter germinal centers, but die by apoptosis within 24 hr (Shokat and Goodnow, 1995). In this case, primed helper T cells in the T cell zone cannot be recalled by the HEL-binding B cells because HEL does not stimulate the DEL-specific T cells. In the absence of T cell help, death of the relocated B cells is likely to be brought about in the same way as that which occurs for naive B cells that are eliminated by follicular exclusion; namely, physical withdrawal from follicular trophic factors and promotion of cell death by continuous antigen receptor signaling (see Section, II,F).

As summarized in Fig. 7, these findings collectively identify two censoring points during memory B cell formation. Self-reactive B cell variants that arise through hypermutation may be rapidly eliminated if they bind more avidly to a self-antigen that is present in the germinal center than they do to foreign antigens immobilized on FDC. It is conceivable that censoring within germinal centers plays an important role in preventing high-affinity antibodies to dsDNA, for example, since a large amount of cell death normally occurs in germinal centers, and apoptotic cells may display nuclear antigens, such as dsDNA, on their surface (Casciola-Rosen *et al.*, 1994). Systemic autoantigens that are not well represented in germinal centers may nevertheless trigger censoring of postgerminal center memory B cells by follicular exclusion, perhaps coupled with induction of anergy and CD95-mediated killing.

3. *Regulation of Switched Memory B Cells*

It will be important in future work to determine whether or not memory B cells that have switched to downstream Ig isotypes are still subject to the peripheral censoring steps (checkpoints 2–7) described above. Antigen receptors composed of membrane IgG, IgA, or IgE differ considerably from IgM or IgD, the most notable feature being extended and highly conserved cytoplasmic tails that are unique to particular downstream isotypes (Rogers *et al.*, 1981; Tyler *et al.*, 1982; Yamawaki-Kataoka *et al.*, 1982; Ishida *et al.*, 1982; Komaromy *et al.*, 1983; Word *et al.*, 1983; Bensmana and Lefranc, 1990; Zhang *et al.*, 1992; Hellman, 1993). The presence of these conserved tails strongly implies a unique intracellular signaling or trafficking function, but none has been identified to date.

Efforts to make transgenic mice expressing switched receptor isotypes have so far met with limited success. The most detailed study published to date is that by Storb's group, who made two lines of Hc-only transgenic mice expressing an IgG2b heavy chain from an antibody to *Pseudomonas aeruginosa* (Roth *et al.*, 1993). The resulting mice had fewer splenic B cells than normal, especially as neonates, and the majority of B cells expressed predominantly or solely an IgM receptor derived from endogenous gene rearrangements. Indeed, few B cells developed if endogenous IgM expression was abolished by a targeted gene disruption. One interpretation of these findings is that IgG2b cannot support maturation of preimmune B cells. However, the rescue of IgG-expressing cells by coexpression of endogenous IgM is comparable to that found for dsDNA-reactive B cells expressing an IgG2b transgene (Iliev *et al.*, 1994). In the latter case, B cells expressing only the transgenic IgG were found, but only if they carried a light chain that did not confer dsDNA reactivity. Thus, an alternative interpretation of the failure of most IgG-only B cells to mature in the studies of Roth is that the V region of the anti-*Pseudomonas* heavy chain yields autoantibodies that are frequently censored at checkpoint 1. The B cells that do develop escape censoring by preferentially expressing an endogenous IgM heavy chain that lacks self-reactivity, analagous to the rescue of dsDNA-specific B cells by light-chain editing (Gay *et al.*, 1993).

To compare the function of IgM and IgG receptors with the same V-region specificity directly, Pogue *et al.* have generated antilysozyme Hc+Lc transgenic mice expressing an IgG_1 heavy chain (S. Pogue, D. Leong, K. Canaan, and C. Goodnow, unpublished data). Lines of mice were obtained containing large numbers of mature HEL-binding

B cells that only expressed the transgenic IgG receptor. This finding establishes that IgG receptors can be sufficient for preimmune B cell maturation in the absence of self-antigen reactivity, consistent with the data of Iliev *et al.* (1994). This approach thus opens up the possibility of exploring how receptor isotype and antigen priming independently affect the regulation of autoreactive memory B cells.

G. Checkpoint 8: Inhibition of Terminal Differentiation

A final tolerance checkpoint in B cell development, at least for responses to T cell-independent antigens, exists at the point of B lymphoblast differentiation into antibody-secreting plasma cells. Thus, when proliferating B lymphoblasts are generated *in vitro* in response to bacterial lipopolysaccharide, clustering their antigen receptors with anti-IgM antibodies augments proliferation but prevents the appearance of antibody-secreting cells (Kearney *et al.*, 1978; Andersson *et al.*, 1978). Antigen receptor cross-linking prevents the appearance of two plasma cell-specific mRNAs encoding the secretory form of IgM and J-chain (Chen-Bettecken *et al.*, 1985; Flahart and Lawton, 1987; Berberich and Schimpl, 1992). The antidifferentiative signal can be mimicked by treating the cells with phorbol ester (Isakson and Simpson, 1984; Berberich and Schimpl, 1992).

An identical block in terminal differentiation occurs when lysozyme-specific B cells from Hc + Lc transgenic mice are stimulated by LPS to proliferate and differentiate in that a critical concentration of sHEL augments proliferation but completely suppresses antibody secretion (Goodnow *et al.*, 1991). Tolerant B cells from Hc + Lc transgenic mice bearing sHEL autoantigen proliferate normally to LPS but form no antibody-secreting cells in the continued presence of sHEL antigen. In the absence of further exposure to sHEL the tolerant cells show a delayed appearance of antibody-secreting cells, suggesting that a differentiation block had been induced *in vivo* and was reversed only after several cell divisions in the absence of further receptor stimulation (Goodnow *et al.*, 1991).

Healy *et al.* have found a close correlation between the antidifferentiative signal and activation of the MAP kinase signaling pathway to the nucleus (J. Healy, M. McMahon, J. Blenis, D. Mack, M.Davis, and C. Goodnow, manuscript in preparation). Activation of MAP kinase and the downstream pp90^{rsk} kinase by sHEL antigen occurs normally in tolerant B cells, despite the profound block in receptor and *syk* tyrosine phosphorylation and calcium flux. The level of MAP kinase activation induced by sHEL in tolerant or nontolerant B cell blasts was equivalent to that induced by treatment with phorbol ester at

concentrations just sufficient to inhibit differentiation. In addition to suppression of mRNA for secretory IgM and J-chain, other plasma cell-specific changes were also suppressed including the induction of syndecan, the extinction of CD72, and the appearance of mRNA encoding BLIMP. The latter is a transcription factor in the Kruppel family of zinc-finger proteins and can induce plasma cell differentiation in lymphoma cells poised to make this step (Turner *et al.*, 1994). Continuous receptor stimulation by self-antigens in autoreactive B cells thus suppresses terminal differentiation through a signaling pathway to the nucleus that directly or indirectly acts on BLIMP to prevent transcription of plasma cell-specific genes.

Interestingly, while this pathway effectively suppresses differentiation promoted by LPS, continuous exposure to sHEL antigen does not prevent T cell-driven plasma cell differentiation (Cooke *et al.*, 1994). Similarly, plasma cell differentiation in response to CD40 cross-linking and T cell-derived lymphokines is not prevented by antigen receptor cross-linking with anti-IgM antibody (Banchereau *et al.*, 1994). This checkpoint may therefore be more important to prevent autoantibody secretion during T cell-independent antibody responses to bacterial antigens such as LPS.

IV. Relevance of B Cell Tolerance Checkpoints for Autoimmune Disease

This chapter summarizes a series of cellular phenomena that potentially censor and remove autoantibody-bearing B cells from the pathway to antibody secretion. Each of these checkpoints has triggering thresholds that depend on the local autoantigen concentration, autoantigen valency, and the B cell's binding affinity, as discussed in detail for elimination of autoreactive B cells in the bone marrow (see Section II,E). It has long been appreciated that these thresholds impose limits on the extent of tolerance (Weigle, 1973,1980; Adelstein *et al.*, 1991). On the one hand, such limits ensure that a sufficient fraction of the repertoire is preserved to mount rapid and effective antibody responses to foreign antigens. On the other hand, the same limits increase the risk of autoimmune disease.

The risk of autoantibody production appears greatest for autoantigens that are restricted to nonhemopoietic organs or confined within cells, as the low concentration of these antigens in the environment of recirculating B cells is unlikely to be sufficient to trigger any B cell censoring mechanism (Weigle, 1973,1980; Adelstein *et al.*, 1991). Akkaraju *et al.* have directly confirmed this notion by showing that

when mHEL is expressed only on thyroid epithelium or pancreatic β cells, high-affinity lysozyme-binding B cells are not detectably censored in the bone marrow or in the circulation (S. Akkaraju, K. Canaan, D. Leong, and C. Goodnow, manuscript in preparation). In the case of organ-specific antigens, therefore, tolerance depends on the absence of T cell help. It is nevertheless unclear to what extent helper T cells are censored to organ-specific antigens in the thymus as opposed to some form of peripheral regulation. Several TCR transgenic models have in fact found no evidence for thymic or peripheral elimination of helper T cells recognizing organ-specific autoantigens (Katz *et al.*, 1993; Goverman *et al.*, 1993; Scott *et al.*, 1994; Lafaille *et al.*, 1994). For autoimmune diseases such as myasthenia gravis, Grave's disease, and insulin-dependent diabetes mellitus, the difference between healthy and afflicted individuals may therefore depend primarily on an apparently delicate balance of different helper T cell subsets (Powrie and Mason, 1990; Fowell *et al.*, 1991; Fowell and Mason, 1993).

Self-antigens that are displayed in reasonable abundance systemically, on the other hand, appear to trigger some or all B and T cell censoring mechanisms except in cells with low affinity for these antigens. Development of autoimmune diseases such as systemic lupus erythematosus, autoimmune hemolytic anemia, and rheumatoid arthritis may therefore require errors at several checkpoints. One clear example of this scenario is the development of autoantibodies in *lpr* or *gld* mice, in which a single genetic defect apparently creates a deficiency at two checkpoints, one that censors autoreactive T cells and one involved in censoring autoreactive B cells (see Section III,E). These twin defects in tolerance nevertheless appear insufficient for pathogenic autoantibody production on their own because a considerable time lag precedes autoantibody production in *lpr* mice and other background genes are needed for development of florid glomerulonephritis (Theofilopoulos and Dixon, 1985; Cohen and Eisenberg, 1991; Watson *et al.*, 1992). In the case of monoclonal antilysozyme Hc + Lc transgenic animals carrying the *lpr* mutation on the B6 background, the mice carry a third defect because they lack competing B cells that could eliminate sHEL-binding autoreactive B cells by follicular exclusion. Despite three defective checkpoints, tolerance is maintained in sHEL/Hc + Lc transgenic *lpr* mice until several months of age, after which anti-HEL autoantibody production develops in a considerable fraction of the animals (Rathmell and Goodnow, 1994). Presumably, tolerance is maintained initially because checkpoints 5, 6, and 8 appear not to be disrupted by either the *lpr* mutation or inade-

quate repertoire diversity. Since genes from the MRL strain background accelerate and exacerbate the effects of *lpr*, it is possible that they affect the remaining censoring steps.

Another interesting example of autoimmunity resulting from disruption of several censoring checkpoints is presented by transgenic mice in which Bcl-2 is constitutively expressed in B cells (Strasser *et al.*, 1990). Constitutive expression of Bcl-2 partially inhibits autoreactive B cell censoring in the bone marrow (Hartley *et al.*, 1993), at the level of follicular exclusion (Cyster *et al.*, 1994; Shokat and Goodnow, 1995), and in germinal centers (Pulendran *et al.*, 1995), yet *Bcl*-2 transgenic mice only develop overt autoantibodies when they carry an undefined set of genes from SJL mice and then only after a lag phase of 9 months (Strasser *et al.*, 1990). The *Bcl*-2 transgene has no effect on checkpoints 3, 4, 5, or 8, presumably accounting for the maintenance of tolerance on other strain backgrounds and the long lag phase on the SJL background.

Escape of autoantibody-secreting clones from all censoring checkpoints in *lpr* mice or *Bcl*-2 transgenic mice may thus resemble the process of neoplasia in animals carrying inherited mutations affecting cell cycle checkpoints, such as deficiency in p53 or a c-*myc* transgene. Neoplastic clones develop in such animals only after a time lag determined by other genes and by environmental factors, during which additional somatically acquired defects in cell cycle control allow rare clones to proliferate in a fully dysregulated manner.

While single locus null alleles like *lpr* are illuminating because they create extreme defects in particular tolerance checkpoints, systemic autoimmunity may nevertheless often arise through a polygenic constellation of milder quantitative alleles, as is the case in the NZB/W strain (Drake *et al.*, 1994; Morel *et al.*, 1994). The biochemical pathways and checkpoints affected by such quantitative traits are nevertheless likely to be the set described in this chapter. As more is understood about the molecules needed to bring about each tolerance checkpoint, it is hoped that many will map to the susceptibility loci for different systemic autoimmune diseases in humans. A convergence of these two fields seems to offer the best chance for understanding systemic autoimmunity and identifying new possibilities for inducing remission.

Acknowledgments

C.C.G. thanks Elaine Ruiz for assistance in preparing the manuscript. Research in C. Goodnow's laboratory was supported by the Howard Hughes Medical Institute and by grants from the NIH, the Searle Scholars/Chicago Community Trust. C. Goodnow is an Assistant Investigator of the Howard Hughes Medical Institute.

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This article was accepted for publication on 9 January 1995.

ADVANCES IN IMMUNOLOGY, VOL. 59

The Regulation of Pulmonary Immunity

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I. Introduction

The human lung is exposed daily to over 10,000 liters of inspired, ambient air and the continuous aspiration of small amounts of nasopharyngeal secretions during sleep (Kikuchi *et al.*, 1994). Depending on the quality of air in the environment or the resident flora in the nasopharynx, the respiratory tree faces the enormous task of oxygenating blood across a moist, thin alveolar–capillary wall (approximately 1 μm) and yet resisting infection. Mechanical mechanisms and other innate host defenses are important in preventing infection, but acquired immunity is essential to prevent recurrent and chronic infection as made strikingly evident by the increased numbers and severity of pulmonary infections in immunocompromised hosts (Newhouse *et al.*, 1976; Mason and Nelson, 1992).

The lung, as is true of other epithelial surfaces that interface with the environment, has developed several strategies to avoid infection. As an important component of these strategies, the host must be able to downregulate both nonspecific and immune-mediated inflammation.

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Failure to regulate local immunity results in diseases such as asthma, hypersensitivity pneumonitis, and perhaps sarcoidosis and idiopathic interstitial pneumonitis (Holt, 1993; Djukanovic *et al.*, 1990; O'Connor and FitzGerald, 1992). Thus, the desire to enhance protective pulmonary immune responses by vaccination and to prevent or control unwanted responses underlie the need to study basic immune mechanisms in the lung. Furthermore, understanding critical immunoregulatory mechanisms may lead to strategies for preventing and controlling lung transplant rejection and immune-mediated lung damage in bone marrow transplant patients.

A number of recent reviews have discussed important issues in the development of pulmonary immune responses (Agostini *et al.*, 1993; Hance, 1993; Lipscomb *et al.*, 1993a; Gyetko and Toews, 1993; Holt, 1993; Bice, 1993). The goal of this chapter is to describe the cells and structures of the lung that participate in pulmonary immunity and to summarize studies that help explain how the lung responds to challenges with foreign antigens, with particular emphasis on animal models that have been developed to explore these issues. Features of the immune apparatus that are unique to the lung will be highlighted, and important questions currently under investigation will be indicated.

II. Immune Cells and Structures of the Lung

An important challenge is to understand how the host protects itself from infection yet regulates immunity to prevent tissue damage. Tissue culture has been a powerful tool for understanding how immunologically relevant cells interact. The study of how various extracellular signals influence gene expression in cultured cells has given important insight into how the milieu could influence cell behavior at various anatomic sites. However, because it is not yet possible to know what all of the influences within a tissue are, hypotheses generated from cells in culture must be tested *in vivo*. For example, alveolar macrophages (AM) exist attached to epithelial cells and migrate within a layer of surfactant rather than attached to plastic in a layer of medium. A recent review in this series discussed the importance of placing lymphocytes in their spatial context within the host to properly understand their function (Kroemer *et al.*, 1993). This consideration is especially important in the lung.

In considering the development of pulmonary immunity in a spatial context, it is useful to divide the evolution of an immune response in the lung into three distinct but overlapping phases (see Fig. 1; Lipscomb, 1993a): (1) in the *afferent phase*, antigen reaches the lung, is

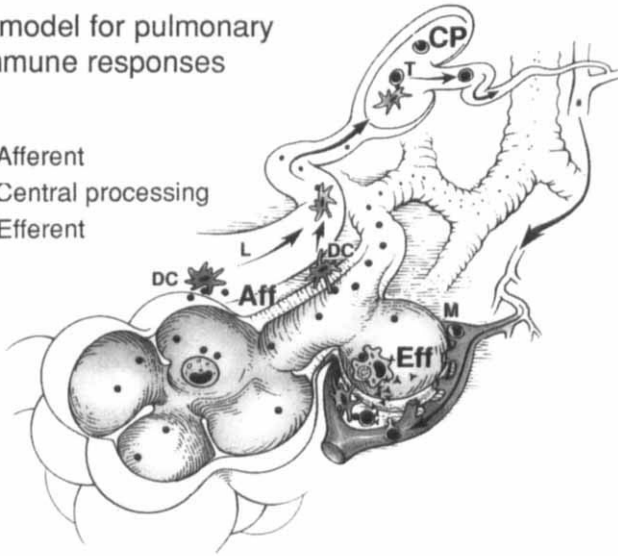

FIG. 1. A model for afferent, central processing, and efferent phases of a pulmonary immune response. In the afferent phase (Aff), antigen that reaches bronchoalveolar spaces can directly enter lymphatics (L) or be processed by intraepithelial or interstitial dendritic cells (DC) that enter lymphatics and migrate to lung-associated lymph nodes (LALN). In the central processing phase (CP) antigen on lung DCs (or, perhaps, antigen processed by resident lymph node DCs) present antigen to T lymphocytes (T) to initiate the expansion of T cell clones that, in turn, may help B cells expand when B cell immunoglobulin receptors recognize "native" antigenic determinants. In the efferent phase (Eff), recently activated T cells and B cells (not shown) leave LALNs and circulate; they are subsequently recruited to the lung at sites of inflammation. Exposure of T cells to relevant antigen results in the release of cytokines that amplify an inflammatory response by recruiting nonspecific effector cells, such as monocytes (M), to the site and activating them to kill and/or growth-inhibit microorganisms.

take up by antigen-presenting cells (APCs), and presented to naive T cells expressing the relevant T cell receptors (TCRs); (2) in the *central processing phase,* specific lymphocyte clones are expanded and differentiate; and (3) in the *effector phase,* effector T cells and B lymphoblasts find their way to pulmonary sites requiring expression of a specific immune response. At each phase, events must be tightly regulated to allow an effective immune response yet avoid excess, potentially destructive inflammation. The location in the respiratory tract where each of these phases occurs is somewhat controversial, but the bulk of evidence indicates that in the normal host after reaching the lungs, antigen is carried on APCs, in phagocytes, or free in lym-

phatic fluid to draining lung-associated lymph nodes (LALNs) (Lauweryns and Baert, 1976–1977; Lehnert, 1992) where central processing occurs. Effector cells are released into the efferent lymph and reach the blood stream where they are recruited from the vasculature into the lung (Berman *et al.*, 1990).

