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Cell Biology of Antigen Processing and Presentation to Major Histocompatibility Complex Class I Molecule-Restricted T Lymphocytes

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I. Introduction

A. Overview of T Cell Recognition and Function

Complex organisms coevolved with a wide variety of microorganisms (viruses, bacteria, simple eukaryotes) that reside within the cells or extracellular fluids of the host. In some cases, hosts and parasites mutually benefit from the relationship. In other cases, parasites are purely destructive. Although the long-term interests of either beneficial or destructive parasites would not generally be served by the extinction of their hosts, this consideration often does not prevent parasites from attempting to maximize their reproduction in the short term. In favor of the parasites are their rapid rates of reproduction and mutation, which together accelerate their evolution, allowing them to respond rapidly to alterations in their host environment. Standing against these multitudes is the vertebrate immune system, which has evolved the capacity to limit specifically the replication of microorganisms, and the means of distinguishing foreign from self proteins.

The immune system evolved two types of protein receptors that function to detect and destroy foreign invaders. One receptor, immunoglobulin, is used largely against extracellular antigens, and consequently is present in body fluids in high concentrations in an extremely stable form. These properties led to its relatively facile discovery, once pioneer immunologists began to examine serum from animals injected with foreign substances. The discovery of the second antigen specific receptor required nearly an additional century. In contrast to immunoglobulin, this receptor is not secreted in biologically active form, but is expressed only on the surface of thymus-derived lymphocytes (T cells). It functions in conjunction with numerous accessory molecules to target lymphocytes to other cells bearing foreign antigens (antigen presenting cells), where lymphocytes either secrete molecules or deliver signals to the antigen presenting cell via

membrane interactions. This can have several outcomes, depending on the nature of the antigen presenting cell. If the antigen presenting cell is harboring an intracellular parasite, T cells can either destroy the cell or alter its metabolism in a way that disfavors parasite replication. If the antigen presenting cell is an immunoglobulin secreting cell (known as B cells, because of their maturation in the bursa of Fabricius, a prominent avian lymphoid organ), T cells can induce the differentiation and growth of the cells, such that large amounts of the most useful antibodies are secreted. If the antigen presenting cell is of monocytic origin, T cells can release molecules that recruit additional immune cells to the site, creating an inflammation.

The targeting of T cells to antigen presenting cells is much more complicated than the simple binding of the T cell antigen receptor to a foreign protein expressed on the surface of the antigen presenting cell. The antigen specificity of interaction is conferred by two subunits of the T cell antigen receptor [α and β chains, or γ and δ chains] that, like antibodies, are expressed clonally by lymphocytes. These subunits interact with a number of other proteins involved in transmitting signals to the cytosol of the lymphocytes. Additionally, a number of other cell surface molecules on T cells and antigen presenting cells can be involved in the interaction (for reviews, see Ashwell and Klausner, 1990; Dustin and Springer, 1991; Matis, 1990; Raulet, 1989; Springer, 1990). Of particular importance functionally, are the interactions of two cell surface glycoproteins, termed CD4 and CD8, whose expression by almost all mature T cells is mutually exclusive, and whose function is to target T cells to different antigen-bearing molecules.

With few exceptions (Macphail and Stutman, 1987; Matsubayashi *et al.*, 1989), T cells expressing CD4 (T_{CD4+}) recognize antigens in conjunction with class II molecules encoded by the major histocompatibility complex (MHC), whereas those expressing CD8 (T_{CD8+}) recognize antigen in conjunction with MHC class I molecules. This targeting is achieved by the direct interaction of CD4 and CD8 molecules with class II and I MHC molecules, respectively, bearing the antigenic determinants recognized by the T cell antigen receptor (Potter *et al.*, 1989; Salter *et al.*, 1990). The expression of CD4 or CD8 molecules on T cells is coordinately regulated with the types of signals that the T cells deliver on antigen recognition. The first signal discovered for T_{CD8+} was the lysis of antigen presenting cells bearing the foreign antigen, which led to their designation as cytotoxic T lymphocytes. Later it was appreciated that these cells also secreted additional molecules (interleukin 2, interferon-γ, and tumor necrosis factor, for example). The first signal discovered for T_{CD4+} cells was the enhance-

ment of antibody production by B lymphocytes. This led to their designation as helper T cells, and is due to the secretion of various lymphokines. More recently, it has become apparent that at least a subset of T_{CD4+} cells are every bit as active as T_{CD8+} cells in lysing antigen presenting cells (Kaplan *et al.*, 1984). Thus, using the term cytotoxic T lymphocyte to refer solely to class I restricted T cells is of historic value only. As this term is potentially misleading, we have chosen to refer to class I-restricted T cells as T_{CD8+} and class II-restricted T cells as T_{CD4+}.