Many investigations have considered the lung in the broader context of mucosal immunity. This conceptual framework is useful, but the respiratory tract has several distinctive features that require that initiation and expression of lung immunity be considered separately from immune responses at other mucosal sites.

A. Concept of an Upper and Lower Respiratory Tract

The respiratory tract shares features of other organs, i.e., skin, gut, and urogenital tract in which an epithelial layer interacts with the environment. For internal organs, the concept of a common mucosal system was developed based on evidence that immune lymphocytes generated at one surface migrated to both homologous and distant mucosal sites (McDermott and Bienenstock, 1979; McGhee *et al.*, 1992). We will return to this concept shortly, but in addition to this concept, the lung must be understood immunologically from the point of view that both an upper and lower respiratory tract system exist (Kaltreider, 1976; Kazmierowski *et al.*, 1977), and each system exhibits distinctive as well as common immune mechanisms.

The upper respiratory tract starts at the nares and extends to the level of the terminal bronchioles. A pseudo stratified to single layered columnar epithelium covers vascularized connective tissue, the lamina propria, which, depending on the level of the airway, also contains variable numbers of mucous glands, smooth muscle, and, in the larger airways, is bounded by cartilage. By contrast, in the lower respiratory tract (which by definition includes the alveolar ducts and alveoli), the epithelium is markedly attenuated and frequently separated from the pulmonary capillary endothelium by only a fused basement membrane. The mechanisms of antigen handling and the types, location, and numbers of immunologically relevant cells differ even within areas of the upper respiratory tract, but are most strikingly different between the upper versus lower respiratory tracts. For example, mucociliary clearance is the major mechanism for clearance of particulates in the upper tract. In contrast, phagocytosis by resident AM which subsequently attain the level of the upper tract to be removed by the mucociliary elevator characterizes particulate clearance in the lower tract (Lauweryns and Baert, 1976–1977; Lehnert, 1992).

Another distinctive feature of the upper versus lower tracts relates

to the organization of lymphoid tissue. In the upper tract, lymphocytes reside in both aggregates and diffusely distributed along the mucosa of the upper tract, and in some animal species may infiltrate the epithelium (Bienenstock *et al.*, 1973a,b; Sminia *et al.*, 1989). In the lower tract, lymphocytes are present in variable numbers both within alveoli and in the interstitium, but, in the normal host, organized aggregates do not occur (Sminia *et al.*, 1989; Pabst, 1992).

Other important features of the upper versus lower tract involve the relative importance of IgA as the protective antibody. Thus, IgA-secreting B cells occur in the mucosa of the upper tract, and IgA is the major immunoglobulin in secretions of the upper respiratory tract; although IgA is present in the lower tract, IgG and IgM predominate in bronchoalveolar lavage (BAL) fluids (Kaltreider, 1976).

The development of immunity in the lung, as elsewhere, requires that relevant cells display appropriate surface molecules for contact and secrete appropriate factors. Some ligand–receptor interactions are specific while others are not, and it is the particular pattern of surface molecules and secreted factors expressed by interacting immune cells that determines the type of immune response that develops during central processing. Furthermore, at sites of pulmonary inflammation, other patterns of adhesion molecules and cytokine/chemokine expression by immune and parenchymal cells direct the tempo and magnitude of accumulation of recruited cells. Many recent studies emphasize the critical importance of cytokines and chemokines and the expression of adhesion molecules in regulating pulmonary inflammation and immunity (Kunkel *et al.*, 1989; Stein-Streilein and Phipps, 1993; Redington *et al.*, 1993; Standiford *et al.*, 1993; Jordana *et al.*, 1993; Lukacs *et al.*, 1994a).

The cells that are the major initiators and regulators of immunity in the lung include macrophages, dendritic cells (DCs), and lymphocytes, each expressing surface molecules and secretory products that depend on perturbations in the environments. However, other cells in the milieu, e.g., epithelial cells, fibroblasts, mast cells, and various recruited blood leukocytes, also play important regulatory roles which will only be briefly examined in subsequent sections. First, however, the special feature of lung macrophages, DCs, and lymphocytes are discussed.

B. Lung Macrophages

Originally proposed as important APCs in the lung (Lipscomb, 1988), this diverse group of cells is now best understood in the context of lung immunity as phagocytes and as regulators of both immunity

and nonspecific inflammation (Holt, 1986; Lipscomb *et al.*, 1993a). Indeed, the bulk of evidence indicates lung macrophages are unlikely APCs in the initiation of primary immune responses. These cells reside within the airways at all levels of the respiratory tract, in the lamina propria, the interstitium, the alveolar regions and pleura, and within pleural spaces; in ruminant species, lung macrophages are found within the pulmonary capillaries (Lehnert, 1992; Brain, 1992). All lung macrophages originate from the bone marrow (van Oud Alblas *et al.*, 1983; Springmeyer *et al.*, 1982; Godleski and Brain, 1972), but maintenance of at least some of these resident populations partly derives from self-replicating pools (Pinkett *et al.*, 1966; Bowden and Adamson, 1980; Sorokin *et al.*, 1984; Tarling *et al.*, 1987; Shellito *et al.*, 1987).

Pulmonary macrophages are both phenotypically and functionally diverse, even within a single compartment. Because of ready access to bronchoalveolar macrophages using BAL, the diversity of these cells has been most frequently studied. Macrophages obtained by lavage include resident AM as well as macrophages that reside within the lumen of the bronchi and bronchioles (intraluminal macrophages); a distinction between these two cell populations cannot be readily made. However, the vast majority of cells obtained during a human lavage are from the alveoli, since the technique is performed with a wedged flexible fiberoptic bronchoscope and the large alveolar surface area relative to the bronchial surface area is sampled (Reynolds, 1987; American Thoracic Society, 1990). In small rodents, e.g., mice and rats, a catheter is typically placed in the trachea and a larger proportion of the recovered cells are from the bronchi and bronchioles, but the majority of cells are still from alveoli if the procedure is performed correctly.

Functional attributes of subpopulations of resident AM have been studied. Most studies exploit the differences in density (Murphy and Herscowitz, 1984; Shellito and Kaltreider, 1985; Oghiso, 1987), predominantly a function of cell size. Another fractionation technique depends on differences in the cells' capacity to be readily lavaged, a function of their adherence to epithelium and/or their residence in the bronchial lumen versus in alveoli (Holt *et al.*, 1982). Within subpopulations, AM differ in expression of class II major histocompatibility antigens (MHC), Fc and complement receptors, phagocytic capacity, responses to chemotactic stimuli, cytotoxicity, cytokine production, and capacity to suppress *in vitro* immune responses (Holt *et al.*, 1982; Murphy and Herscowitz, 1984; Shellito *et al.*, 1983; Shellito and Kaltreider, 1985; Oghiso, 1987). Furthermore, the size, function, and phenotype of AM shifts during an inflammatory response (van Oud Alblas

et al., 1983). Although alternate explanations for these changes have been offered, the most likely one is that AM recently arrived from the peripheral blood are similar in the size and phenotype to circulating monocytes, the precursors of AM. Thus, in the study of AM and their subpopulations and, indeed, in any study of lung immunity, it has become a dictum that investigators must be careful to avoid low levels of chronic inflammation by housing experimental animals in specific pathogen-free environments. Because respiratory infections are so common in nearly all animal species and can markedly affect experimental results, this consideration cannot be overstated.

Interstitial macrophages (IM) when compared with AM also exhibit size, functional, and phenotypic differences. These differences may reflect the stage of differentiation from blood precursors, but more likely reflect the environment and physiological roles of phagocytes in these two distinct locations. Important membrane molecules that determine the function of macrophages include complement, Fc, mannose, and scavenger receptors, as well as class I and II MHC, adhesion, and other signaling molecules. Important differences in many of these exist depending on the location of lung macrophages. For example, in mice, IM express C3 receptors, whereas most resident AM do not (van Oud Alblas and Van Furth, 1979).

Few studies exist on the role of interstitial, pleural, or intravascular lung macrophages in immune responses. However, numerous studies have examined the role of AM in stimulating mitogen, alloantigen, and antigen stimulated responses *in vitro*. Not surprisingly, these studies have often reached conflicting conclusions, a result of the differing animal species used, the level of superimposed AM suppressive activity, and the assay procedure that was used (Holt, 1986; Lipscomb, 1988). Most studies support the concept that AM are poor APC for priming T cells even when they express high levels of class II MHC, as human AM do (Holt, 1979; Ansfield *et al.*, 1979; Toews *et al.*, 1984a; Lipscomb *et al.*, 1986). Bronchoalveolar cells, which contain up to 90% AM, fail to act as effective APC *in vitro* because they are either actively suppressive or because they fail to express some other poorly understood accessory function which may manifest as poor lymphocyte-accessory cell binding (Shellito *et al.*, 1983; Lyons *et al.*, 1986; Kradin *et al.*, 1987). Most current evidence indicates that DCs are the most efficient APC in stimulating naive T cells, especially CD4 T cells, although class II MHC-positive cells of many cell types are capable of stimulating recently primed T cells (Steinman, 1991; Croft, 1994). Variable contamination of cells with lung DCs (Pollard and Lipscomb, 1990) and the presence of recently activated T cells in

responder populations may well explain reports that AM can function as effective APC (Rich *et al.*, 1987).

Mechanisms utilized by populations of BAL cells to suppress immune responses are likely to depend on variables of the assay systems and are particularly dependent on animal species. In dogs, PGE_2 production (Demenkoff *et al.*, 1980) and the synergistic activity of PGE_2 and oxygen radicals (Kaltreider *et al.*, 1986) were shown to be important. In humans, AM/lymphocyte contact leading to inhibition of receptor-induced intracellular calcium increases in responder T cells has been described (Yarbrough *et al.*, 1991). Resident murine macrophages, particularly at high numbers relative to numbers of stimulator lung DC, suppress a mixed lymphocyte reaction (MLR) by secreting TGFβ (Lipscomb *et al.*, 1993b). Furthermore, nitric oxide (NO) made by murine AM may inhibit the development of potent APC cell function of lung DC (Holt *et al.*, 1993). Evidence that AM suppression plays a role *in vivo* has been indirectly shown by depleting AM with an intratracheal dose of liposomes containing a macrophage cytotoxic drug (dichloromethylenediphosphonate) followed by immunizing via the respiratory tract. The animals demonstrated increased numbers of antibody-forming cells (AFCs) in LALNs compared to immunized controls pretreated with only liposomes (Thepen *et al.*, 1989). IgG, IgA, and IgE AFCs were all increased.

AM-suppressive activity is important to prevent the development of hypersensitivity reactions, but in circumstances in which lung immunity is important for protection, AM-suppressive activity could be counterproductive. However, suppressive activity of murine macrophages can be inhibited by exposure to GM-CSF and to a lesser extent by other selected cytokines (Bilyk and Holt, 1993). The major role of AMs seems to be to phagocytose and remove potentially dangerous particulates and soluble antigens from the alveoli and to inhibit local lung immune responses. However, immunity may develop in the presence of otherwise suppressive AMs by the recruitment of leukocytes to the alveolus with opposing activity or by strong environmental influences that result in cytokine secretion that diminishes AM suppressor function.

C. Lung Dendritic Cells

Immunologists have long recognized that adherent cells are required for optimal *in vitro* immune responses whether one measures T cell lymphoproliferative responses, T cell cytokine production, or T-dependent B cell responses. They have also developed an increasing appreciation of the role of DCs in priming naive T cells since they

were first identified in the spleen (Steinman and Cohn, 1973). Since then, the role of DCs in immune responses initiated at the epithelial surface is being clarified. Little doubt exists that DCs play a pivotal role in initiating immune responses in the skin (reviewed by Steinman, 1991). Furthermore, substantial support has developed for the concept that intraepithelial DCs are functionally different from those that have migrated into regional lymph nodes. Thus, freshly isolated Langerhans cells are capable of processing antigen and stimulating T cell clones, but they require "maturation" *in vitro* before they are fully effective in stimulating naive T cells in an MLR; at this time they have a markedly reduced capacity to process antigen (Romani *et al.*, 1989). Data indicate that lung DCs share a number of characteristics of skin DCs and likely also play a critical role in initiating lung immunity (Holt, 1993).

APCs must not only process antigen and express it in the context of class II MHC, but also express appropriate accessory molecules to enhance the interaction of the APCs with T cells. Recent studies emphasize the importance of the interaction of B7 on the APC cell surface with CD28 and/or CTLA4 expressed on responder T cells to deliver a second signal (reviewed by June *et al.*, 1994). Failure to trigger this second signal may cause T cells interacting with APCs via only the TCR–peptide/MHC II interface to become anergic (Harding *et al.*, 1992; Boussiotis *et al.*, 1993; Chen and Nabavi, 1994) and produce a state of tolerance *in vivo* (Van Gool *et al.*, 1994). B7 is a receptor family of at least two molecules in the immunoglobulin supergene family. B7-1, (CD80), and B7-2 (CD86) are both expressed constituitively on DCs in contrast to macrophages and B cells that must be activated to express these molecules (Vandenberghe *et al.*, 1993). Other important accessory molecules, usually identified phenotypically by standard immunocytochemistry and functionally by the ability of specific antibodies to block an immune function, include CD40, CD54 (ICAM-1), and CD58 (LFA-3) (Steinman, 1991).

1. *Isolation and Characterization*

Like DC from other sites, lung DC constituitively express both class I and II MHC, are light density, loosely adherent, poorly or nonphagocytic, and demonstrate long dendritic processes both in tissue sections and in cell suspensions (Holt *et al.*, 1985;1988; Sertl *et al.*, 1986; Rochester *et al.*, 1988; Nicod *et al.*, 1987; Pollard and Lipscomb, 1990). Lung DCs fail to express pan T, natural killer (NK) cell, B cell and *many* macrophage markers. The critical accessory molecules CD54 and CD58 (Xia *et al.*, 1991; Nicod and El Habre, 1992) are

present on many lung DCs, but data are incomplete on the expression of CD40, CD80, and CD86 family.

The high levels of constitutive class II MHC and the dendritic shape of DCs has been exploited to examine their location in tissue sections of lung by a number of investigators. DCs form an interdigitating network in the airway epithelium of all species in whom they have been investigated (Sertl *et al.*, 1986; Holt, 1993) similar to the network described for skin Langerhans cells. Intraepithelial DCs are particularly dense in the trachea and gradually diminish in concentration as the airways branch, but are increased at sites of chronic inflammation (Schon-Hegrad *et al.*, 1991). DCs also exist in the connective tissue surrounding bronchi and bronchioles, in perivascular connective tissue, in alveolar septa, in the pleura, and in very small numbers in alveolar spaces (Sertl *et al.*, 1986; Holt and Schon-Hegrad, 1987; Kradin *et al.*, 1991; Havenith *et al.*, 1992; van Haarst *et al.*, 1994). In rats, intraperitoneal injections of IFNγ increased the numbers of intraepithelial and septal DCs without increasing their accessory cell function (Kradin *et al.*, 1991), and inoculation of Bacillus Calmette–Guerin (BCG) increased the numbers of DC that could be lavaged from the alveolus (Havenith *et al.*, 1992). Thus, the numbers of lung DCs in various anatomic sites depend on signals delivered that are coincident with inflammation.

Lung DCs have been isolated with a variable degree of success utilizing adherence, density, and class II MHC expression properties; their function and other phenotypic features have been studied. Lung macrophages are the most difficult cells to separate from DCs in single cell suspensions of lung cells. Effective procedures for isolating fairly pure DC populations require exploiting the phagocytic and autofluorescent properties of lung macrophages (Nicod *et al.*, 1987,1989a; Pollard and Lipscomb, 1990; Havenith *et al.*, 1993a). Using these techniques, the function of DC-enriched lung cells has been assessed. Functional assays have included stimulation of periodate-treated lymphocytes, MLRs, and antigen-induced stimulation of either memory T cells or lymphoblasts. All have shown that lung DCs function as well or better than DCs from other sources (Nicod *et al.*, 1987; Rochester *et al.*, 1988; Pollard and Lipscomb, 1990).

2. *Phenotypic Heterogeneity*

DCs isolated from whole lung preparations are phenotypically heterogeneous, and cell markers differ somewhat among animal species and even within individuals of a species. An example of this latter variation relates to CD1a (OKT6), expressed by human Langerhans

cells in the skin, which has been variously described as being present on from less than 1% (Sertl *et al.*, 1986; Nicod *et al.*, 1987) to 30% (van Haarst *et al.*, 1994) of lung DCs in man. This antigen may be important in stimulating $\gamma\delta$ T cells, a potentially important interaction for host defenses in the lung in view of observations that $\gamma\delta$ T cells may recognize heat-shock proteins of *Mycobacterium tuberculosis* (Mtb) (Born *et al.*, 1991, Kaufmann and Kabelitz, 1991).

Variation in expression of cytoplasmic and surface markers also occurs *within* populations of lung DCs. For example, in the mouse, the interdigitating cell antigen (NLDC-145) and CR3 are present on about half of lung DCs, while the majority of lung DCs express CD25 and the heat-stable antigen (as defined by J11D; Pollard and Lipscomb, 1990). The latter two markers are also uniformly expressed on murine thymus and skin DCs, but are absent from the majority of splenic DCs (Crowley *et al.*, 1989), indicating the likelihood of a closer relationship of lung DCs to tissue DCs rather than to DCs that primarily home from bone marrow to lymphoid tissue. Additional evidence that lung DCs are distinct from the majority of splenic DCs is that lung DCs fail to express the splenic DC marker recognized by the monoclonal antibody 33D1 (Pollard and Lipscomb, 1990).

In both rat and mouse, another heterogeneous marker is FcRII, positive on about half of lung DCs (Pollard and Lipscomb, 1990; Xia *et al.*, 1991). Interestingly, Langerhans cells freshly isolated from the skin express FcRII which is downregulated as the cells mature in culture. Thus, it is possible that the FcR expression in the lung denotes a population similar to freshly isolated skin DCs. In the rat, DCs in the epithelium lining the airways are more likely to be FcR^+, whereas parenchymal DCs are nearly uniformly FcR^- (Gong *et al.*, 1992). Murine DC in the epithelium of the trachea also express FcRII (Sertl *et al.*, 1986). This suggests the possibility that intraepithelial DCs are poised to take up and process antigens at the epithelial/environment interface and enter the interstitium to traffic to LALN. Thus, at least some interstitial DCs which fail to express FcR may be in transit. On the other hand, the presence of FcR^- DCs in lung parenchyma, including alveolar septa, may indicate distinct immune functions for this subset of lung DCs.

No difference was found in mice in the capacity of the FcR^+ or FcR^- subsets to stimulate an MLR (Pollard and Lipscomb, 1990). However, in the rat, the FcR^- population was a more potent stimulator of naive T cells in an MLR and in responses to lectins, but FcR^- and FcR^+ were equally capable of presenting soluble and particulate antigens to antigen-sensitized T cells (Kradin *et al.*, 1993). Of interest

in these later studies was that 70% of FcR^- and only 39% of FcR^+ cells expressed the adhesion molecule CD54 (Kradin *et al.*, 1993), an observation that might partly explain the increased ability of the FcR^- cells to stimulate an MLR. In elegant studies in which lung intraepithelial DCs were isolated from the rat, these cells were shown to present antigen to primed T cells more efficiently than did parenchymal DCs. In man, further enrichment for FcR^+ decreased the ability of lung cells enriched in DC to stimulate an MLR (Nicod *et al.*, 1987), although these studies are complicated by the large numbers of contaminating FcR^+ macrophages in the DC populations. Nevertheless, FcR positivity has been a useful marker for identifying subsets of lung DCs and the majority of data are consistent with the concept derived from Langerhans cells that a population of FcR^+ intraepithelial DCs may take up antigen from the bronchial lumen and differentiate into cells that can stimulate naive T cells upon migration into draining lymph nodes.

3. *Origin*

DCs in all tissue sites originate from the bone marrow (Steinman, 1991). They can be cultured from precursors in bone marrow and peripheral blood using GM-CSF and, for adult human DCs, from peripheral blood with IL4 (Inaba *et al.*, 1992,1993; Thomas *et al.*, 1993; Sallusto and Lanzavecchia, 1994). In one study, Ia^+ lung DCs were first recognized in rat lung parenchyma in fetal life at Day 15 of gestation (McCarthy *et al.*, 1992). Intraepithelial DCs were present by Day 17 and continued to increase during postnatal development (McCarthy *et al.*, 1992). Comparison of fetal lung DCs demonstrated that they were not as efficient as adult DCs in stimulating an immune response, but were fully functional at birth. In contrast, another study failed to detect Ia^+ DCs in rat lung epithelium or parenchyma until birth, and the numbers increased rapidly until 3 weeks of age when they approximated those found in adults (Nelson *et al.*, 1994). Ia^+ DCs were first observed in the nasal turbinates and intensity of Ia staining increased in time, first in the trachea and later in lung parenchyma, compatible with environmental exposure effecting the change. Using another marker for DC, OX62 (which also detects $\gamma\delta$ T cells), these same authors found CD3-negative, OX62-positive DCs at all levels of the lung in fetal rat lung and speculated that environmental influences upregulated Ia expression and subsequent function of these cells. Consistent with this speculation was that IFNγ increased the numbers of Ia^+ cells in airway epithelium while steroid inhalation decreased the numbers relative to control rats. The conflicting findings of the

two groups may be based on the differences in the housing environments for the two groups of rats. Thus, Nelson and collaborators (1994) used dust-free bedding in contrast to McCarthy *et al.* (1992). It was quite likely that *in utero* influences affected the numbers and function of lung DCs.

4. *In Vivo Function*

Numerous studies have demonstrated that DCs from spleen, lymph node, and skin have extraordinarily potent ability to immunize recipient animals following inoculation either iv or subcutaneously (Knight *et al.*, 1983; McKinney and Streilein, 1989; Sornasse *et al.*, 1992). Lung DCs have not been used to immunize experimental animals to date, but several lines of evidence suggest that they have an important role *in vivo*. As discussed, their location within the epithelium places them in an optimal position to take up and process antigens that breach the epithelial barrier; perivascular and septal DCs should be poised to process antigens that reach the lung via the vasculature. Studies have shown that splenic DCs instilled into the lung may reach LALN (Havenith *et al.*, 1993b) and if pulsed with antigen may induce an immune response (Havenith *et al.*, 1993c). In this latter study, AMs pulsed with antigen were also capable of initiating an immune response, but in contrast to DCs heat-killed AMs were also able to immunize suggesting that AMs stimulated responses by having the antigen reprocessed by the host's own APCs. Additionally, explanted lung DCs had gained the ability to stimulate primed T cells following the intratracheal delivery of the relevant antigens (Holt *et al.*, 1993). Last, consistent with the dynamic activity of epithelial DCs in carrying environmental antigens into LALN, irradiation of rats resulted in a loss of 85% of resident DCs by 72 hr and reconstitution of tracheal DCs from bone marrow precursors by 10 days (Holt *et al.*, 1994). Lung DCs most surely play a major role in regulating lung immunity and, as discussed previously, are likely themselves regulated by multiple environmental factors. It remains to be determine whether antigen presented by lung DCs is more likely to result in TH1 versus Th2 responses in the lungs and whether bypass of DCs in immunization of the host is more likely to deliver a tolerogenic signal, or whether other factors are more important in determining these outcomes.

D. Lung Lymphocytes

T cells, B cells, and NK cells have all be described in various lung compartments (Holt and Schon-Hegrad, 1987; Stein-Streilein, 1988; Pabst, 1990; Agostini *et al.*, 1993). Their phenotype and function have

been described either by evaluating markers *in situ* or by obtaining cells from BAL or collagenase digestion of lung tissue. As with lung macrophages and DCs, lung lymphocytes are a dynamic population with the capacity to enter and leave the lung depending on influences in the milieu.

1. *Location*

In several species including rat, rabbit, and chicken, large numbers of lymphocytes are located in organized bronchus-associated lymphoid tissue (BALT), which is discussed below. However, BALT is not constitutive in all species and not regularly seen in hamsters, mice, and humans. In these latter species, the majority of lung lymphocytes are present in the interstitium, diffusely scattered in the mucosa, alveolar septa, or pleura. Also, depending on the species, there may be an intraepithelial and/or an intravascular pool of lymphocytes (Pabst, 1990). A population of lung lymphocytes exists in the bronchoalveolar spaces and is recovered by BAL. In animals kept in specific pathogen-free environments, lymphocytes range from 5 to 10% of cells recovered by BAL (Agostini *et al.*, 1993; Pabst, 1990). In normal, nonsmoking humans the figures vary from less than 8 to 20% (Daniele *et al.*, 1975; Davidson *et al.*, 1985; Becker *et al.*, 1990).

The proportion of T cells to B cells and NK cells varies to some degree with the compartment in which the lymphocytes are present. The composition of lymphocytes in BAL fluids is generally representative of the proportion of T cells, including CD4 and CD8 T cells, and B cells found in peripheral blood. Interestingly, in humans, differences in the phenotype and function of NK cells occur between the peripheral blood and the alveolus. Thus, NK cells in human alveoli fail to express cytolytic activity and are largely negative for CD16, although CD16-expressng NK cells with cytolytic activity exist in the interstitium (Weissler *et al.*, 1987). Relatively high numbers of cytolytically active NK cells also exist in the interstitium of mice. Fifteen to 20% of cells fractionated on nylon wool columns following isolation from enzyme-digested lungs express the allotypic NK1.1 marker, detectable in C57BL/6 mice (Stein-Streilein *et al.*, 1983).

When leukocytes are isolated from the lung parenchyma of guinea pigs, mice, rats, and humans, the relative percentage of lymphocytes varies from 15 to over 50%. The variance likely depends on the strain of animal, whether they are kept specifically pathogen-free, and the rigor by which small monocytes are excluded (much of the data are derived from examining Wright–Giemsa-stained cytospin preparations) (Lipscomb *et al.*, 1982; Stein-Streilein *et al.*, 1983; Holt and

Schon-Hegrad, 1987; Nicod *et al.*, 1989a,b). Flow cytometric analysis of T cell and B cell populations of lung lymphocytes indicates that the relative proportion of B cells is either increased or the same, and the CD4/CD8 ratios are either decreased or similar compared to peripheral blood (Holt *et al.*, 1986; Abraham *et al.*, 1990; Marathias *et al.*, 1991; Huffnagle *et al.*, 1994).

Intraepithelial lymphocytes are common in the gut mucosa and exist in lung epithelium of some animal species, but are relatively less common than those in the gut (Holt and Schon-Hegrad, 1987; Fournier *et al.*, 1989). In humans, no B cells were found in airway epithelium, and CD8 outnumbered CD4 T cells (Fournier *et al.*, 1989). In an examination of epithelium of the upper respiratory tract (in the nose and covering the tonsil and adenoids), both B cells and T cells were found. Notably, $\gamma\delta$ T cells occurred in aggregates where they comprised up to 30% of the total T cells, although in general 80–90% of T cells expressed the $\alpha\beta$ TCR (Graeme-Cook *et al.*, 1993).

In studies examining cells isolated from human lung parenchyma, $\gamma\delta$ T cells made up less than 5% of T cells and were $CD3^+$, $CD4^-$, and $CD8^-$ (Abraham *et al.*, 1990; Marathias *et al.*, 1991). In a study of mice, on the other hand, 8–20% of resident lung lymphocytes were $CD3^+$ and $\alpha\beta$ TCR^-, and were presumably $\gamma\delta$ T cells. $\gamma\delta$ T cells increased following aerosol delivery of *M. tuberculosis* (Augustin *et al.*, 1989).

2. *Traffic*

The regulation of movement of lymphocytes into and out of the lung is still incompletely understood. Nevertheless, with increased information about the role of adhesion molecules in regulating the traffic of lymphocytes (Springer, 1994), investigators are beginning to unravel what regulates steady-state movement of cells into the lung as well as possible preemptive signals that occur with inflammation. Research on emigration of cells into the lung has centered on the accumulation of neutrophils within pulmomary vasculature and their immigration into the air spaces of the lung because these cells are likely to play a critical role in the development of the acute respiratory distress syndrome. Recent information indicates that the adhesion molecules, P- and E-selectin, the integrins, and ICAM-1 and ICAM-2 play important roles in the accumulation of neutrophils in the lung (Pilewski and Albelda, 1993), The relevance of each adhesion molecule likely depends on the inflammatory stimulus that provokes the recruitment (Hellewell *et al.*, 1994; Doerschuk *et al.*, 1990; Doerschuk, 1992; Mulligan *et al.*, 1993a–e).

The regulation of lymphocytes into the lungs is less well understood even though, as mentioned above, evidence exists that lymphocytes isolated from LALN or collected from the efferent lymphatics of LALN have a predisposition to return to the lungs (McDermott and Bienenstock, 1979; Spencer and Hall, 1984; Joel and Chanana, 1987). Several studies have demonstrated that T lymphocytes isolated from lung lavages of normal humans predominantly express a memory phenotype, e.g., they are $CD45RO^+$, $CD45RA^-$ cells (Saltini *et al.*, 1990; Becker *et al.*, 1990). Other important markers for naive versus memory T cells are the hyaluronic acid receptor (CD44) and L-selectin. Naive T cells express low levels of CD44 and high levels of L-selectin, memory T cells express high levels of CD44 and low levels of L-selectin (reviewed in Sprent, 1994). Thus, with the recognition that memory T cells accumulate preferentially in the lung, the issue becomes what is the stimulus for their entry and why do naive cells fail to accumulate.

The role of naive T cells with their multitude of diverse receptors is to continuously recirculate through secondary lymphoid organs, i.e., spleen and lymph nodes, so that antigen-bearing APCs can interact with appropriate T cells at that site. This APC–T cell interaction results in clonal expansion and subsequently provides challenged tissues with a population of cells able to specifically react and protect the host. Thus, naive T cells have receptors that allow them to migrate to secondary lymphoid organs (Butcher *et al.*, 1990; Jutila 1994). In contrast, lymphocytes that must enter challenged or inflamed tissues might be expected to express a different set of homing receptors. Indeed, this is the case. Although accumulation in markedly inflamed sites seems to be nonspecific, when only low levels of inflammation exist, where immune cells accumulate may be determined in the draining lymph nodes from which they derive (Picker, 1994). Teleologically, this would be a more efficient way for the immune system to selectively redirect cells to sites where they are needed. Indeed, evidence suggests memory lymphocytes bearing the cutaneous lymphocyte-associated antigen seem to specifically home to the skin (reviewed in Picker, 1994). Studies also suggest that memory cells in the lung may have a unique set of homing receptors compared to memory cells in the skin or at mucosal sites (Picker *et al.*, 1994). Thus, $CD3^+$, CD45RO high/CD45RA low T cells in the skin were E-selectin$^+$ and CLA^+, but were $\alpha4\beta7^-$ and $\alpha_e\beta7-$, while lung memory T cells were E-selectin$^-$, CLA^-, and $\alpha4\beta7^-$, but 50% of the cells were $\alpha_e\beta7^-$. The lung phenotype was different from the overall memory T cell phenotype in blood, suggesting that there might be an unidentified receptor on lung T cells that specifically selected them for emigration into the lung.

An important issue is whether any naive lymphocytes traffic through the lungs. Naive T cells migrate into peripheral lymph nodes via high endothelial venules (HEV) using an L-selectin/peripheral lymph node addressin interaction. Some animal species have BALT in which HEV are present. Thus, in these animals, naive cells could enter the lungs and be available for primary immune responses to develop at these sites. However, evidence to support this possibility does not exist. Despite scanty information about the migration of naive and long-term memory T and B cells into the lungs, several studies have noted that lymphoblasts (or recently divided lymphocytes) have a tendency to migrate into both inflamed and uninflamed lung (Daniele *et al.*, 1977; Berman *et al.*, 1990). The location and mechanisms of lymphocyte transmigration are under investigation.

E. Organized BALT

Macrophages, DCs, and lymphocytes are diffusely distributed throughout all of the compartments of the lung. In addition, in most animal species examined, at least some organized lymphoid tissue may be found in variable amounts lining bronchi and bronchioles. These structures have been recognized for many years, but were first carefully described in 1973 (Bienenstock *et al.*, 1973a,b). More recently, some controversy about these structures has been raised because of the inability to readily detect them in all species. Thus, they are relatively rare in normal humans, cats, and young pigs (Pabst, 1990,1992). When first described they were compared to intestinal Peyers patches; they had the appearance of a follicle without a capsule. Furthermore, lymphocytes infiltrates the overlying bronchial epithelium which demonstrated alterations in morphology compared to adjacent epithelial cells. They were originally described in the bronchial mucosa of all species examined, e.g., rabbits, guinea pigs, rats, mice, dogs, pigs, chickens, and man (Bienenstock *et al.*, 1973a). These early studies revealed that neonatal thymectomy failed to affect the normal development of BALT in rats and chicks, and that tritiated thymidine labeling and autoradiography indicated that there was rapid cell proliferation of cells constituting BALT (Bienenstock *et al.*, 1973b). Transplantation of fetal lungs into extrapulmonary sites did not interrupt the development of the BALT, although it was not as cellular, suggesting that antigenic stimulation was required for full development.

Since these early studies, two comprehensive reviews have summarized the morphology and function of BALT (McDermott *et al.*, 1982; Sminia *et al.*, 1989). An individual aggregate of BALT or bronchus-associated lymphoid unit (BALU), a term defined by Sminia and col-

leagues, consists of a focal area of T and B cells admixed with fibroblasts, reticulum cells, macrophages, interdigitating cells which are comparable to Ia-positive DCs, and follicular dendritic cells. BALUs have no capsule, subcapsular sinuses, nor afferent lymphatics. Nevertheless, they have peripheral sinus-like lymphatics which subsequently drain into lymph nodes (Lauweryns and Baert, 1976–1977). Furthermore, there are arterioles, capillaries, and venules which include high endothelical venules (HEV) (Otsuki *et al.*, 1989). The structure of a BALU with the demonstration of HEV indicates the likelihood that naive T cells might migrate to these structures and initiate a primary immune response against an antigen translocated from the bronchial lumen, comparable to the role for Peyers Patches in the gut. Although early studies failed to demonstrate antigen could cross the overlying epithelium, more recent studies have suggested that soluble antigens might be translocated from the bronchial lumen across the epithelium overlying a BALU (Fournier *et al.*, 1977; Myrvik and Ockers, 1982; van der Brugge-Gamelkoorn *et al.*, 1985).

In BALUs lymphocytes are partitioned into B and T cell areas with central B cells surrounded by T cells. The dome over these areas and underneath the epithelium is a mixture of B and T cells (Sminia *et al.*, 1989). In BALUs, IgM- and IgG-bearing cells are present in significant numbers together with IgA-positive cells which contrasts with Peyers Patches in which IgA cells predominate (Sminia *et al.*, 1989). In rats, BALT has been examined with monoclonal antibodies to determine the type of T cells present; CD4-positive cells outnumber CD8-positive cells, but in the B cells aggregates nearly all of the T cells are CD4-positive cells (Sminia *et al.*, 1989).

Important studies relating to the traffic of T and B cells to BALUs have been published (van der Brugge-Gamelkoorn and Kraal, 1985) demonstrating *in vitro* binding of equal numbers of T and B lymphocytes to both rat and guinea pig BALUs. This finding is clearly different from Peyers Patch binding of T and B cells in which the numbers of B cells that bind are much greater than T cells that bind at a ratio of five B cells for every T cell (Stevens *et al.*, 1982). This finding corresponds to the increase in B cells in Peyers patches relative to the numbers of B cells in BALUs (Crawford and Miller, 1984). Taken together, these studies indicate that the specificity of HEV in BALUs is different from that in Peyers patches and more closely resembles the specificity of the HEV in mesenteric lymph nodes (van der Brugge-Gamelkoorn and Kraal, 1985).

It is important to address the issue of a similarity of BALT with gut-associated lymphoid tissue. BALT is prominent in certain species

including chicken, rabbit, and rat, but is clearly much less prominent in other species, including man. In some members of these species, BALT may be completely absent (Pabst, 1990). Nevertheless, even in man, other studies have shown that after birth, there is a gradual increase in loose aggregates of lymphoid cells that collect beneath the epithelium, particularly at points of bifurcation of bronchi. In man, mouse, and hamsters, these collections of lymphoid cells do not involve the epithelium nor modify the epithelium over the aggregate. In view of the relative lack of prominence of these structures in certain species, it suggests that their role in protective immune responses in the lung is not essential. Since an important function in the gut is for these structures to initiate IgA responses, it is possible that the lack of well-organized BALT in some species predicts that local initiation of IgA responses is not required for health. It also suggests that the common mucosal system in which IgA B cell precursors are developed in the gut and migrate to the lung may function to successfully protect the host from lung infections.

III. Lung Immunity to Noninfectious Particulate and Soluble Antigens

Since the late 1960s, investigators have been systematically exploring mechanisms in the development of immune responses to particulate and soluble protein antigens in the lung (Pepys, 1969). A major impetus was to understand what caused hypersensitivity lung diseases, such as asthma and hypersensitivity pneumonitis, to develop in some individuals, but not in others, although antigenic exposures were the same. Models to examine immune responses to various respiratory antigens were developed in many animal strains, including mice, rats, hamsters, guinea pigs, ferrets, dogs, monkeys, horses, and cattle; antigens were delivered via aerosol, intranasal, intratracheal, or intrabronchial instillation. The end point for immunity in experimental animals was generally measured by assessing the development of hypersensitivity disease clinically and morphologically, measuring serum and/or bronchoalveolar antibody, or characterizing some aspect of cell-mediated immunity (CMI) such as migration inhibition, delayed-type hypersensitivity (DTH) via skin test, or lymphoproliferation (Richerson, 1972; Newhouse *et al.*, 1976; Kaltreider, 1976; Kazmierowski *et al.*, 1977; Ganguly and Waldman, 1977).