Although it is clear that the effector functions of T_{CD4+} and T_{CD8+} overlap to some extent, this should not obscure the fundamental differences between their roles in host immunity. The primary function of T_{CD8+} cells is to limit the replication of intracellular parasites. Consistent with this role, class I molecules are constitutively expressed on most types of cells (neurons being a prominent exception). By contrast, class II molecules are constitutively expressed largely by cells of immune lineage, particularly B cells, macrophages, and dendritic cells (although class II expression is induced in many cells by interferon-γ). This corresponds with the major function of T_{CD4+} in enhancing humoral or cellular immune responses.

To use the distinct functions of T_{CD8+} and T_{CD4+} optimally, vertebrates evolved separate pathways for monitoring intracellular versus extracellular environments for the presence of foreign proteins. Intracellular proteins are sampled by transporting cytosolic proteins, or more likely, their peptide fragments, into the exocytic compartment of cells. Here, antigenic peptides form a complex with class I molecules and are transported to the cell surface for recognition by T_{CD8+}. Extracellular proteins are monitored by internalization into the endosomal compartment of cells, where they are unfolded and fragmented into peptides. Such peptides then complex with class II molecules and are transported to the cell surface for recognition by T_{CD4+}. The means by which cells generate antigenic peptides from proteins and display them on the cell surface in a complex with MHC class I or II gene products is termed antigen processing and presentation. The purpose of this article is to present a detailed review of the current state of knowledge regarding the processing and presentation of antigens in association with MHC class I molecules.[1] To help nonspecialists ap-

[1] A number of more concise reviews of antigen processing and presentation are available; some also describe recent progress of processing and presentation of antigens in association with class II molecules (Braciale and Braciale, 1991; Brodsky and Guagliardi, 1991; Harding and Unanue, 1990; Lanzavecchia, 1990; Neefjes *et al.*, 1991; Rötzschke and Falk, 1991; Townsend and Bodmer, 1989; Yewdell and Bennink, 1990).

preciate this topic, in this introductory section we provide some historical and background information on the nature of the class I molecule–antigen complex recognized by T_{CD8^+}.

B. Brief History of the Discovery of MHC Restriction

In the late 1960s, it was found that lymphocytes from virus infected animals could lyse tissue culture cells infected with the same but not different viruses. This lytic activity was puzzling, however, because it was inconsistently observed between studies. The reason for this variability was provided by the seminal studies of Zinkernagel and Doherty on T cell recognition of lymphocytic choriomeningitis virus, which indicated that antigen specific lysis of virus infected cells by lymphocytes required that target cells and lymphocytes be of the same MHC haplotype, or in other words, it was MHC restricted. In prior studies, unsuspecting investigators did not concern themselves with the genetic background of the target cells used, the primary criteria being the infectibility of the cells by the virus of interest and the suitability of the cells in the chromium-51 release assays commonly used as a measure of target cell lysis. Within a short time, similar findings were reported for T cell recognition of numerous other antigens, including other viruses, haptenylated cells, and minor histocompatibility antigens [see Zinkernagel and Doherty (1979) for a classic review of the findings that preceded the discovery of MHC restriction and the information explosion it ignited].

The findings of Zinkernagel and Doherty clearly demonstrated that T cells and antibodies recognize antigens in a fundamentally different manner. While antibodies simply bind to free antigens, T cell recognition somehow involves MHC gene products. These gene products were known for many years to be involved in transplantation rejection, and it was through this phenomenon that the polymorphism of MHC products was first appreciated. Through painstaking breeding of mice, it proved possible to map transplantation rejection to chromosome 17, and to make a fairly detailed genetic map of this region, which was termed H-2 (the human MHC is known as HLA, and is located on chromosome 6). Within H-2 are two or three loci encoding gene products that elicit rapid rejection of transplanted tissue. These are termed K, D, and L (in humans the equivalent loci are termed HLA-A,-B, and -C). Alleles at these loci are designated with superscript letters (e.g., K^d). The combination of alleles present on one chromosome is termed the MHC haplotype. Two loci with the same superscript indicate that

these alleles are derived from the haplotype of a commonly used mouse strain. In humans, alleles are designated numerically. As class I molecules encoded by different loci are nearly as similar to each other genetically and functionally as different alleles at the same locus, it is useful to refer to the various gene products encoded by the class I loci as allomorphs.