Among the important findings of these early studies were that soluble antigens instilled into the lungs, in contrast to particulate antigens, often failed to produce immunologic lung damage (Schatz *et al.*, 1977; Fink, 1988), and that soluble antigen repeatedly instilled into the lung

could lead to local tolerance (Ratzjczak *et al.*, 1980; Holt and Leivers, 1982). Interpretation of early studies did not benefit from the current perspective that the type of immune response that develops, i.e., CMI versus antibody (including the predominant isotype of the antibody), is regulated by cytokines secreted by T cells and other cells present at sites of antigen deposition (Mosmann and Coffman, 1989). More recent studies have expanded the important concepts derived from earlier studies by focusing on the regulatory mechanisms in the development of lung immunity.

In order to understand how various forms of antigen might reach the immune apparatus and, therefore, antigen clearance is discussed. Then, while T and B cell immunity are clearly interdependent, studies that have focused on measuring specific T cell responses versus immunoglobulin synthesis in response to noninfectious lung antigens are summarized. Models that specifically examine the lung's response to infectious agents and alloantigens or lead to hypersensitivity disease are covered later.

A. Lung Clearance of Antigens

Many studies have shown that the LALNs are responsible for primary immune responses after lung immunization (Bice *et al.*, 1980b; Kaltreider *et al.*, 1983; Stein-Streilein *et al.*, 1979; Stein-Streilein and Hart, 1980; Lipscomb *et al.*, 1982). LALNs function as effective filters to remove particulate materials cleared from the lower respiratory tract via the lymphatics (Brain *et al.*, 1978; Green *et al.*, 1977; Morrow, 1972). Although LALNs are largely responsible for the induction of immunity after primary immunization, the mechanisms responsible for the clearance of antigen from the lung to LALNs are not completely understood. Most antigen deposited in the lung is cleared by phagocytosis by AMs and neutrophils that transport foreign material up the mucociliary escalator and out of the lung, although some antigen is transported to LALNs where an immune response is produced. At least some of this latter antigen is carried free in lymphatic fluid and apparently occurs in circumstances of limited inflammation (Lauweryns and Baert, 1976–1977).

The induction of pulmonary inflammation by antigen exposure appears particularly important in the translocation of immunogens from the lung to LALNs. Exposure of the lung to noninflammatory doses of antigen often fails to induce immune responses (Yoshizawa *et al.*, 1982; Bice *et al.*, 1991). It is possible that an immune response to airway antigens requires a dose that overwhelms normal phagocytic and clearance mechanisms (Bice and Muggenburg, 1988; Bice *et al.*, 1991). The observation that elevated immune responses are produced

in LALNs if antigen is deposited in the lungs of animals that have inhaled inflammagens further supports the importance of pulmonary inflammation in the translocation of antigen from the lung to the LALNs (Bice *et al.*, 1985,1987b). It is possible that inflammation may also alter the relative proportion of antigen reaching LALNs in cells as opposed to antigen free in lymphatic fluid.

In dogs inoculated via the airways with sheep red cells, a large number of neutrophils enter the lung from the vasculature with a peak response about 1 day after instillation of antigen (Bice *et al.*, 1989). Furthermore, neutrophils can phagocytize particles in the alveoli and migrate to the LALNs carrying the particles (Harmsen *et al.*, 1987). AMs can also phagocytize particles in the lung and transport them to LALNs (Corry *et al.*, 1984; Harmsen *et al.*, 1985). The relative contribution of neutrophils and AM in the translocation of antigen from the lung to LALNs is not known. However, neutrophils with phagocytized particles reach LALNs earlier than AMs and, may be more important for antigen transport to LALNs than AMs. Antigen transported to the LALNs may be released from both neutrophils and AMs and reprocessed by resident APCs to initiate pulmonary immunity. In addition to AMs and neutrophils, as previously discussed, lung DCs also likely carry antigen to the LALNs, and recent studies in which pulsed splenic DCs inoculated into the trachea were capable of immunizing the hosts supports this concept (Havenith *et al.*, 1993c). Whether antigen or lung DC initiates a different type of T helper subset response than antigen arriving in phagocytes or free in lymph has not been determined. Nevertheless, if lung inflammation enhances the transport of antigens from the lung to the LALNs, it is possible that inhalation of materials that induce pulmonary inflammation might lead to increased recognition of airborne antigens. Thus, pulmonary inflammation caused by inhaled pollutants (Osebold *et al.*, 1980) and passive cigarette smoke (Murray and Morrison, 1988; Ehrlich *et al.*, 1992) might increase the immune recognition of allergens and be responsible for increasing rates of asthma (Evans *et al.*, 1987; Platts-Mills *et al.*, 1991). In addition, inflammation induced by pulmonary viral infections may also be important in the induction of immunity to low levels of environmental antigens, e.g., allergens responsible for asthma (Castleman *et al.*, 1990; Duff *et al.*, 1993).

B. T Cell-Mediated Lung Immunity

Several important questions relate to the development of CMI in the lung and include (1) do antigens instilled into the lung cause local and/or systemic CMI? (2) Does the form of antigen, i.e., soluble, particulate, expressed by viable microorganisms influence the out-

come? (3) Does iv or subcutaneous inoculation of similar antigens generate similar degrees of CMI in the lung? (4) What redirects the immune T cells back into the lungs?

Inoculation of antigens into the lung can result in both local and systemic CMI (Kaltreider, 1976). As discussed previously, both soluble and particulate antigens can induce immunity, but soluble antigens induce a less easily detectable CMI response than a similar antigen delivered in particulate form as shown by experiments in which either soluble or aggregated human serum albumin were used as the immunogen (Burrell and Hill, 1975; Hill and Burrell, 1979). Infectious organisms which replicate *in situ* are even more capable of producing CMI both locally and systemically (Waldman *et al.*, 1972; Spencer *et al.*, 1974; Ganguly and Waldman, 1972; Lipscomb *et al.*, 1982). Haptens, such as trinitrobenzene (Stein-Streilein, 1983), or metals (Parker and Turk, 1978), such as beryllium oxide (Haley *et al.*, 1989), may also induce CMI in the lung following direct instillation into appropriate animal models. Haptens and metals are agents that lead to sensitization of humans exposed to these agents in the workplace and are associated with hypersensitivity reactions.

A number of early studies addressed the issue of whether subcutaneous, iv, or direct lung instillation of antigens resulted in differences in the expression of CMI. In three separate studies, guinea pigs injected via the lung with human γ-globulin (HGG), DNP–HGG, or heat-killed influenza virus accumulated specific T cells among lymphocytes recovered from the lungs as measured by migration inhibition factor release or by antigen-induced lymphoproliferation assays. However, subcutaneous or iv injection of these antigens failed to result in measurable specific T cell accumulation in the lungs, although systemic CMI could be measured in lymphocytes from nodes draining the subcutaneous inoculation site or from spleen (Waldman and Henney, 1971; Nash and Holle, 1973; Lipscomb *et al.*, 1982). However, the initiation of a mild inflammatory response in the lungs resulted in the accumulation of immune T cells in the lungs of the animals immunized via the extrapulmonary route (Waldman *et al.*, 1972; Nash and Holle, 1973; Lipscomb *et al.*, 1982).

Using a live, attenuated rubella vaccine or another live strain of rubella virus, the kinetics of a local CMI response was studied following the inoculation of guinea pigs either subcutaneously or by an intranasal inoculation (Morag *et al.*, 1974). In these studies, migration inhibition factor activity was the parameter for measuring CMI responses and was initially detected in the lungs 2 weeks after immunization, peaked at 4 weeks, but was no longer detectable by 6 weeks.

When primary immune responses are generated in the LALN, what

stimulus recruits cells back to the lungs? Lymphoblasts rapidly exit lymph nodes during developing immune responses (Joel and Chanana, 1987). These cells enter the circulation and, under the control of adhesion molecules and locally generated chemotactic and other adhesion molecule-stimulating cytokines, are recruited into inflamed lungs (Berman *et al.*, 1990). As previously discussed, although markedly inflamed lungs nonspecifically recruit both T cells and B cells, memory T cells generated in LALNs may also have homing molecules that specifically direct their return to the lung (Picker *et al.*, 1994). An important factor in retaining recruited immune-specific cells is the continued presence of specific antigens within tissue and at least two groups have shown that this can occur (Lipscomb *et al.*, 1982,1983; Lyons and Lipscomb, 1983; Emeson *et al.*, 1982). Thus, T cell blasts enriched for two different antigens and labeled with two distinguishable radioisotopes were shown to be retained in lung lobes nonspecifically, but with an additional selectivity in lung lobes containing the relevant antigen (Lipscomb *et al.*, 1982). Selective retention was induced by antigen carried by APC deposited in the lungs and was class II MHC restricted, suggesting that the T cells were retained in the lung lobes by binding to the APC *in vivo* (Lyons and Lipscomb, 1983). These studies added validity to a concept that four general mechanisms relate to recruitment and retention of immune cells in the lungs: (1) recently activated T and B cells, i.e., lymphoblasts, from any lymphoid tissue are nonspecifically recruited into inflamed lungs; (2) cells recently activated in LALNs express adhesion molecules that are uniquely designed to target their binding to lung endothelium; (3) matrix and lung parenchymal cell adhesion molecules expressed under the control of local environmental perturbations facilitate the emigration of immune cells; and (4) antigen expressed on the appropriate MHC in the lung leads to preferential retention and further expansion in the lung. Specificity of recruitment for B cells has not been shown (see below), but in the presence of retained antigen and specific T helper cell recruitment, specific B cells could divide and differentiate.

C. B Cell-Dependent Lung Immunity

1. *Primary Immune Responses*

Antigen-specific antibody produced in LALNs after a primary lung immunization is released into blood (Bice *et al.*, 1980a; Shopp and Bice, 1987). In addition, large numbers of antigen-specific IgG, IgA, and IgM AFCs produced in LALNs also enter the blood after lung immunization of sevral species, e.g., dogs, cynomolgus monkeys, chim-

panzees, humans (Bice *et al.*, 1980a,1982b; Kaltreider *et al.*, 1981; Mason *et al.*, 1985; Weissman *et al.*, 1994). In studies of larger animals, AFCs in blood are recruited into a lung lobe exposed to antigen, but significantly fewer AFCs are found in the other lung lobes of the same animal that are exposed to saline or nothing.

There appear to be two factors that control the entry of AFCs into the lung. First, AFCs must have been recently produced in an immune response (Bice *et al.*, 1989). The lymphoid tissue in which they are produced exerts no control on their entry into the lung because AFCs produced in the popliteal lymph nodes enter the lung at the same rate as AFCs produced in LALNs (Hillam *et al.*, 1985). Second, AFCs enter sites of inflammation produced by instillation of antigen into the lung (Bice *et al.*, 1982a; Hillam *et al.*, 1985). However, the recruitment of AFCs into inflammatory sites in the lung is not antigen specific because they also enter lung lobes inflamed by instillation of particles or other inflammatory agents.

Plasma cells are found in the alveoli and interstitial lung tissues of immunized lung lobes suggestig that AFCs that enter the lung mature to plasma cells (Bice *et al.*, 1987a). Most antigen-specific IgM, IgG, and IgA antibody in the lung after a primary exposure to antigen is produced locally by these cells (Hill *et al.*, 1983). The few AFCs in control lung lobes exposed to saline also actively produce antigen-specific antibody (Bice *et al.*, 1980a,1989). The results of several studies show that AFCs in the lung after a primary immunization are recruited into the lung and are not produced locally (Mason *et al.*, 1985; Bice *et al.*, 1989). In addition, cell numbers are not amplified by interaction with antigen that might have been retained in the lung after primary immunization (Bice *et al.*, 1982a). In addition to AFCs, large numbers of other lymphocytes enter the lung with a peak response occurring between 7 to 14 days after immunization with a mean of 25% of the total lavage cells being lymphocytes (Bice *et al.*, 1989).

Species differences exist in the release of AFCs into the blood from LALNs and in the recruitment of AFCs into the lung. Dogs (Bice *et al.*, 1982b), nonhuman primates (Bice *et al.*, 1982a, Mason *et al.*, 1985), and humans (Stevens *et al.*, 1979; Lue *et al.*, 1988; Weissman *et al.*, 1994) all have large numbers of AFCs in their blood after immunization, and blood AFCs enter the lung. In contrast, data from a single immunization of the lungs of rats, guinea pigs, rabbits, and mice suggest that few or no AFCs are released into blood, and that relatively few AFCs appear subsequently in the lung (Bice and Shopp, 1988). However, the use of adjuvants and large doses of antigen appears to increase the number of AFCs in the lung of guinea pigs and mice

(Shopp and Bice, 1987; Curtis and Kaltreider, 1989). It is possible that the strain of species being evaluated may also be important, although no data are available that compare pulmonary B cell responses in different strains of laboratory animals.

2. *Memory Responses*

Although primary immune responses are not produced in the lung independently of secondary lymphoid tissues, data suggest that memory responses may be detected in the lung to antigen challenges that are independent of LALNs (Mason *et al.*, 1985; Jones and Ada, 1986, 1987; Bice *et al.*, 1991). The most logical explanation for the production of AFCs and antibody in the lung after an antigen challenge is that immune memory cells are recruited into and/or develop in the lung after a primary immunization. Unlike a primary immune response, most specific IgG and IgA antibody produced in an immunized lung after a rechallenge with antigen appears to come from local immune memory B cells and localized production of AFCs, rather than by AFCs recruited into the lung from blood. Only minimal specific IgM is produced in the lung after antigen rechallenge (Bice *et al.*, 1991).

3. *Long-Term Antibody Production in the Lung*

Specific antibody continues to be produced in the lung for several years after the last exposure to antigen (Bice *et al.*, 1991). Lavage fluid from immunized and challenged lung lobes contained significantly more specified IgG several years after the last exposure to antigen than was present in lavage fluid from control lung lobes. Thus, once an intense, localized antibody response was established in the lung, immune mechanisms supported continued localized antibody production for several years after the last exposure to antigen, but only at the site of antigen exposure.

Although AFCs were identified in lavage fluid from exposed lung lobes several years after antigen challenge, it was possible that cells in interstitial lung tissue, as well as in LALNs or distant lymphoid tissues, were all important in long-term antibody production. However, the evaluation of antibody production in lung and various extrapulmonary tissues showed that most long-term antibody production occurred in interstitial tissue in the immunized lung lobe (Bice *et al.*, 1993). Cells from control lung lobe tissue, from LALNs that received lymphatic drainage from the immunized lung lobes, from spleen, gut-associated lymph nodes, or popliteal lymph nodes did not produce significant levels of antibody 2 years after the last antigen challenge. Therefore, immune cells retained in lung tissue previously exposed

to antigens may be an important source of antibody to protect the lung. In addition, the absence of antibody production in LALNs or in other distant lymphoid tissue suggests that antibody produced in lung tissue exposed to antigen could possibly enter the bloodstream and provide immune protection for unexposed lung lobes and extrapulmonary tissues.

Two possible mechanisms could be responsible for long-term antibody production in lung lobes previously exposed to antigen. First, antigen retained in the lung, possibly on follicular dendritic cells, could stimulate antibody production by antigen-specific memory B lymphocytes that migrate through the lung. Alternatively, B lymphocytes recruited into or produced in the lung in response to the initial antigen challenge might live for several years and continuously secrete antibody. Because continuous antibody production occurs only in lung lobes exposed to antigen, an antigen depot may be essential. Data have been published that support both possibilities (Tew *et al.*, 1990; Peeters and Carter, 1981).

In summary, studies suggest that pulmonary humoral immunity can be maintained both by continued long-term spontaneous antibody production and by antigen challenge restimulating local pulmonary memory B cells to secrete antibody.

IV. Models for Immunity in Lung Infections

Despite the ability of the lung to express both natural and acquired immunity, respiratory tract infections are the most common type of infections experienced by humans. Certainly the common cold alone wins this competition hands down! Vaccination has been a powerful intervention to protect against many respiratory tract infections including the bacteria *Bordetella pertussis*, *Corynebacteria diphtheriae*, *Hemophilus influenzae*, *Streptococcus pneumoniae*, and *M. tuberculosis* and viruses, including one virus that primarily infects the respiratory tract, i.e., influenza, and several which initially infect via the respiratory tract, rubeola, rubella, and mumps. A major impetus for current research in infectious diseases is to learn more about natural host defenses in infections and how the immune system amplifies these defenses (Mason and Nelson, 1992). By better understanding these strategies, the hope is to optimize vaccination of immunocompetent hosts and perhaps even hosts who are immunosuppressed, yet retain some capacity to respond immunologically. Alternatively, in immunosuppressed individuals, once we are better able to understand how immunologically derived cytokines function in the normal host

during infections, recombinant forms might be administered as replacement.

The use of animal models has considerably enhanced our understanding of lung infections and the role of immunity in controlling them. In most pneumonias caused by extracellular bacteria, recruited phagocytes and opsonins, especially antibody and complement, are required to effectively control infections, even when antibiotics are used. Thus, the goal for vaccination in these pneumonias is to raise the level of local antibody. For chronic pneumonias and pneumonias caused by intracellular microorganisms, the goal of vaccination is less clear, although protection against the viruses listed previously correlates with serum antibody levels. The best evidence supports the probability that enhancing CMI would be protective for immunization against many obligate and facultative intracellular bacteria, fungi, and parasites (Lipscomb, 1989; Campbell, 1993).

In this section, examples of animal models of infectious disease that address how pulmonary immunity develops to various etiologic agents are discussed, and the type(s) of immunity that afford protection are indicated. While investigators have used experimental models to study nearly all of the infectious agents that produce respiratory infections, space dictates that only a few representative studies be included here.

A. Acute Bacterial Pneumonias

1. *Streptococcus pneumoniae*

The administration of *S. pneumoniae* into the lung leads to a rapid accumulation of neutrophils in the alveolar space. The development of this local inflammatory response during *S. pneumoniae* pulmonary infections was noted in early histopathological studies (Loosli, 1940; Wood, 1941; Loosli, 1942). Subsequent studies confirmed the requirement for intact granulocyte function in the host to eradicate the pulmonary infection (Wood *et al.*, 1946; Heidbrink *et al.*, 1980). Studies have examined the mechanisms responsible for neutrophil recruitment during the early stages of *S. pneumoniae* pulmonary infection (Vial *et al.*, 1984; Bruyn *et al.*, 1992). Several groups demonstrated that animals systemically decomplemented with cobra venom factor had an impaired ability to recruit neutrophils and to clear the organisms from the bronchoalveolar space. A role for C5 in the recruitment of neutrophils in response to intratracheally delivered *S. pneumoniae* was assessed by using congenic C5-sufficient ($C5^+$) and C5-deficient ($C5^-$) mice (Toews and Vial, 1984). The results indicated that C5 was important in producing optimal, early neutrophil recruitment and bacte-

rial clearance in response to *S. pneumoniae*, but other chemotaxins must be involved, because chemotactic activity and neutrophil recruitment was found in both $C5^+$ and $C5^-$ mice.

Once phagocytic effector cells were recruited into the lung, neutrophils and macrophages required the opsonins, immunoglobulin and complement, for efficient phagocytosis of the *S. pneumoniae* (Guckian *et al.*, 1980; Coonrood and Yoneda, 1981). The presence of C3b on the surface of the pneumococci is vital for phagocytosis. C3b can be deposited on *S. pneumoniae* by either the classical complement pathway through interaction with antibodies or through the alternative complement pathway (Winkelstein, 1981; Joiner *et al.*, 1980). *Streptococcus pneumoniae* that are not killed by the initial pulmonary inflammatory reaction drain to LALNs and eventually enter the systemic circulation resulting in a bacteremic phase (Austrian, 1981). During this phase, type-specific antibody to capsular polysaccharide is produced. It has been demonstrated through passive immunization studies that the presence of type-specific antibody (IgG and IgM) in the serum is protective against severe pneumococcal infection (Musher *et al.*, 1990). Nontype-specific antibodies to *S. pneumoniae* are made during infection, including antibodies to cell wall components (Brown *et al.*, 1983), surface protein A (Szu *et al.*, 1983), and the F polysaccharide (Au and Eisenstein, 1981). In general, most animal studies indicate that these latter antibodies play a minimal role in providing effective protection against infection by *S. pneumoniae* (Szu *et al.*, 1986; Brown *et al.*, 1983).

The role of secretory IgA in the prevention of pneumococcal disease is unclear, although one mouse model demonstrated that *S. pneumoniae*-specific IgA could "arm" lung lymphocytes which subsequently demonstrated antibacterial action against *S. pneumoniae* (Sestini *et al.*, 1988). IgA has been reported to fix complement and act as an opsonin (Hiemstra *et al.*, 1988; Gorter *et al.*, 1989) and thus could play a role preventing *S. pneumoniae* infection. Finally, humoral factors other than antibody have been implicated in protection against *S. pneumoniae*, particularly C-reactive protein which can activate complement and act as an opsonin when bound to the capsule. Although a role for C-reative protein has been demonstrated in clearing *S. pneumoniae* from the bloodstream (Volanakis and Kaplan, 1971; Horowitz, *et al.*, 1987), a role for C-reactive protein in the pulmonary stages of infections has not been demonstrated.

2. *Haemophilus influenza*

Models for acute pulmonary infection with *H. influenza* have been developed in the rat (Wallace *et al.*, 1989) and mouse (Esposito and

Pennington, 1984; Toews *et al.,* 1984b). The latter method delivers a reproducible bolus of organisms to the lower respiratory tract via an endobronchial catheter. Using this technique it was determined that the clearance of both typable and nontypable *H. influenza* from lungs occurred at a very similar rate. The clearance of the organisms appeared to occur in two phases. During the initial 6 hr postinoculation, the organisms increased in numbers three- to fivefold, while during the next 18 hr the organisms were rapidly cleared (Toews *et al.*, 1984b). Studies indicated that the rapid clearance phase corresponded to the influx of neutrophils into the lung and that the presence of these leukocytes was vital for effective clearance (Toews *et al.*, 1985). The effect of specific antibody to the *H. influenza* on the rate of pulmonary clearance was examined using both active immunization and passive administration of immune sera. The results indicated that the presence of specific antibody in the serum and the BAL fluid of immunized mice correlated with an increased rate of clearance from the lung. That systemic IgC could provide protection in the lower respiratory tract of animals was also demonstrated by experiments showing enhanced clearance of *H. influenza* from the lungs of mice that had received immune sera. Taken together, these results indicated that in the presence of elevated titers of serum IgG, protective antibodies could enter the airways of infected lungs to provide protection against pulmonary pathogens.

3. *Staphylococcus aureus*

In contrast to the organisms discussed previously, previous immunization with *S. aureus* does not appear to enhance clearance or provide protective antibody in pulmonary infections (Jakab, 1976). Recent studies suggest that the pulmonary clearance of this organism may be dependent on locally produced opsonins that enhance phagocytosis by AM. Surfactant protein A, produced by type II pneumocytes, can bind to *S. aureus* and increase phagocytosis, while this protein does not enhance uptake of *S. pneumoniae* by AM (McNeely and Coonrood, 1993).

B. Chronic Bacterial and Fungal Pneumonias

Lung infections with two microorganisms, Mtb and *Cryptococcus neoformans* (Cne), are discussed in this section as examples of infections requiring intact CMI for resolution. Animal models of chronic lung infections with several other important pathogenic organisms have been studied, including *Pneumocystis carinii* (Walzer, 1984; Shellito *et al.*, 1990; Harmsen and Stankiewicz, 1990; Boylan and Current, 1992), *Histoplasma capsulatum* (Baughman *et al.*, 1986; Defaveri and

Graybill, 1991; Fojtasek *et al.*, 1993; Allendoerfer *et al.*, 1993), *Blastomyces dermitiditis* (Morozumi *et al.*, 1982; Moser *et al.*, 1988; Frey *et al.*, 1989; Williams *et al.*, 1994), *Paracoccidiodes braziliensis* (Brummer *et al.*, 1984; Defaveri *et al.*, 1989), *Coccidiodes immitis* (Cox *et al.*, 1988), *Chlamydia trachomatis* and *psittaci* (Williams *et al.*, 1988), *Rhodococcus equi* (Kanaly *et al.*, 1993), and *Mycobacterium avium-intracellulare* (Takashima and Collins, 1988). Although infection with *Legionella pneumophila* can cause an acute pneumonia in susceptible hosts, it is a facultative intracellular bacterium; CMI is thought to be necessary for resolution of the infection. An animal model to study this infection has also been developed (Skerrett and Martin, 1991).

A central role for CMI (in which T cells recruit and activate macrophages) in controlling intracellular bacterial infections was first proposed by George Mackaness using a *Listeria monocytogenes* murine infection model (Mackaness, 1964). After finding an important role for CMI in controlling an aerogenous *Listeria* infection in mice, Mackaness extended his studies to propose that cell-mediated hypersensitivity might be an important cause of lung disease (Mackaness, 1971). However, he and his collaborators observed that, in contrast to protection afforded by active immunization, adoptive transfer of *Listeria* immune splenocytes seemed to afford only minor protection against an aerosolized infection (Truitt and Mackaness, 1971). In retrospect, adoptive immunity might have been transferred more successfully if LALNs or lung lymphocytes from aerosol-infected mice had been used instead of spleen cells from systemically immunized mice; these latter cells likely homed inefficiently to the infected lung (Huffnagle *et al.*, 1991b). Nevertheless, in experiments with virulent Mtb, when organisms were given iv, the lung developed effective resistance, although less effectively than spleens and livers (Mackaness, 1971). These early studies suggested that CMI in the lung might be more rigidly downregulated, perhaps to prevent excessive damage to delicate structures. However, Mackaness offered an additional explanation, e.g., lung infections with Mtb may be more difficult to control locally because organisms are sequestered in AM, macrophages that, in contrast to recruited monocytes, might resist activation signals delivered by T cells.

1. *Mycobacterium tuberculosis*

The role of pulmonary immunity during Mtb infection has been analyzed in a variety of animal models (Smith and Wiegehaus, 1989) including rabbits (Lurie, 1964), mice (Orme and Collins, 1984; North and Izzo, 1993), and guinea pigs (Smith and Harding, 1977). Initial

experiments involved intranasal or intratracheal inoculation and the development of the Middlebrook chamber (Middlebrook, 1952) provided a means of aerosolizing Mtb into animals. Early studies examined the number of Mtb required for a reproducible infection in animals. Mice exposed to a mist of virulent Mtb developed discreet lesions that were progressively fatal over a 10–21 week period (Schwabacher and Wilson, 1937). These initial studies were extended by comparing aerosol versus intranasal delivery of Mtb and it was found that both routes produced similar pathology. It was observed that a deposited inoculum of about 100 organisms was required for reproducible infection, while a dose of approximately 12,000 organisms resulted in death (Glover, 1944).

Resident AM undoubtedly play a role during an Mtb infection. Mtb deposited into the lung are rapidly taken up by AM. Evidence for a role for AM in defense against Mtb partly comes from epidemiologic studies examining Mtb infections in individuals with silicosis (Snyder, 1978). Silica exposure results in the uptake of silica particles by AM. These silica particles remain in the phagolysosomes of AM throughout the life of the individual (Allison and D'Accy Hart, 1968) and likely affect their function. Essentially all epidemiological studies examining the incidence of Mtb infections in a silica-exposed population have concluded that the incidence of Mtb infections in this group is significantly higher than the incidence of Mtb infection in a non-silica-exposed population (Snyder, 1978).

Several studies have shown that both human and/or mouse AM are stimulated to produce chemotactic factors and cytokines in response to Mtb or components of the Mtb cell wall (Barnes *et al.*, 1992; Chatterjee *et al.*, 1992). These released products may represent an early native defense system against Mtb. Thus, chemotactic factors can act to recruit neutrophils and monocytes from the circulation, while AM-released cytokines, such as TNFα, can activate both local AM and newly recruited cells. Indeed, some studies indicate that cytokine-activated AM and/or monocytes can inhibit the growth of or kill Mtb (Crowle, 1990; Rastogi, 1990; Denis, 1991a). More recent data suggest that macrophage cytokines, including IL12, may enhance the development of the Th1 subset leading to protective immunity (Hsieh *et al.*, 1993). Data indicating that avirulent Mtb can elicit a greater cytokine response from macrophages than virulent Mtb have lead to the hypothesis that the observed differences in Mtb virulence may be due to an intrinsic ability of virulent Mtb to prevent or decrease the release of factors by AM (Barnes *et al.*, 1992; Chatterjee *et al.*, 1992; Roach *et al.*, 1993). Ethnic differences observed in susceptibility to Mtb (Coultas *et*

al., 1993) might be due to a genetic disposition for a poor initial response by AM to Mtb.

A role for CMI was demonstrated for protection against Mtb infection (Suter, 1961; Leveton *et al.*, 1989). In a guinea pig model, it was demonstrated that in animals given a low dose of Mtb, the organisms replicated in a log phase until Days 19 or 20, after which exponential growth ceased (Smith and Harding, 1977). The decrease in growth coincided with the onset of tuberculin skin test sensitivity and the development of detectable bacillemia. Bacteriostasis ensued over the next 40–50 days after which the numbers of Mtb in lung were gradually reduced. Although these data were consistent with a role for the development of an acquired CMI response for resolution of the Mtb infection, it was not until Orme and Collins (1984), by examining the immune response in a mouse model, that direct evidence was provided for a role of T cells in pulmonary immunity against Mtb. In a series of adoptive transfer experiments, they removed splenic T cells from a mouse that had received an iv inoculation of *Mycobacterium bovis* or BCG. After injecting these BCG-immune T cells into thymectomized, sublethally irradiated nonimmune mice, the mice were challenged with an aerosol dose of Mtb that deposited 10^4 organisms into the lungs. Two important findings in these studies were (1) adoptively transferred immune T cells enhanced clearance of Mtb from the lung; and (2) by differentially removing subsets of T cells with specific antisera, the skin test tuberculin sensitivity was dissociated from protective antituberculous immunity which indicated that separate populations of T cells may be responsible for the two events. In a follow-up study (Orme, 1987), only Mtb-immune CD8 T cells adoptively transferred protection to mice challenged with a lethal aerosol inoculum (1.5×10^5 organisms) of Mtb, while either CD4 or CD8 cells could transfer protection to mice exposed to a low dose (500 organisms) of Mtb.

In comparison to the pulmonary inoculation studies, models using intraperitoneal or iv routes of inoculation have produced different results. In an *intraperitoneal* model, an Mtb-reactive CD4 T cell clone provided both a DTH response and protection as measured by the growth of Mtb in the peritoneum (Pedrazzini and Louis, 1986). Similarly, an Mtb-immune CD4 T cell clone provided protection, as measured by reduced splenic CFU, following an iv Mtb infection. Another study using *in vivo* depletion of T cell subsets demonstrated that depletion of CD4 T cells decreased resistance to iv infection, while depletion of CD8 T cells did not have a significant effect (Pedrazzini *et al.*, 1987). In contrast, in a similar model, transgenic mice incapable

of producing CD8 T cells were shown to have a decreased resistance to Mtb compared to normal mice (Flynn *et al.*, 1992).

A role for $\gamma\delta$ T cells in pulmonary defenses against Mtb is unresolved (O'Brien *et al.*, 1989; Kaufmann and Kabelitz, 1991). Because $\gamma\delta$ T cells release IFNγ, it is tempting to speculate that these T cells represent an initial defense mechanism in the lung to provide activating cytokines to enhance local effector mechanisms to help control the infection until the development of protective immunity by $\alpha\beta$ T cells. Initial studies demonstrated an increase in the number of lung $\gamma\delta$ T cells after an intratracheal dose of PPD (Janis *et al.*, 1989). Other studies suggested that many $\gamma\delta$ T cells responded to the heat-shock protein of Mtb (Born *et al.*, 1991; Kaufmann and Kabelitz, 1991). Other studies suggested they may play a role in granuloma formation (Modlin *et al.*, 1989). However, in humans with active Mtb infections, there was no increase in $\gamma\delta$ T cells in the granuloma as determined by immunohistochemical staining (Tazi *et al.*, 1991). More work in animal models and human natural infections is required to define the role of $\gamma\delta$ T cells in mucosal immunity, particularly regarding their role in Mtb infections.

Complex interactions exist in the development of protective immunity by T cells and the type of cytokines produced during an infection. Similar to the studies that show an important protective role for Th1 cells that preferentially secrete IFNγ in *Leishmania* infections (Locksley *et al.*, 1991), it is likely that mechanisms for production of appropriate cytokines are critical in the development of protective immunity against Mtb (Flesch, 1990; Denis, 1991b; Kawamura *et al.*, 1992; Barnes *et al.*, 1993; Orme *et al.*, 1993) as well as in the maintenance of a resistant state during the chronic infection stage. An important role for IFNγ in Mtb resistance was recently demonstrated in both an aerosol and an iv Mtb infection model. Comparing infected normal and IFNγ knockout mice (Cooper *et al.*, 1993; Flynn *et al.*, 1993), it was demonstrated that a lack of IFNγ resulted in a significant increase in Mtb susceptibility. However, since IFNγ was absent throughout the course of infection, it was unclear at what stage in the immune response IFNγ was required (Flynn *et al.*, 1993). Indeed, IFNγ may be important for all aspects of the response to Mtb including T cell development, cell recruitment, and activation of effector mechanisms.

Other studies in mice examined the granulomatous response to iv-injected BCG in animals that had received neutralizing antibody to TNFα (Kindler *et al.*, 1989). These and other studies (Amiri *et al.*, 1992) indicate that TNFα also plays a critical role in protection against Mtb, particularly in the development and maintenance of granulomas.

Attempts to vaccinate animals with avirulent or killed Mtb have

provided important data regarding potential vaccines. In general, studies suggest that to enhance the immune response against virulent Mtb, viable organisms must be used (Larson and Wicht, 1962). The route of immunization with viable organisms can be either iv or by aerosol. Further, vaccination does not prevent infection, but rather limits tissue destruction and the degree of hematogenous dissemination (Harding and Smith, 1977). Immunization with nonviable cellular elements does not afford protection.

In summary, the development of protective immunity to a pulmonary infection with Mtb requires the coordinated activity of multiple cell types, particularly macrophages and T cells. The continued study of the Mtb pulmonary infection should aid in understanding the mechanisms for developing effective CMI in the lung and suggest strategies to enhance pulmonary defenses.

2. *Cryptococcus neoformans*

Cne is an encapsulated yeast found in desiccated form in soil, particularly in areas contaminated by pigeon feces. Cne usually causes only an asymptomatic infection in humans following inhalation. Normal individuals typically clear the organisms, but in those who are susceptible, particularly those with defects in CMI, the organism may disseminate via the bloodstream and produce an extrapulmonary infection, usually meningitis. Mice have been used as experimental models for studying the host defenses against this microorganism, which because of the capsule resists endocytosis and thus typically replicates in tissues in an extracellular location. In murine models the organism was frequently inoculated iv or ip, although it had been established many years ago that mice housed on contaminated bedding (Smith *et al.*, 1964), exposed to aerosols (Karaoui *et al.*, 1977), or that received intranasal inoculations of the organism (Ritter and Larsh, 1963) developed infection. These early studies validated the concept that the organism was acquired by the respiratory tract. Murphy and her colleagues have contributed substantially to the understanding of immune and natural defense mechanisms in protection against this yeast and demonstrated that animals inoculated intranasally developed pulmonary infection that disseminated, but following the development of DTH gradually cleared the infection (Lim *et al.*, 1980a). Furthermore, these investigators demonstrated that transfer of T cell-enriched splenocytes from mice immunized by an intranasal infection was capable of protecting mice against an iv challenge (Lim *et al.*, 1980b). Of importance was that in these studies, passive transfer of serum failed to protect mice.

We and others developed an intratracheal inoculation infection

model with Cne to study pulmonary immune mechanisms in mice (Hill and Harmsen, 1991; Huffnagle *et al.*, 1991a; Huffnagle and Lipscomb, 1992). In our model, the organism is inoculated in small amounts directly into the trachea and yeasts in the lungs are quantitated by homogenizing the organ and measuring colony-forming units (CFU). Over an initial 7 days, the organisms grow rapidly followed by a gradual decrease in CFU in appropriate mouse strains, a process referred to as "lung clearance" (Huffnagle and Lipscomb, 1992). An important aspect of this model, as is true of many other lung infection models, is that both the strain of microorganism and the strain of mouse determine whether the infection will be cleared from the lung and at what rate (Huffnagle *et al.*, 1991a). In studies using a relatively low virulence encapsulated yeast, athymic nude mice, mice with severe combined immunodeficiency (SCID), or mice depleted of CD4 and CD8 T cells were incapable of pulmonary clearance (Huffnagle *et al.*, 1991a,b; Huffnagle and Lipscomb, 1992; Hill and Harmsen, 1991). Furthermore, protection of the lung was adoptively transferred to SCID mice by splenic lymphocytes, but adoptive immunity was more effective if lymphocytes isolated from the lungs and LALNs of animals that had been immunized during a lung infection were used (Huffnagle *et al.*, 1991b). Interestingly, using a more virulent organism, CD4 T cells were responsible for increased resistance to the highly virulent organism following extrapulmonary spread, but did not demonstrate an effect in controlling the infection within the lung (Mody *et al.*, 1990). These latter studies demonstrated, as did the earlier studies of Mackaness, a dichotomy between the ability of immunized animals to demonstrate effective immunity in the lung compared to extrapulmonary organs.