The discovery of the MHC class I-restricted nature of T_{CD8+} recognition of virus-infected cells caused great excitement, as it represented the first function to be ascribed to MHC class I gene products since transplantation rejection. Prior to the evolution of surgeons and immunologists, transplantation was not a common occurrence in nature, and it was obvious that the primary function of the MHC lay elsewhere. Virus infections, on the other hand are commonplace, and although the mechanism of MHC-restricted recognition remained obscure, it was clear that at least one of the major functions of the MHC had been discovered.

Of the many clever models proposed to account for the phenomenon of MHC restriction, most proposed that the viral antigens recognized by T_{CD8+} were membrane glycoproteins. All of the viruses used in the initial studies were membrane viruses that expressed such proteins as part of their infectious cycles. As there was every reason to believe that T_{CD8+} recognition was based on its interaction with cell surface structures, it was a natural assumption that only those viral antigens expressed on the surface would be recognized. This hypothesis received considerable support from a number of experimental approaches. Additional findings obtained using viruses representing a number of families indicated, however, a curious discrepancy between T_{CD8+} recognition of virus-infected cells and antibodies specific for viral glycoproteins: T_{CD8+} often recognized cells infected with closely related viruses in a far more cross-reactive manner than antibodies.

For more than a decade immunologists struggled toward a molecular understanding of T_{CD8+} recognition of foreign antigens that would explain both MHC restriction and the cross-reactive nature of recognition. This entailed defining both the T cell antigen receptor and the nature of the antigen recognized on virus-infected cells. These formidable tasks were made tractable by the application of three technologies introduced or popularized during this period: monoclonal antibody (mAb) production by B cell hybridomas, cloning and expression of genes by genetic engineering methods, and the production of synthetic oligopeptides. With these tools, it proved possible to identify the constituents of the complex recognized on the virus-infected cell by

T_{CD8+}. In parallel, crystallographic methods were used to define precisely the structure of a soluble class I molecule–antigen complex.[2]

C. What T_{CD8+} Recognize

1. Structure of Class I Molecules

Class I molecules consist of two noncovalently bound chains. The α chain [relative molecular mass (M_r) 44 kilodaltons (kDa)] is the polymorphic glycoprotein encoded by MHC class I loci. α Chains are encoded by eight exons. Exon 8 encodes a carboxy terminal cytoplasmic domain of 1 to 10 residues. The bulk of the rest of the cytoplasmic domain (approximately 24 residues) is encoded by exons 6 and 7. Exon 5 encodes the transmembrane anchor and linkers of 8 or so residues at each end. The extracellular domain is encoded by exons 2, 3, and 4, which encode the α1, α2, and α3 domains, respectively. Most of the polymorphism between class I allomorphs occurs in the α1 and α2 domains. The first exon encodes the leader sequence that targets newly synthesized α chains to the endoplasmic reticulum (ER), and is proteolytically cleaved from the mature protein shortly thereafter. Bound to the extracellular domain, is β2-microglobulin (β_2m) [M_r 12 kDa], a nonpolymorphic protein,[3] which as its name implies is present in serum, although only in trace amounts.

Association of β_2m with α chains occurs in one of the early compartments of the exocytic pathway, either the ER, the intermediate compartment believed to exist between the ER and the Golgi complex (GC), or the proximal portions of the GC. Assembled class I molecules then traverse the more distal reaches of the GC, before being delivered to the plasma membrane by vesicles that probably bud from the trans-GC network. During its transport to the cell surface, the one to three N-linked oligosaccharides are modified from the simple high mannose form added to α chains in the ER to the galactose- and sialic acid-containing complex forms characteristic of many plasma membrane glycoproteins.

[2] It has proven somewhat more difficult to obtain structural information regarding the T cell antigen receptor. Although most, if not all, of the components of the T cell receptor complex have been identified, the multisubunit nature of the complex (and possibly the low affinity of the complex for antigen) has frustrated attempts to study the specificity of the receptor in cell-free form. Although there has been some recent progress in this area (Matusi *et al.*, 1991), solving the structure of the T cell receptor–ligand complex by crystallographic methods remains a distant goal. At present, information regarding the interaction of the T cell receptor with its ligands must be derived from indirect methods.

[3] There is actually some very limited polymorphism in mouse β_2m. Whatever functional significance this polymorphism might have is undetermined.

Treatment of cells with papain cleaves many class I molecules at a single site near their attachment to the plasma membrane. Purified papain-released HLA-A2 molecules were used to determine the structure of class I molecules by crystallographic methods (Bjorkman *et al.*, 1987a,b, reviewed in Bjorkman and Parham, 1989). This revealed that the portion of the molecule most distal from the membrane comprises the $\alpha 1$ and $\alpha 2$ domains folding together to form an antigen binding groove consisting of two parallel α helices separated by 18 Å, lying atop a platform of eight antiparallel β sheets to form a 25-Å-long cleft 10 Å deep. The $\alpha 1$ and $\alpha 2$ regions do not form extensive contacts with other parts of the molecule. Beneath the antigen binding region lies the $\alpha 3$ region, which folds similarly to immunoglobulin domains and forms extensive contacts with β_2m, which also forms an immunoglobulin-like fold (these folding patterns were expected based on sequence homologies between these domains and immunoglobulin domains).

Many of the polymorphic residues in class I molecules are located in the α helices or the β sheets that form the floor of the pocket. Substitutions in some of these residues have been shown to affect the specificity the binding of exogenous peptides (Gotch *et al.*, 1988; Hogan *et al.*, 1988, 1989; Mattson *et al.*, 1989; McMichael *et al.*, 1988; Robbins *et al.*, 1989), as well as peptides naturally processed from cell proteins (Van Bleek and Nathenson, 1991). Only the polymorphic residues in the α helices (and a few in loops exposed laterally on the molecule), however, affect the binding of monoclonal antibodies specific for $\alpha 1\alpha 2$ region. This makes perfect sense both structurally and functionally, as these residues would be accessible to antibodies and these antibodies generally have the capacity to block T_{CD8^+} recognition of target cells. Although the residues that form the binding site clearly will have the greatest impact on the specificity of peptide binding, it is likely that some residues outside the site will also influence peptide binding (Muller *et al.*, 1991).

Solution of the structure of HLA-Aw68, which differs from HLA-A2 by substitutions in 13 residues (a normal number of substitutions between allomorphs) revealed a structure startlingly similar to that of HLA-A2 (Garrett *et al.*, 1989). Ten of the amino acid alterations extend into the antigen binding groove. Although none of the alterations affect the overall folding of the binding site, the net effect of the ten changes is to alter the shape and charge of localized regions of the groove. Most striking was the creation of a negatively charged pocket in Aw68 occupied by a residue from the peptide ligand present in the crystallized structure. While the peptides present in A2 and Aw68 could not be resolved at high resolution (probably because of their heterogeneity),

considerable structural information could be gleaned from the peptides present in HLA-B27 crystals (Madden *et al.*, 1991) (discussed in the next section).

2. *Nature of Antigens Bound to Class I Molecules*

The very first indication that T_{CD8+} recognize peptides derived from viral proteins was the finding that peptides with a $M_r < 5$ kDa produced by enzymatic and chemical cleavage of Sendai virus proteins stimulated Sendai virus-specific T_{CD8+} proliferation *in vitro* (Guertin and Fan, 1980). This did little to shake the widely held conviction that T_{CD8+} recognize intact proteins. Cracks in the edifice began to appear, however, when it was realized that class II molecules present peptides of 15 or so residues to T_{CD4+} (Babbitt *et al.*, 1985; Buus *et al.*, 1986; Schwartz, 1985). The dogma was finally overturned by the elegant series of studies by Townsend and colleagues (1984, 1985, 1986a), culminating in the demonstration that cells incubated with synthetic peptides of 14 or more residues corresponding to sequences in influenza virus nucleoprotein-sensitized target cells for recognition by T_{CD8+}. Using synthetic peptides, is has been possible in the past 5 years to define precisely the determinants recognized by T_{CD8+} specific for many different proteins. Indeed, to our knowledge, there is no published evidence that T_{CD8+} recognize anything but short peptides. Rather, in the past year there have been numerous reports that provide direct evidence that determinants processed naturally by cells are in fact peptides, and in an unexpected twist, of quite uniform length.