What is the role of CD4 and CD8 T cells in immune protection? The absence of either reduced the numbers of inflammatory cells, including macrophages, but they were even more profoundly decreased when both were absent (Huffnagle *et al.*, 1994). A role for CD8 T cells was repeatedly shown in strains of mice that demonstrated acquired resistance to low-virulence Cne, i.e., BALB/c, C.B-17 (congenic to BALB/c, but with the IgH locus of C57BL/6 mice), and CBA mice (Hill and Harmsen, 1991; Huffnagle *et al.*, 1991a; Mody *et al.*, 1993). A role for CD8 T cells in this infection was particularly curious, although CD8 cells clearly participate in the development of immunity to intracellular organisms. Mechanisms proposed include secretion of IFNγ or lysis of infected targets following recognition by CD8 T cells of peptides in the context of class I MHC (Kaufmann, 1988). However, it is not known how CD8 cells function in murine Cne disease in which

the organism is primarily extracellular. One important mechanism may be related to their capacity to enhance either the clonal expansion or the recruitment of CD4 cells to the lung (Huffnagle *et al.*, 1994). Thus, CD8 T cell depletion of Cne-infected mice reduced the numbers of CD4 T cells in infected lungs. A second role may relate to the finding that lung cells isolated from CD4 T cell-depleted animals were capable of secreting IFNγ in mitogen-stimulated cultures, suggesting CD8 cells in this setting could also contribute to IFNγ production and play a role in macrophage activation. It was also demonstrated that CD8 cells played a critical role in the development of DTH to Cne in Cne-infected mice and could adoptively transfer DTH (Mody *et al.*, 1994). This is an important observation because it proves CD8 T cells can recognize antigens of extracellular organisms in the context of class I MHC. Thus, it is possible that CD8 T cells might lyse AM that phagocytose the organism, but cannot kill it, so that activated macrophages or other effector cells may play a role.

C.B-17 mice were particularly adept in the development of a Th1 response characterized by clearing Cne from their lungs, and this ability was related to enhanced secretion of IL-2 and IFNγ by LALN cells early in infection (Hoag *et al.*, 1994). The heightened resistance in C.B-17 mice correlated with expression of the inducible nitric oxide synthase (iNOS) gene in the lungs, was accompanied by secretion of NO by lung cells during the early clearance phase, and was completely abrogated by both anti-IFNγ treatment and feeding animals an inhibitor of NO production (Lovchik *et al.*, 1995). Thus, in C.B-17 mice, clearance in the lung was related to the capacity of the animals to make IFNγ and NO.

If T cells are necessary to protect lungs from *Cne* infections, is the effector mechanism mediated mainly by activation of macrophages? The answer to this question is still uncertain, but rat AM activated by IFNγ were able to inhibit the growth of Cne (Mody *et al.*, 1991). Furthermore, prolonged incubation with GM-CSF also activated AM for Cne growth inhibition (Chen *et al.*, 1994). Murine macrophages from the peritoneum of BCG-immunized mice inhibited the growth of Cne *in vitro* by an arginine-dependent mechanism and were related to NO production (Granger *et al.*, 1988; Alspaugh and Granger, 1991) consistent with the *in vivo* data of Lovchik (1995). An important aspect of growth inhibition by this NO-dependent mechanism was that it did not require endocytosis, although endocytosis enhanced the growth inhibition (Granger *et al.*, 1986). Others have demonstrated that IFNγ-activated mouse macrophages kill Cne, but determined that a secreted protein was important (Flesch *et al.*, 1989). Activated human macro-

phages make little if any NO unlike rat and mouse macrophages leaving open the question of what effector mechanism human macrophage may use to growth inhibit Cne.

Human neutrophils and macrophages not only inhibited growth, but killed, Cne in cultures that include fresh complement (Miller and Mitchell, 1991). The organism fixes complement by the alternate pathway resulting in C3bi binding to the yeast capsule and allowing phagocytosis by CR3-positive neutrophils and macrophages (Kozel and Pfrommer 1986; Kozel *et al.*, 1988). This mechanism was shown to play an important role in clearing Cne from the pulmonary vasculature during fungemic states in mice (Lovchik and Lipscomb, 1993). However, Cne in tissues tend not to provoke brisk inflammation, and bronchoalveolar spaces (and cerebrospinal fluid) do not contain significant complement. Furthermore, AM may not express CR3. Thus, T cells must amplify effector systems by recruiting and/or activating nonspecific effectors or by themselves becoming direct effectors.

Recent studies indicated that human NK cells and T cells had direct activity *in vitro* against Cne (Levitz *et al.*, 1994; Murphy *et al.*, 1993), although there was conflicting evidence that human NK cells had no growth-inhibiting activity unless antibody against the organism was present (Miller *et al.*, 1990). We demonstrated that murine NK cells had a minor effect against the organism following iv inoculation, but failed to play a role in *early* lung clearance if the organism was inoculated via the trachea (Lipscomb *et al.*, 1987). Recently a T cell-independent, partially protective host defense mechanism was found in lung clearance in SCID mice and BALB/c mice depleted of CD4 and CD8 T cells. A Thyl$^+$, CD4$^-$, CD8$^-$, asialo GM1$^-$ cell was responsible (Hill and Dunn, 1993). Further studies are clearly indicated to examine the role of this cell in animal models and to identify its origin, particularly in view of the possible importance of these cells in human Cne infections.

Histologic examination of murine lungs during the clearance phase demonstrated that yeasts were surrounded by macrophages with an activated appearance (Hill, 1992; Huffnagle and Lipscomb, 1992). This appearance was similar to that seen in the lungs of humans with cryptococcomas who are known to be able to resolve their infections without antibiotic therapy. When the lung becomes inflamed and complement is available, neutrophils may play some role in killing Cne in the lung, although the relative importance of neutrophils over T cells and activated macrophages remains to be clarified.

Continued studies using a murine Cne lung infection model should help elucidate the mechanisms that lead to the development of a

Th1-like response in LALNs early during infection and subsequent recruitment of lymphocytes and macrophages into the lung. A closer examination of what effector mechanisms are at work in the lungs of animals that clear a Cne infection, particularly in resistant mouse strains that may not utilize NO from activated macrophages, may help elucidate host defense mechanisms in man.

C. Viral Pneumonias

Viruses are intracellular organisms that usurp host cellular machinery to replicate. Viral entry into cells can be blocked by antibodies. However, once inside the cell, the virus is resistant to both antibody and T cell recognition until viral peptides are presented in the context of class I MHC antigens on the cell surface and allow specific cytotoxic T cells to lyse the infected cell (Zinkernagel, 1993). As viral replication ensues and particles are released from the cell surface, antibody again has an opportunity to block the further spread of the virus. In general, cytotoxic T cells play an important role in controlling local viral replication, while antibody can *prevent* initial infection and *extracellular* spread within the host. In some viral infections, there seems to be a relatively minor role for CD4 T cells (Zinkernagel, 1993). However, in a number of viral infections in mice, depletion of CD4 T cells increased mortality and reduced the rate of clearance. The role for CD4 cells may relate to providing help for cytolytic T lymphocyte (CTL) development (Reiss and Burakoff, 1981) and for B cell production of high-affinity IgG and IgA antibodies. The role of $\gamma\delta$ T cells, NK cells, and macrophages in acute viral infections is still not clear. Furthermore, whether memory CTLs play an important role in preventing recurrent infection is also uncertain (Zinkernagel, 1993). It is also uncertain whether persistence of long-term memory T or B cells against viruses requires the continued presence of virus or viral particles (Zinkernagel, 1993; Sprent, 1994).

A large number of viruses infect the respiratory tract, including rhinoviruses, coronaviruses, adenoviruses, influenza, and parainfluenza viruses, respiratory syncytial virus (RSV), measles, mumps, and rubella viruses. Good models in mice exist for both RSV and influenza A infections and are discussed to highlight experimental models that have provided insight into immune defenses against viral respiratory tract infections.

1. *Respiratory Syncytial Virus*

Immunization against influenza A with killed or fractionated viral antigens protects against influenza, but immunization against RSV has

been problematic (Salk and Salk, 1977; Wright *et al.*, 1982; Graham *et al.*, 1993; Alwan *et al.*, 1994). Protection against RSV, which produces a bronchiolitis in infants and is the most common cause for hospitalizing infants in Western countries, was not afforded by immunization with formalin-inactivated virus. Subsequent infection after such immunization sometimes resulted in unusually severe infections and even death (Kapikian *et al.*, 1969; Kim *et al.*, 1969). The mechanism is unknown, but various theories include immune complex disease, a CD4 T cell-mediated DTH reaction, or a CTL-mediated pneumonitis (Graham *et al.*, 1993). Recent efforts using RSV infections in mice have sought to understand what the mechanisms for protection might be, and why immunization might lead to enhanced pathogenicity with a subsequent challenge.

Depleting mice of either CD4 or CD8 cells reduced the disease in the lung following an initial RSV infection, but also enhanced virus replication (Graham *et al.*, 1991). Thus, control of viral replication during even a primary infection resulted in lung pathology. At least two groups attempted to determine whether various viral subunits might initiate protective immunity, yet cause minimal pathology. Mice vaccinated either parenterally or by intranasal inoculation, followed by nasal RSV challenge, lead to the expression of cytokine mRNA in the lungs (Graham *et al.*, 1993). The specific cytokine mRNA detected was dependent on whether live, heat-killed, or subunit vaccines were given. Inactivated virus or subunit fusion (F) protein induced cytokine expression that suggested a Th2-like lymphocyte response with increased IL-4 mRNA relative to IFNγ expression. In contrast, when mice were primed with parenteral or nasal live virus, Th1 responses were prominent. Formalin-fixed virus and the F protein component were somewhat protective. However, the most effective protection was induced by immunizing intranasally with the live virus. Furthermore, this immunization protocol resulted in the least lung pathology after rechallenge.

Experiments were designed to determine which T cell types caused pathology and whether specific RSV subunits evoked specific pathology-producing immune T cells. Cell lines were developed from immune lymphocytes of mice immunized against the F protein, the major surface glycoprotein (G), and a 22-kDa matrix protein expressed by recombinant vaccinia virus (Alwan *et al.*, 1994). F protein lead to the development of both CTL and CD4 T cells with a Th1 phenotype. G protein facilitated the development of CD4 cells with a Th2 phenotype. Immune cells from the 22-kDa protein-immunized mice resulted in predominantly CD8 CTL. Representative cell lines from each of

these groups transferred both protection and pathogenic effects to RSV-infected mice, but the Th2 cells seemed to be the most damaging. Furthermore, combinations of lines afforded the greatest protection. Thus, protection is often synonymous with pathology and it may be difficult to dissociate the two.

2. *Influenza Virus*

It has been clear for some time that serum antibody correlates with protection against influenza viruses. Influenza viruses exhibit antigenic drift and shift that requires individuals be immunized yearly for protection against the prevalent virus strain (Zinkernagel, 1993; Salk and Salk, 1977). While immunization against influenza has been successful, it is possible that new immunization protocols might be developed that would be broadly protective. In contrast to B cell epitopes, T cell epitopes may be cross-reactive; cytotoxic T cells seem to play an important role in controlling influenza infections (Zinkernagel and Althage, 1977).

Early studies established that recovery of mice from infections with influenza A required the development of a CTL response to the virus, and protection was afforded by the adoptive transfer of immune cells into naive-infected hosts (Yap *et al.*, 1978; Lukacher *et al.*, 1984). Further studies indicated that both class I and II MHC-restricted T cell clones could promote recovery from a lethal pulmonary infection (McDermott *et al.*, 1987). These T cell clones were preferentially retained in lungs of influenza-infected mice, independent of any viral antigenic specificity, and migrated from the pulmonary vessels into the bronchiolar lumens. Thus, the immune cells accumulated at a site appropriate to provide protection against a viral challenge.

Mice die within 6 days of lethal influenza infections. An array of cytokines could be detected in BAL fluids in these mice, but none that were unequivocally indicative of a T cell response (Hennet *et al.*, 1992). Thus, while IL-1α, IL-1β, IL-6, TNFβ, GM-CSF, IFNγ, and leukotriene B_4 were identified in lavages, IL-2, IL-3, and IL-4 were not. On the other hand, in sublethal infections in which the influenza infection was resolved, examination of cells from lavages as well as from LALNs demonstrated T cell cytokine production (Carding *et al.*, 1993; Sarawar *et al.*, 1993). In a primary infection, the kinetics of cytokine mRNA was compatible with an initial response occurring in the regional lymph nodes with the effector T cells appearing later in the lungs. Among the $\alpha\beta$ T cells, transcripts for IFNγ and TNFβ were predominantly found in CD8 cells, but there was a tendency for IL-4 and IL-10 to appear in CD4 cells. Interestingly, $\gamma\delta$ T cells were identified and expressed IL-2, IL-4, and IFNγ. During a secondary

response, T cell cytokine mRNA was found almost simultaneously in LALNs and in the lung (Carding *et al.*, 1993). In related studies, mRNA was detected by *in situ* hybridization and by cytokine production identified in individual cells by ELISPOT. The majority of cells in lavages produced IL-2, IL-4, and IFNγ with relatively little TNF and IL-10. Depletion of CD4 and CD8 cells caused a significant reduction in IL-2- and IL-4-producing cells, but IFNγ-producing cells remained and were likely $CD4^-$, $CD8^-$ $\alpha\beta$, or $\gamma\delta$ T cells; both populations were present during the infection.

These studies are typical of recent studies examining cytokine patterns in various lung infections in the lung attempting to learn what determines the type of immune response that develops to airway antigens. B2-Microglobulin-deficient mice were used to determine the effect of IFNγ on influenza clearance. Mice did not develop CD8 CTL because of the absense of class I MHC, but residual CD4 cells were capable of mediating clearance, possibly related to the development of antibody of the IgG2a subclass (Sarawar *et al.*, 1994). Treatment of the mice with anti-IFNγ antibody delayed clearance for at least 3 days, whereas antibody to IL-4 had no effect. However, all mice survived and eventually cleared the virus. Notably, neither antibody to IFNγ nor IL-4 altered the cytokine profiles detected in freshly isolated lung lymphocytes. The conclusion was that although IFNγ played an important role in viral clearance, its role was not to drive CD4 cells to become Th1 T cells.

γδ T cells developed as a prominent component of the late inflammatory process during murine influenza infections (Carding *et al.*, 1990). These cells expressed all known γ genes, although some predominated at times. A suggested role for these cells was that they recognized heat shock proteins on inflammatory macrophages and decreased their numbers. However, close examination of the data failed to show an inverse relationship of the numbers of γδ T cells and lung macrophages (Carding *et al.*, 1990). In another study, γδ T cells were found to be noncytolytic, but expressed mRNA for IFNγ, GM-CSF, and TNFβ (Eichelberger *et al.*, 1991). The hypothesis was presented that these cells, through their capacity to make cytokines, provided nonspecific protection against secondary infections. Thus, although γδ T cells are a component of the host response to viral lung infections, their role remains unknown.

D. Implications for Vaccination

A National Institutes of Allergy and Infectious Diseases (NIAID) Blue Ribbon Panel on Vaccine Research was convened in 1993 by Anthony Fauci, Director of the NIAID, to assess the long-term goals

for vaccine research and to recommend immediate priorities for the institute. Among the panel's recommended priorities was the development of vaccines for respiratory infections of children, and to improve the current vaccines for pertussis and measles. An additional priority was to develop vaccines for reemerging infectious diseases, including influenza because of its inherent problems of antigenic drift and shift.

The Jordan Report, a publication of the Division of Microbiology and Infectious Diseases of the NIAID, has reviewed on a yearly basis the progress in vaccine research. In the 1993 report, respiratory tract infectious diseases for which vaccines were either being developed or improved were listed and discussed. They included the bacteria Groups A and B Streptococci, *H. influenzae* type B, nontypable *H. influenzae, Neisseria meningitidis, S. pneumoniae, B. pertussis, Pseudomonas aeruginosa,* and *M. tuberculosis;* the viruses Rubeola, rubella, adenoviruses, influenza, and parainfluenza viruses, and respiratory syncytial virus; *Mycoplasma pneumoniae;* and the fungi, *H. capsulatum, C. immitis,* and *C. neoformans.* Thus, an enormous scientific effort is being directed at designing vaccines to protect the host from respiratory pathogens. And yet the report also highlighted the gaps in our knowledge about normal host defenses at mucosal surfaces, what immune defenses we should attempt to enhance with vaccinations, and the best methods for immunization.

Important issues for vaccine research have been discussed in recent publications (Lambert, 1993) including reviews on the possibilities of immunization against tuberculosis (Kaufmann and Young, 1992) and on novel approaches to vaccination such as inoculating polynucleotides encoding antigens directly into muscle (Donnelly *et al.*, 1994).

Important general goals for any vaccine are that it be efficacious, easy to store, easy to give, and be free of side effects. Specific goals for vaccines for respiratory infections are (1) the immune response generated must be protective (a corollary is that the immune response to a microbial challenge should not cause lung pathology.); (2) the immune response must be quickly available in the respiratory tract and at the site within the respiratory tract where the microorganism is likely to seek entrance and/or produce disease; and (3) immune memory should be long term.

The study of the type of protective immune responses that develop during natural infections in man or induced infections in experimental animals has provided important clues to what responses should be enhanced by immunization. This has been a productive approach for viral infections and those caused by intracellular pathogens. However, host responses that can prevent a second infection may differ from the

immune responses that bring an acute infection under control. For example, in many viral infections, although cytotoxic T cells may bring a primary infection under control, antibody may prevent reinfection. Another example of this principle is that although CMI controls *Cne* infections in mice, in special circumstances, antibodies to *Cne* can be protective (Mukherjee *et al.*, 1992). The isotype of the predominating antibody in preventing infection may also be extremely important, e.g., upper tract infections may be more dependent on IgA responses, while in the alveolar spaces, IgG may be more effective.

As already discussed, an important consideration for viral vaccines is whether subunit vaccines are as effective as live attenuated organisms in initiating the desired type of immune response. An innovative strategy for generating CMI responses against nonviable antigens is to inoculate a regulatory cytokine at the time of antigen delivery (Afonso *et al.*, 1994). The vaccine could even consist of a fusion protein of the antigen and the cytokine (Tao and Levy, 1993).

The immune response at the time of challenge must develop in the correct site. If the organism infects the upper respiratory tract, local IgA is important. If infection is initiated in the lower respiratory tract, it may be sufficient that protective cells and antibody are available in circulation if recruitment can occur immediately following challenge. However, if significant inflammation is required before recruitment of immune cells occurs, some clinical manifestations of infection must necessarily develop, before the protective response neutralizes the infection. Feasible strategies for generating IgA in the upper respiratory tract are to aerosolize the antigen, to deliver the relevant antigens on the surface of a nonpathogenic microorganism, such as *Streptococcus gordonii*, that have the potential to colonize the nasopharynx (Pozzi *et al.*, 1992), or to immunize with oral vaccines with antigens either chemically bound to cholera toxin B-subunit (McGhee *et al.*, 1992), enclosed in a liposome or biodegradable microsphere (Mestecky and Eldrigde, 1991), or encoded in a plasmid carried by live attenuated *Salmonella* spp. (Cárdenas and Clements, 1992). Ample evidence has documented that IgA precursors generated in the gut home to bronchial mucosa (Weisz-Carrington *et al.*, 1987; Chen *et al.*, 1987; Ruedl *et al.*, 1994), although the converse does not appear to occur to an appreciable extent (McDermott and Bienenstock, 1979; Joel and Chanana, 1987; VanCott *et al.*, 1994). However, a cautionary note in relationship to immunizations that enhance IgA responses is that they could also stimulate IgE responses and lead to allergic responses in the lungs. Oral immunization with protein antigen and cholera toxin resulted in anaphylaxis in mice following an intraperitoneal antigen challenge (Snider *et al.*, 1994).