Two approaches were used to identify natural peptides associated with class I molecules. Rammensee and colleagues initially found that antigenic peptides could be isolated from cells simply by disrupting cells in acid solution, and separating material of $M_r < 5$ kDa by reverse-phase high-performance liquid chromatography (HPLC) (Rötzschke *et al.*, 1990a). Antigenic peptides were then identified in fractions by their capacity to sensitize target cells for lysis by antigen-specific T_{CD8+}. Next it was shown that the existence of antigenic peptides in acid extracts usually requires the expression of the class I allomorph that presents the peptides (Falk *et al.*,1990)[4] (see Section II.C.2.e for discussion of this important finding). Finally, peptides were isolated from influenza virus-infected cells that sensitized target cells for recognition by K^d- or D^b-restricted T_{CD8+} specific for previously (Bodmer *et al.*, 1988; Townsend *et al.*, 1986a) defined determinants derived from

[4] It was later reported that the amount of peptides from minor histocompatibility antigens recovered from different organs was proportional to class I expression in the organ (Griem *et al.*, 1991).

the viral nucleoprotein (Rötzschke *et al.*, 1990b). The behavior of these peptides on HPLC was compared with that of synthetic peptides that, as it happened, were longer than the natural peptides. This revealed that most of the antigenic activity in preparations of peptides 12 or 16 residues resided with peptides eluting in different fractions than the bulk of the peptide preparation. Much of the antigenic activity, then, was due to the presence of minor contaminants produced during peptide synthesis. By sequencing the contaminants that coeluted with peptides obtained from virus-infected cells, it was found that the natural peptides likely consisted of nine residues. In support of this conclusion, it was shown that the natural peptide and a synthetic peptide of the same nine residues coeluted in HPLC performed with various eluants (Falk *et al.*, 1991a).

A more direct though more arduous approach was used by Van Bleek and Nathenson (1990), who acid-eluted radiolabeled peptides from immunoaffinity-purified K^b derived from radiolabeled cells infected with vesicular stomatitis virus. Material with a M_r of < 1500 kDa was fractionated by reverse-phase HPLC, and the single prominent radioactive peak uniquely present in virus infected cells relative to uninfected cells was sequenced. This revealed that the natural peptide was derived from residues 50–57 of the viral nucleocapsid protein. A synthetic peptide corresponding to this sequence was found to coelute with the radioactive natural peptide in HPLC.

By the use of peptides acid eluted from immunoaffinity-purified HLA-B27 molecules it proved possible to obtain sufficient material in HPLC fractions to sequence a number of unique peptides derived from self proteins (Jardetzky *et al.*, 1991). All consisted of nine residues and had arginine in position 2 (numbering from the amino terminus). Several of the other positions demonstrated biases toward specific residues or types of residues. These findings agreed perfectly with a prior study (Falk *et al.*, 1991b), in which peptides eluted from purified mouse and human class I molecules were pooled from HPLC fractions and sequenced. The pooled peptides were homogeneous in length (nine residues for K^d, D^b, or HLA-A2.1, eight residues for K^b), and featured two "dominant anchor positions" in which one or two amino acids were heavily overrepresented. Other positions often demonstrated preferences ranging from strong to weak for specific amino acids. Comparison of the sequences of natural peptides to those of defined antigenic peptides revealed a nearly perfect correlation in the types of residues present at each position (Falk *et al.*, 1991b; Jardetzky *et al.*, 1991; Romero *et al.*, 1991). Moreover, the amazing uniformity in length correlates with a large number of studies reporting that the optimal length of peptides for binding to class I molecules (assessed by

either direct binding assay, potency of sensitizing target cells for T_{CD8+} lysis, or ability to block sensitization of antigenic peptides in competition assay) is eight [K^b] or nine [D^b, K^d, L^d] residues (Cerundolo *et al.*, 1991; Deckhut *et al.*, 1992; Elliot *et al.*, 1991; Gould *et al.*, 1991; Palmer *et al.*, 1991; Reddehase *et al.*, 1989; Romero *et al.*, 1991; Schulz *et al.*, 1991; Schumacher *et al.*, 1991).

These findings provide compelling evidence that class I molecules at the cell surface contain peptides of eight to nine residues. Although it is clear that peptides of this length occupy a sizable majority of the stably assembled class I molecules expressed at the cell surface, the failure to routinely recover longer peptides from cells or purified class I molecules does not necessarily indicate that longer peptides do not contribute to T_{CD8+} recognition. While such complexes would be expected to be short lived at the cell surface, they might reach sufficient steady state levels to enable T_{CD8+} recognition, particularly as only 200 or so complexes per cell are necessary to trigger T_{CD8+} recognition (Christinck *et al.*, 1991). The dissociation of longer peptides from class I molecules could contribute to the many cell surface class I α chains that appear not to be associated with peptides or β_2m (see Section III.B.). Ultimately, the relevance of recognition of longer peptides will have to be established by the isolation of T_{CD8+} that require residues at the termini of such peptides derived from endogenously synthesized proteins.