An important issue for any vaccine is whether long-term memory is possible. Based on the data derived from immunization with subunit vaccines or killed microorganisms, it seems unlikely that sufficient T cell memory could be induced for long-term CMI protection against respiratory tract infections. However, in the presence of continuous antigen, such as would occur with low levels of replicating attenuated viruses or retained intracellular microorganisms in Ia-positive APC, memory T cells should persist. Furthermore, follicular dendritic cells are present in BALT, and long-term B cell memory could also persist due to the retention of nonviable antigens in the form of antigen–antibody complexes on these cells. Evidence that long-term B cell memory occurs in the lungs of dogs has been obtained as discussed previously. However, it is important to remember that this observation was made in animals in which the antigen was directly instilled into the lung. Therefore, it is not obvious that extrapulmonary immunization would induce retention of long-term local B cell memory, and suggests that lung immunity might be more effective if primary immune responses to pulmonary pathogens were boosted by intranasal or aerosol antigen delivery.

V. Models for Hypersensitivity Lung Disease

Animal models of human lung disease have been used to test hypotheses under well controlled conditions and to dissect mechanisms of injury, inflammation, and repair. These models have been particularly useful in distinguishing direct lung toxicity from injuries that result from immune mechanisms.

To prove that a lung injury is immune mediated requires previous exposure to an appropriate agent, evidence of a specific immune response, and evidence that the injury involves recognized immunologic mechanisms. Early work concentrated on dissecting isolated aspects of the immune response, i.e., the role of antibody, antibody plus complement, or T cells, emphasizing *in vivo* analogs of *in vitro* events. Current models of immune-mediated injury emphasize the interrelationships among these aspects of the immune response. In particular, the importance of T cells (especially CD4 T cells) in regulating the type of the immune response is better appreciated.

A. Early Models

Early studies of immune-mediated lung disease in animals were performed by administering different antigens via different routes and with different adjuvants to guinea pigs (Richerson, 1972). Immune

responses varied depending on the type of antigen, method of immunization, and the presumed predominant response. Animals immunized with ovalbumin (OVA) in complete Freund's adjuvant (CFA) developed specific complement-activating antibody and hemorrhagic pneumonitis with a predominant neutrophil response after aerosol antigen challenge. In contrast, animals immunized with ABA-*N*-acetyltyrosine in CFA exhibited DTH without demonstrable serum antibody. Later, OVA aerosol challenge in sensitized animals produced scattered focal areas of alveolitis with thickening and increased cellularity of alveolar septa and alveolar filling with mononuclear cells. Other work (Brentjens *et al.*, 1974) confirmed the hemorrhagic nature of immune complex-mediated lung disease. Hemorrhagic neutrophilic pneumonitis could be transferred with serum and suppressed by administration of cobra venom factor, which depletes C′ *in vivo* (Roska *et al.*, 1977).

Richerson and colleagues described the results of aerosol OVA exposure of rabbits systemically sensitized to OVA in CFA. Acute exposure lead to transient foci of acute pulmonary inflammation (Richerson *et al.*, 1971), whereas chronic exposure caused decreasing inflammation (Richerson *et al.*, 1978). T cells were prominent in both the acute and the chronic lesions (Upadrashta *et al.*, 1988). Inhalation of muramyl dipeptide could substitute for systemic immunization with OVA in Freund's adjuvant (Richerson *et al.*, 1982). The pulmonary inflammatory response could be decreased by administration of cyclosporin A at the time of aerosol challenge (Kopp *et al.*, 1985), implicating DTH in the etiology of pulmonary inflammation in the rabbit OVA model.

Diminution in the pathologic response in the lungs despite continuing challenge could be produced by either repeated iv or aerosol exposure of rabbits to OVA and was not associated with decreased antigen-specific lymphocyte proliferation nor decreased blood or BAL antibody response (Richerson *et al.*, 1981; Butler *et al.*, 1982). A similar decrease in pulmonary inflammation has been observed in rabbits, guinea pigs, and mice subjected to repeated exposures to *Micropolyspora faeni*, the agent that causes farmer's lung disease in humans (Schuyler *et al.*, 1983,1987,1992), and *Thermoactinomycetes vulgaris*, which causes humidifier lung (Takizawa *et al.*, 1989).

The decrease in pulmonary inflammation during continued challenge has been attributed to desensitization, defined as suppression of preexisting DTH by administration of homologous antigen. However, desensitization is clearly not present in the above models of lung disease, because lymphocyte proliferation and antibody responses were not depressed. Since desensitization in other systems can be achieved by administration of an antigen by an unusual route, such

as orally (Weigle, 1973), it is rather surprising that immunologic desensitization was not evident in models of repetitive pulmonary instillation of antigen. Two other possible mechanisms, increased degradation of inhaled antigen or increased suppression of lymphocyte proliferation by AM, were not present in these models (Schuyler and Schmitt, 1985; Kopp *et al.*, 1988). Additional possibilities, including decreased exposure of the lung to antigen due to changes of clearance mechanisms or change of T lymphocyte subtypes, were not investigated.

B. Immune Complex-Mediated Lung Injury

Ward and colleagues extended these studies on immune complex-mediated lung injury using intratracheal instillation of antibody to bovine serum albumin (BSA), followed by iv administration of BSA. Lung injury was measured using morphology, leakage of labeled intravascular protein and red blood cells into the lung, and quantitation of the neutrophil enzyme, myeloperoxidase, in the lung (Johnson and Ward, 1974). Marked differences were shown in responses to instilled IgG versus IgA immune complexes.

IgG immune complex-mediated damage was characterized by neutrophil infiltration into the lung and evidence of increased pulmonary vascular permeability. It was neutrophil dependent (Warren *et al.*, 1991) and required IL-1β and platelet-activating factor (Warren, 1992), which were likely produced by TNFα-stimulated macrophages (Warren *et al.*, 1990). Expression of VLA-4 and CD18 (Mulligan *et al.*, 1993b,c), functioning CR1 receptors (Mulligan *et al.*, 1992a), and CD11a (but not CD11b), and ICAM-1 expression, were also involved (Mulligan *et al.*, 1993c). There was upregulation by ELAM-1 on pulmonary venules and capillary endothelium, perhaps mediated through a rat analog to IL-8 (Mulligan *et al.*, 1991,1993a), and upregulation of ICAM-1 expression modulated through TNFα (Mulligan *et al.*, 1993a).

In contrast, IgA immune complex lung injury was characterized by accumulation of mononuclear cells (Warren *et al.*, 1991), perhaps mediated through monocyte chemoattractant protein 1 (MCP-1) (Jones *et al.*, 1992). The injury was neutrophil and TNFα independent and was not modulated by increases of ELAM-1 expression (Mulligan *et al.*, 1992c) or TNFα secretion, despite a TNFα-induced increase of ELAM-1 expression. Iga-mediated injury was similar to IgG immune complex injury in that it was VLA-4, CD18, and ICAM-1 dependent, but was dissimilar in that it is more dependent on CD11b than CD11a expression (Mulligan *et al.*, 1993d). IgA immune complex lung injury was apparently mediated through nitric oxide or its derivatives (Mulligan *et al.*, 1992c). IL-4 and IL-10 protected against the pulmonary

response to IgG immune complexes, whereas only IL-10 protected against IgA immune complex-mediated injury (Mulligan *et al.*, 1993e).

C. Asthma Models

There are multiple animal (e.g., primate, sheep, guinea pig, dog, rabbit, rat) models of asthma which have been used to explore pathophysiologic aspects of asthma (Wegner *et al.*, 1991; Abraham, W; Murray *et al.*, 1991; Lukacs *et al.*, 1994b; Yamaya *et al.*, 1990; Du *et al.*, 1992; Waserman *et al.*, 1992; Coyle *et al.*, 1990) and as a method to test the effectiveness of various therapeutic agents. Although all have some resemblance to human asthma, there are substantial differences, especially in regard to the physiologic response to airway challenge and in methods to induce immune hyperreactivity. Typical protocols use intraperitoneal administration of antigen with aluminum hydroxide and/or Bordetella adjuvants.

Guinea pigs immunized intraperitoneally with OVA in aluminum hydroxide adjuvant, often with the addition of pertussis (Handley *et al.*, 1992; Mauser *et al.*, 1993), can be induced to form IgE and IgG_1 antibodies, exhibit airway and blood eosinophilia, and display early and late-phase bronchoconstriction (Cerasoli *et al.*, 1991) when reexposed to OVA. These animals also demonstrate increased bronchial reactivity to histamine or acetylcholine administered iv. Using this method of sensitization, guinea pigs exhibit strain differences in blood eosoniphilia and bronchial hyperreactivity (Winthereik *et al.*, 1992). Increased responsiveness to acetylcholine after antigen challenge and late-phase bronchoconstriction correlates with BAL neutrophilia (Asano *et al.*, 1994). This method of immunization is very different from that which occurs in humans, and the pulmonary physiologic response of guinea pigs is dissimilar to human asthma. Guinea pigs exposed to various parasite antigens also exhibit airway hyperreactivity (Yamaya *et al.*, 1990).

Nonhuman primates exposed to *Ascaris suum* antigen via the airway exhibit immediate skin test reactivity and either early or both early and late increases in airway resistance (Patterson and Harris, 1992). Dual responses are associated with more BAL eosinophils and a greater increase of BAL neutrophils (Gundel *et al.*, 1992).

Hirshman and colleagues found that *Ascaris*-sensitized basenji greyhound dogs exhibited a number of changes similar to those in humans with asthma including increased specific and nonspecific airway reactivity and increased numbers of BAL mast cells (Hirshman *et al.*, 1980, 1986). Spontaneous and induced histamine release from BAL mast cells was increased compared to control animals (Hirshman *et al.*,

1988). Tracheal muscle from these animals exhibited impairment of the usual increase in cyclic AMP in response to isoproterenol (Emala *et al.*, 1993), and coincident with the measurement of collateral airway resistance, high resolution CT scanning detected airway narrowing (Herold *et al.*, 1991; Corddry *et al.*, 1991). Chronic treatment with methylprednisolone decreased nonspecific airway reactivity and BAL eosinophil number (Darowski *et al.*, 1989).

Others have induced an asthma-like syndrome with increased bronchial reactivity and reaginic antibody in dogs immunized intraperitoneally as puppies with hapten–carrier complexes in aluminum hydroxide adjuvant (Kepon *et al.*, 1977). Later work extended this model by using ragweed antigen (Baldwin and Becker, 1993; Becker *et al.*, 1989). Immunized dogs exhibited immediate- and late-phase skin test reactivity (Becker *et al.*, 1988), increased antigen-specific and nonspecific bronchial reactivity, and increased BAL mast cells, eosinophils, and histamine (Becker *et al.*, 1989).

T cells appear to be important in animal models of asthma. Frew and colleagues demonstrated a substantial influx of non-CD8 (presumably $CD4^+$) T cells into bronchial wall mucosa and adventitia of aerosol antigen challenged guinea pigs undergoing late-phase bronchoconstriction (Frew *et al.*, 1990). Using picryl chloride epicutaneous sensitization, mice challenged with intranasal hapten exhibited peribronchiolar cellular infiltration and increased pulmonary resistance *in vivo* (Garssen *et al.*, 1991). Hypersensitivity to carbacol was present in tracheas from such animals and could be transferred with T cells from sensitized animals. This phenomenon could not be produced in athymic mice (Garssen *et al.*, 1991). Cyclosporin A and FK506 administration prevented the development of both the late asthmatic response and bronchial hyperresponsiveness after antigen challenge (Fukuda *et al.*, 1991). IL-4-deficient and class II MHC-deficient mice which lack mature $CD4^+$ T cells could not express peribronchiolar inflammation or BAL lymphocytosis and eosinophilia when exposed to OVA (Brusselle *et al.*, 1994). Exposure of mice to certain parasites, e.g., *Schistosomes*, also caused the appearance of intrapulmonary and BAL eosinophilia via an IL-4-dependent mechanism (Lukacs *et al.*, 1994b), perhaps by altering the balance of Th2 versus Th1 T cell numbers in the lungs.

Antibody to IL-5 can ablate the eosinophilic airway response to OVA exposure in sensitized guinea pigs and can even block the OVA-induced increased sensitivity to substance P (Chand *et al.*, 1992; Mauser *et al.*, 1993). Eosinophil infiltration into the tracheas of sensitized mice after aerosol antigen challenge is dependent on CD4 cells and

IL-5 (Nakajima *et al.*, 1992) and can be blocked by inoculations of IFNγ (Iwamoto *et al.*, 1993).

Gelfand and colleagues (Renz *et al.*, 1992) developed a model of asthma in BALB/c mice (typically high IgE responders) induced by repetitive inhalation of OVA. These animals exhibited increased specific IgE production, increased sensitivity to iv methacholine, and evidence of sensitized cells in LALNs and spleen capable of producing specific IgE and IgG1. Isolated trachea from sensitized animals were hyperresponsive to electrical field stimulation. Specific IgE antibody and increased airway reactivity could be induced in naive recipients by transfer of sensitized cells from LALNs, but not spleen cells, followed by a single aerosol OVA exposure. Low-IgE responding SJL/L mice failed to develop either IgE antibodies or increased bronchial responsiveness, although they developed specific IgG antibodies (Larsen and Wicht, 1962). Local airway challenge, as well as systemic sensitization, was required for the development of airway hyperreactivity (Saloga *et al.*, 1994). This suggested that local factors in addition to systemic sensitization were required for bronchial hyperreactivity. In this model, IFNγ administration during OVA sensitization both decreased specific IgE production and ablated increased airway reactivity. The effect of IFNγ was dependent on the route of administration. Systemic administration decreased serum-specific IgE, but not LALN-specific IgE production. Perhaps most importantly, the OVA-induced increase of airway reactivity was ablated by airway, but not systemic, IFNγ administration (Lack *et al.*, 1994). These results were compatible with an IFNγ-induced shift from a predominant Th2 to a Th1 T cell response and also gave evidence for compartmentalization of both systemic and airway immune responses. Thus, airway hyperresponsiveness correlated with LALN, but not systemic sensitization.

D. T Cell-Mediated Hypersensitivity

T cell-mediated inflammation in the lung can result in pulmonary fibrosis or hypersensitivity pneumonitis (HP).

1. *Hapten-Immune Model*

Exposure of mice to a hapten instilled into the lungs caused systemic sensitization as measured by ear swelling after reexposure to the sensitizing hapten (Stein-Streilein, 1983). Furthermore, following epicutaneous sensitization with lipophilic trinitrophenylchlorobenzene and an intratracheal challenge with the water-soluble hapten, trinitrophenyl, pulmonary fibrosis developed. Intratracheal rechallenge with an unrelated hapten (dinitrophenol) did not produce pulmonary fibrosis

(Stein-Streilein *et al.*, 1987). This model was similar to previous models of contact sensitivity using the skin for both sensitization and challenge and was consistent with a T cell-mediated DTH process (Polack, 1980). Different inbred mouse strains of animals exhibited coincidence of skin reactivity and the ability to develop pulmonary fibrosis (Kimura *et al.*, 1992). Induction of tolerance by injection of hapten-coupled splenocytes before sensitization depressed both the skin and the pulmonary responses following tracheal challenge (Kimura *et al.*, 1993). The inflammatory and fibrotic responses to intratracheal hapten challenge were transferred with immune lymphocytes, but not with immune serum. *In vivo* administration of anti-CD4 and anti-CD8 antibodies to sensitized mice prevented or ameliorated the inflammatory and fibrotic responses to tracheal challenge. The development of pulmonary fibrosis is associated with BAL IL-2 activity and lymphotoxin mRNA in BAL nonadherent cells (Garcia *et al.*, 1992). An increased ratio of procollagen type I:III mRNA in the fibroblasts from immunized, challenged animals developed, indicating that qualitative as well as quantitative collagen differences occurred (Stein-Streilein *et al.*, 1992).

2. *Bleomycin-Induced Pulmonary Fibrosis*

Bleomycin is a mixture of glycoproteins from *Streptomyces verticullus* used clinically for its antineoplastic properties, but which predictably causes pulmonary fibrosis. In a hamster model, intratracheal administration of a single dose of bleomycin to experimental animals caused pulmonary fibrosis which resembled clinical pulmonary interstitial fibrosis, albeit with some differences in the pattern of fibrosis from usual interstitial fibrosis (Snider *et al.*, 1978).

Evidence has accumulated that cytokines are important mediators in animal fibrosis models. Increased TGFβ (Khalil *et al.*, 1989), TNFα (Piguet *et al.*, 1989a), IL-1 and IL-6 (Jordana *et al.*, 1988), MCP-1 (Brieland *et al.*, 1993), and macrophage-derived growth factor for fibroblasts (Denholm and Phan, 1989) have been detected in lungs or pulmonary cells derived from animals exposed to bleomycin. Administration of anti-TNFα can prevent fibrosis (Piguet *et al.*, 1989a).

Although many of the cytokine studies concentrated on the role of AM, recent reports indicate that pulmonary endothelial cells constitutively produced IL-6 which is increased by exposure to bleomycin (Karmiol *et al.*, 1993). MCP-1 is produced by fibroblasts (Rolfe *et al.*, 1992) and TGFβ is produced by pulmonary artery endothelial cells (Phan *et al.*, 1991,1992) or lung fibroblasts (Breen *et al.*, 1992). Therefore, the source of cytokines in bleomycin-induced pulmonary fibrosis

could include nonmacrophage pulmonary cells as well as macrophages.

Despite the evidence of toxicity of bleomycin-induced macrophage-derived cytokines, several studies have suggested that T cells may also be important. Different strains of mice responded differently to intratracheal bleomycin (Schrier *et al.*, 1983a). Pulmonary fibrosis did not occur in athymic nude mice (Schrier *et al.*, 1983b) and depletion of both CD4 and CD8 T cells prevented fibrosis (Jordana *et al.*, 1988).

3. *Experimental Hypersensitivity Pneumonitis*

Cormier and colleagues described a model of HP associated with alveolitis and fibrosis, and which was caused by repeated pulmonary instillation of *M. faeni*. It was associated with increased BAL IL-1α, IL-6, and TNFα (Denis *et al.*, 1991). Cyclosporin A administration blocked pulmonary fibrosis, but not alveolitis, and BAL IL-1α and TNFα, but not IL-6 (Denis *et al.*, 1992a), which suggested a role for T cells in producing fibrosis. Pulmonary fibrosis was also prevented by anti-TNFα antibody (Denis *et al.*, 1991). Evidence was also found for a role for AM-secreted TGFβ in promoting fibrosis (Denis and Ghadirian, 1992a). Secretion of TNFα, which probably originated from pulmonary macrophages, was fostered by CSF-1 and GM-CSF secreted by lymphocytes exposed to *M. faeni* (Denis and Ghadirian, 1992b). In contrast to an adoptive transfer model of HP to be described, *in vivo* depletion of T cells did not substantially affect the pulmonary histologic response to *M. faeni* (Denis *et al.*, 1992b). This might be related to repetitive challenges with an agent which has adjuvant effects (Bice *et al.*, 1974), so that inflammation in this model was macrophage, rather than lymphocyte, driven. Pulmonary fibrosis induced by repeated challenges with *M. faeni*, but not an increase of BAL inflammatory cells, was reduced by administration of anti-CD11a, implicating integrins in the processes that lead to fibrosis in this model (Denis and Bisson, 1994).

Lymphocytes can be implicated in other models of HP. Cyclosporin A administration ameliorated pulmonary lesions in animals subjected to airway challenges with *T. vulgaris* (Takizawa *et al.*, 1988). Nude mice did not exhibit pulmonary lesions of HP after exposure which was able to produce lesions in T cell-sufficient littermates. The ability to express pulmonary lesions could be transferred with T cells from sensitized mice (Takizawa *et al.*, 1992).

Schuyler and colleagues have developed an adoptive transfer murine model of experimental HP using *M. faeni*. Cells for adoptive transfer were obtained from spleen, peripheral lymph nodes, LALNs,

and peritoneal exudate from immunized animals. The cells were restimulated in culture with relevant antigen and could then transfer to naive recipients a susceptibility for increased lung inflammation following an intratracheal rechallenge with antigen (Schuyler *et al.*, 1991). IFNr and IL-2 were present in substantial quantities in cultures (Fei *et al.*, 1993), and CD4 cells were required at the beginning of culture to generate effective cells for the adoptive transfer (Schuyler *et al.*, 1994). The transferred cells were a mixture of naive and memory CD4 T cells, as defined by CD44, CD45RB, and L-selectin expression (Schuyler *et al.*, 1994a,1992). Successful transfer was also dependent on the presence of CD4 T cells in the recipient (Schuyler *et al.*, 1994b), suggesting the necessity of an important interaction between host and recipient CD4 T cells.

VI. Models for Lung Transplantation and Graft versus Host Disease

A. Lung Allograft Rejection and Graft versus Host Disease (GVHD) in the Lung: A Pathologic Comparison

Despite different methods to develop models for lung allografting or GVHD, the immunologic responses in the lung show similar histologic patterns. Several studies have documented these similarities (Atkinson *et al.*, 1971; Emeson *et al.*, 1982; Piguet *et al.*, 1989b; Randhawa and Yousen, 1992; Stein-Streilein *et al.*, 1981; Yousem *et al.*, 1990). The histoincompatible allografted lung is recognized as "foreign" by the recipient and, therefore, is subject to rejection (Randhawa and Yousem, 1992). In GVHD, donor immunocompetent allogenic cells recognize the recipient as foreign (Farrara and Deeg, 1991).

In both of these disorders, pulmonary manifestations of lung allograft rejection and GVHD may be separated into acute and chronic changes. In acute lung allograft rejection, the initial pathologic lesions of perivascular mononuclear cell infiltrates is termed "minimal rejection" or grade 1 (Yousem *et al.*, 1990). A more severe perivascular mononuclear cell infiltrate consisting of activated lymphocytes, plasma cells, and macrophages is called "mild acute rejection" or grade 2 (Yousem *et al.*, 1990). In some instances rare eosinophils are present (Yousem *et al.*, 1990). Vascular changes may include degeneration of the endothelium (endothelialitis) (Yousem *et al.*, 1990; Randhawa and Yousem, 1992), and lymphocytic infiltration of the bronchioles may be present (Yousem *et al.*, 1990). In grade 3 acute lung cancer allograft rejection, also known as moderate acute rejection, infiltrates progress and become more apparent around pulmonary veins, arterioles, and peribron-

chiolar areas (Yousem *et al.*, 1990). In grade 4 or "severe acute rejection" mononuclear cell infiltrates extend into air spaces and involve vessels and bronchioles. Necrotizing vasculitis and parenchymal necrosis may also be visible (Randhawa and Yousem, 1992).

In contrast to acute lung allograft rejection, the pulmonary pathology of acute GVHD has not been assigned histologic grades. Beschorner *et al.* (1978) first described the acute changes of GVHD in the lung in recipients of bone marrow transplants. In these patients, the pathology was limited to that of lymphocytic bronchitis (Beschorner *et al.*, 1978). More recently, Atkinson *et al.* (1991) reported an acute pulmonary syndrome after bone marrow transplantation that resembled acute GVHD of the lung. The lesions included lymphocytic peribronchial infiltrates, bronchial epithelial degeneration, and lymphocytic perivascular infiltrates (Atkinson *et al.*, 1991). Relative to the histology of acute lung allograft rejection, the pulmonary changes observed in acute GVHD of the lung are analogous to a grade 2 rejection response.

In animal models, investigators have reported acute "GVHD-like" changes in the lung (Piguet *et al.*, 1989b; Stein-Streilein *et al.*, 1981; Wilkes *et al.*, 1994a). In these studies the histologic lesions included alveolitis, lymphocytic bronchitis, and vasculitis. The histologic changes of acute GVHD were analogous to grade 4 or severe acute rejection in a lung allograft. In both allograft rejection and GVHD of the lung, the pathologic lesions of the airway began at the level of the bronchioles and extended into alveolar spaces.

The chronic stage of lung allograft rejection and GVHD is associated with the development of bronchiolitis obliterans (BO) (Farrara and Deeg, 1991; Rhandhawa and Yousem, 1992; Yousem *et al.*, 1990). Bronchiolitis obliterans is not a lesion specific to allograft rejection or GVHD and, in fact, has been associated with a variety of conditions (Epler, 1988) including toxic fume inhalation, rheumatoid arthritis, penicillamine use, postinfectious etiologies, as well as idiopathic causes. The histology of BO shows granulation tissue plugs within the lumens of the small airways, epithelial cell damage, mononuclear cell infiltrates, and, at times, complete obstruction of the airways (Epler, 1988; Randhawa and Yousem, 1992). Bronchiolitis obliterans observed in chronic lung allograft rejection and GVHD involve the membranous and respiratory bronchioles, and possibly involve more proximal airways (Randhawa and Yousem, 1992). In contrast to the pathology observed in the acute disease, lymphocytic perivascular infiltrates are present in only 40% of BO cases associated with chronic lung allograft rejection (Randhawa and Yousem, 1992). The degree of vascular involvement in BO associated with chronic GVHD is unknown but is likely less than that of acute GVHD.

B. Models of Lung Transplantation

Both canine and rat models have been utilized to study lung transplantation and the immunopathogenesis of rejection (Benfield, 1976; Prop *et al.*, 1985a,b). However, the availability of more immunological reagents has allowed the rat model developed by Marck, Prop, and Wildevuur to be more extensively studied (Marck *et al.*, 1983). Orthotopic transplantation of the Brown Norway rat lung allografts (RT^n) into Lewis (RT^1) rats resulted in histological and immunological changes analogous to that of human lung transplantation (Marck *et al.*, 1983; Prop *et al.*, 1985a,b). The rejection process occurred in four phases (Prop *et al.*, 1985a,b): (1) the latent phase which occurred immediately after transplantation in which no immunological activity was described in the graft (Day 1 after transplantation); (2) the vascular phase, characterized by infiltration of BALT and perivascular tissue by lymphocytes (Days 2 or 3 after transplantation); (3) the alveolar phase, with mononuclear cell infiltration of the alveolar walls (Days 4 or 5 after transplantation); and (4) the destruction phase, characterized by intraalveolar edema and destruction of airways and vessels by infiltrating mononuclear cells (Day 6 after post-transplantation). Significantly, these four phases resemble somewhat the four grades associated with acute rejection in humans (Yousem *et al.*, 1990).

While these studies described the histological changes associated with acute rejection, only two reports currently exist in the literature describing animal models of chronic rejection, known as BO (Hertz *et al.*, 1993; Uyama *et al.*, 1992). Utilizing the previously described rat lung allograft model, rats made tolerant to their allografts by cyclosporin developed the typical changes of BO around 6 months after transplantation. The airway lesions were associated with upregulated class II MHC expression on the epithelium in the large airways, aggregates of DCs in the submucosa, and ulcerated epithelium (Uyama *et al.*, 1992). In a murine model utilizing hetertopically transplanted airways, the characteristic lesions of BO developed in the allograft after 21 days (Hertz *et al.*, 1993).

A significant difference between the rat models and that of clinical transplantation is that only one or two doses of the immunosuppressant drug, cyclosporin, results in indefinite acceptance of the donor rat lung (Uyama *et al.*, 1992). Human lung allograft recipients usually require life-long therapy to prevent rejection (Trulock, 1993). However, without cyclosporin, the rat lung allograft undergoes a rapid rejection process which usually results in the destruction of the allograft in 7 or 8 days posttransplantation (Prop *et al.*, 1985a,b).

Although lung transplantation has become an increasingly utilized modality for the treatment of many endstage lung diseases (Trulock, 1993), the lung allograft, in both animal models and humans, is more prone to rejection than other solid organs (Prop *et al.*, 1985a,b; Trulock, 1993). The presence of many immunocompetent cells present in the donor lung that can stimulate a rejection response may be the explanation (Prop *et al.*, 1985a,b; Trulock, 1993). Notably, despite the large numbers of T lymphocytes present in the lung and thus carried into the recipient, the clinical syndrome of systemic GVHD has not been reported in human lung allografted individuals. However, GVHD in lung transplantation was reported in an animal model in which the recipient was rendered severely immunoincompetent by total body irradiation (Prop *et al.*, 1989).

Acute lung allograft rejection is believed to be initiated by donor lung APC, i.e., DCs and perhaps macrophages, interacting with recipient lymphocytes (Winter *et al.*, 1989). Although there is no direct evidence that these accessory cells mediate allograft rejection, several studies suggest their role in the rejection responses. Acute rejection episodes commonly occur at a time when there is an abundance of donor DCs and lung macrophages, i.e., the first 8 to 12 weeks after transplantation, and diminish when these cells are replaced by those of the recipient (Paradis *et al.*, 1985; Uyama *et al.*, 1993). Utilizing a murine model of renal transplantation in which DCs had been depleted, Lechler demonstrated that repletion of DCs resulted in the rejection of the allograft (Lechler and Batchelor, 1982). Similarly, blocking antibodies to DCs resulted in the prolongation of survival of murine pancreatic islet allografts (Faustman *et al.*, 1984). As discussed previously, DCs exist within the epithelium and subepithelial areas of the bronchi/bronchioles, areas that are involved in both acute and chronic rejection. Additionally, IFNγ, a cytokine crucial to the rejection process (O'Connell *et al.*, 1993), was shown to upregulate the number of DCs in the interstitium surrounding pulmonary capillaries, within the alveolar interstitium, and in the bronchial epithelium (Kradin *et al.*, 1991). Finally, DCs accumulate in areas of BO during the course of chronic allograft rejection (Uyama *et al.*, 1992). Collectively, these studies suggest a central role for DCs in the pathogenesis of lung allograft rejection. Lung macrophages, although suppressive of many immune cell functions, may also be involved in the initiation of the rejection response.

Lung accessory cell–lymphocyte interactions occur through cytokines and intercellular signals and result in upregulated cellular and humoral immunity (Wilkes and Weissler, 1994). Cellular immunity is

crucial in lung allograft rejection (Prop *et al.*, 1985a,b) and may result from the differential stimulation of Th1 versus Th2 cells. Th1 lymphocytes play a significant role in the pathogenesis of solid organ allograft rejection (Jordan *et al.*, 1991; O'Connell *et al.*, 1983). For example, in a murine example of pancreatic islet rejection, allograft infiltrating lymphocytes preferentially expressed mRNA for IL-2 and IFNγ, and not IL-4 (O'Connell *et al.*, 1983). Similarly, in rat lung allografts, IFNγ mRNA was expressed during the rejection episodes (Jordan *et al.*, 1991). The clinical importance of Th1 lymphocytes in allograft rejection is exemplified by the fact that the primary immunosuppressive agent used in recipients of human lung allografts is cyclosporin, which preferentially inhibits IL-2 and IFNγ production from lymphocytes (Cockfield *et al.*, 1993). In contrast, Th2 lymphocyte activity, i.e., production of IL-4 and IL-10, which downregulates Th1 activity, has been strongly associated with prevention of allograft rejection (Gorczynski and Wojcik, 1994). Both allogeneic AM and parenchymal lung DCs were potent inducers of IFNγ, but not IL-4, from lymphocytes (Wilkes and Weissler, 1994). Collectively, these data suggest that allograft rejection is in part mediated by lung macrophages and DCs stimulating Th1 lymphocytes.

Cytokines from Th1 and Th2 lymphocytes can both result in specific immunoglobulin production (Kitani and Strober, 1993). Therefore, the upregulated Th1 lymphocyte activity observed in allograft rejection might be responsible for the enhanced local immunoglobulin production observed during the rejection process (Wilkes *et al.*, 1994b). IFNγ, a Th1 cytokine, can stimulate IgG2a from murine B lymphocytes (Kitani and Strober, 1993). Furthermore, IFNγ production, induced by human lung macrophages, selectively stimulated IgG2 production from allogeneic peripheral blood mononuclear cells (Wilkes and Weissler, 1994). In recipients of lung allografts undergoing rejection, Wilkes (1995) demonstrated that local production of IgG2 was selectively upregulated and, thus, served as a marker for the rejection response (Wilkes *et al.*, 1994b). Few studies have demonstrated a role for alloantibodies in mediating the process of lung allograft rejection. Coronary atherosclerosis secondary to murine cardiac allograft rejection was in part mediated by antibodies directed against the donor coronary epithelium (Russell *et al.*, 1994). Similarly, IgG2, but not IgG1, IgG3, or IgG4, produced locally during human lung allograft rejection, preferentially bound to perivascular and peribronchial extracellular connective tissue matrices which are the anatomic locations involved in the rejection process (Wilkes, manuscript in preparation). Taken together,

these studies suggest a role for a Th1-dependent humoral responses in the pathogenesis of lung allograft rejection.

While lung accessory cell–T lymphocyte interactions initiate organ rejection (Winter *et al.*, 1989), the production of proinflammatory cytokines, IL-6 and TNFα, has been identified as a mediator of the rejection process (DeMeester *et al.*, 1993; Saito *et al.*, 1993). Evidence that TNFα was involved in rejection was demonstrated by DeMeester who reported that TNFα mRNA and protein were upregulated in lung tissue during acute rejection of rat lung allografts (DeMeester *et al.*, 1993). Significantly, anti-TNFα antibodies reduced the vasculitis and hemorrhagic lesions in rejecting lung allografts (Saito *et al.*, 1993). Similarly, IL-6 was upregulated in the lung during rejection and was postulated to be clinically important in following the activity of the rejection process (Rolfe *et al.*, 1993).

C. Models of Graft versus Host Disease in the Lung

GVHD is a systemic process, and relatively few have described pulmonary involvement in animal models of GVHD (Piguet *et al.*, 1989b; Stein-Streilein *et al.*, 1981). Stein-Streilein *et al.* (1981), utilizing a murine model, reported GVHD "reactions" in the lung. In these studies, suspensions of parental (MHA) lymph node cells were instilled into the trachea of F1 hybrid (MHA × CB) recipient hamsters. The histology observed in the recipient lungs showed mononuclear cell infiltration in the interstitium, alveolar, peribronchiolar, and perivascular areas. Some of the animals developed thymic atrophy and splenomegaly which suggested a systemic component to the disease process (Stein-Streilein *et al.*, 1981). Interestingly, when the cells were given iv or intracutaneously, the animals developed systemic GVHD without any distinctive pulmonary pathology (Stein-Streilein *et al.*, 1981).

Piguet also studied the pulmonary disease associated with GVHD. In these studies, irradiated F1 hybrid (CBA × B10) mice were injected with either parental T lymphocyte-depleted bone marrow cells or with parental bone marrow cells together with suspensions of lymph node cells as a source of T lymphocytes. In addition to the induction of systemic GVHD, the histology of the lung was similar to that reported by Stein-Streilein. Additionally, these investigators demonstrated the central role of T lymphocytes in the lung pathology of GVHD in that injection of T lymphocyte-depleted bone marrow cells did not induce pulmonary pathology (Piguet *et al.*, 1989b).

Wilkes *et al.* (1994a) recently reported that allogeneic (C57BL/6) BAL accessory cells (>90% macrophages), when instilled intratracheally into the lungs of normal BALB/c mice weekly for 4 weeks, induced a lymphocytic alveolitis, bronchitis, and vasculitis analogous to GVHD of the lung or acute lung allograft rejection. Unlike other animal models of GVHD (Piguet *et al.*, 1989b; Stein-Streilein *et al.*, 1981), these recipient mice had no evidence of systemic disease. Additionally, if no further allogeneic challenges were performed, the pulmonary lesions eventually healed.

As previously stated, the clinical manifestations of chronic GVHD in the lung are associated with the development of BO (Farrara and Deeg, 1991; Randhawa and Yousem, 1992; Yousem *et al.*, 1990). In contrast, no animal models of chronic GVHD have reported an association with this type of pulmonary disease.

The immunopathogenesis in animal models of GVHD has been well described (Antin and Farrara, 1992; Piguet *et al.*, 1989b). Similar to acute lung allograft rejection, acute GVHD has been associated with the production of several proinflammatory cytokines including TL-1, TNFα, and IL-6 (Antin and Farrara, 1992; Piguet *et al.*, 1989b). In fact, IL-1 receptor antagonist was shown to significantly inhibit GVHD (McCarthy *et al.*, 1991). Relative to the lung, anti-TNFα antibodies partially prevented the pulmonary pathology of GVHD (Piguet *et al.*, 1989b). While proinflammatory cytokines are involved in the pathogenesis of GVHD, T lymphocytes initiate the process (Antin and Farrara, 1992). Th1 lymphocytes are crucial in acute GVHD. In a murine model of GVHD, IFNγ and IL-2 were preferentially produced in the course of acute GVHD (Allen *et al.*, 1993) with similar findings to those reported by other investigators (Antin and Farrara, 1992). Therefore, the immune response to alloantigens in both lung allograft rejection and GVHD seems to be associated with upregulated Th1 lymphocyte activity.

A role of humoral immunity in GVHD has not been well defined. However, Wilkes *et al.* reported (1994a) that allogeneic BAL cells instilled into murine lungs resulted in the predominant local production of IgG2a. Additionally, only IgG2a was shown to be deposited in the perivascular and peribronchiolar extracellular connective tissues, the same anatomic locations involved in lung allograft rejection and GVHD of the lung. These data suggest that locally produced immunoglobulins recognize component(s) of the extracellular connective tissue matrix and may be involved in the pathogenesis of GVHD of the lung.

VII. Summary

No evidence has emerged which suggests that the principles of immunity derived from studies on cells from other body sites are contradicted in the lung and its associated lymphoid tissue. What is clear, however, is that the environment dictates the types of cells, their relationship to one another, and what perturbing events will set in motion either the development of an "active" immune response or tolerance. Investigating mechanisms for the development of lung immunity has increased our understanding of how human diseases develop and is continuing to suggest new ways to manipulate pulmonary immune responses. Demonstration that lung cells regulate both nonspecific inflammation and immunity through the expression of adhesion molecules and the secretion of cytokines offers hope for ways to design more effective vaccines, enhance microbial clearance in immunosuppressed hosts, and to suppress manifestations of immunologically mediated lung disease. Important lung diseases targeted for intensive research efforts in the immediate future are tuberculosis, asthma, and fibrotic lung disease. Perhaps even the common cold might be conquered. Considering the pace of current research on lung immunity, it may not be too ambitious to predict that these diseases may be conquered in the next decade.

ACKNOWLEDGMENTS

The authors thank their secretarial staff for their excellent help in preparing the manuscript, specifically Patricia Sanchez, Becky Snyder, Rebecca Flores, Gerri Chavez, and Christine Cave.

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This article was accepted for publication on 16 January 1995.

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