3. *What Does the T Cell Antigen Receptor See?*

The relatively ordered structure of the peptides in the antigen binding groove of HLA-B27 provided the first direct structural examination of the peptide class I interaction (Madden *et al.*, 1991). Consistent with the sequencing of eluted peptides, the peptides present in B27 are largely, if not exclusively, nonamers. The peptides bind to the site with uniform polarity, and assume a largely extended conformation, with a kink between positions 3 and 4 from the amino terminus. Importantly, there are numerous interactions between main chain atoms in the last two residues of the amino and carboxy termini of the peptides (including the terminal amino and carboxy groups themselves) with groups of residues at each end of the binding groove that are highly conserved between class I molecules. This provides a ready explanation for the increased binding affinity for class I molecules of peptides of the proper (nonameric) length (see Section II.E.2.b). The observed preferences for certain residues in various positions of B27-associated peptides are consistent with the shape and charge of regions of the binding site that interact with the favored residue. Most striking, the arginine at position 2, which was present in all high-affinity B27 binding peptides,

was accommodated by a long and narrow pocket that is not present in HLA-A2. At least four of the peptide side chains were oriented away from the floor of the binding site and could potentially interact with the T cell antigen receptor.

Although further structural refinements of the peptide–class I molecule complex will no doubt be forthcoming, and determining the structure of T cell antigen receptor bound to the class I–peptide complex remains as perhaps the ultimate achievement in immunological crystallography, the puzzle of MHC restriction has largely been solved. Class I molecules evolved to bind a large variety of peptides with relatively high affinity by interacting in a highly specific manner with one or two residues and in a less specific manner with several others. The other four or five residues are displayed in an extended conformation to the T cell antigen receptor which can decide whether these residues are derived from self or foreign proteins.[5]

II. Antigen Processing

A. Preliminaries

Antigen processing can be divided into five steps: (1) entry of proteins into the pathway, (2) unfolding and proteolysis, (3) transport of antigens into an exocytic compartment, (4) assembly of the class I–peptide complex, and (5) transport of the assembled complex to the cell surface. The sequence of these steps is inevitable, with the notable exception of step 2, which as discussed below, could occur either before or after steps 3 and 4, or, for that matter, in multiple parts of the sequence.

Before launching into a step-by-step analysis of antigen processing, it is useful to introduce several mutant cell lines that have proven invaluable in dissecting and understanding antigen processing. The first mutant cell line appreciated to be deficient in antigen processing was produced by Kärre and colleagues (1986; Ljunggren and Kärre, 1985) from a Rauscher virus-induced T lymphoma line (RBL-5, H-2^b) by chemical mutagenesis, followed by selection for resistance to lysis by anti-H-2 antiserum plus complement. The mutagenized but nonselected cells (RMA) expressed normal levels of K^b and D^b, whereas selected cells expressed 5–10% of the normal amount of class I molecules. α Chains and β_2m are produced at normal levels, but fail to be efficiently assembled and transported to the cell surface. Subsequently, it was found that RMA/S cells efficiently present synthetic

[5] It also seems likely that subtle conformational alterations in the α helices are induced by the binding of some peptides, and contribute to self, nonself discrimination.

peptides to T_{CD8+}, but are deficient in their capacity to present influenza virus nucleoprotein to T_{CD8+} (Townsend *et al.*, 1989). The antigen processing deficit in RMA/S cells cannot be attributed to mutations in class I α genes or β_2m, as H-2^b-restricted antigen presentation and class I surface expression are rescued by fusion of cells with L929 cells (Ohlen *et al.*, 1990b).[6] On the basis of a number of independent studies, it is clear that RMA/S cells retain some antigen processing capacity. RMA/S cells have been shown to present antigens to allo-, minor-(Ohlen *et al.*, 1990a), and viral-specific T_{CD8+} (Esquivel *et al.*, 1992). Indeed, RMA/S cells presented both influenza virus nucleoprotein and vesicular stomatitis virus nucleocapsid to T_{CD8+}, which demonstrates that the cells are capable of processing cytosolic antigens, although presentation required more antigen, occurred with slower kinetics, and, like allo-recognition, was more easily inhibited by anti-CD8 antibody (Esquivel *et al.*, 1992). This latter observation is consistent with either quantitative or qualitative deficiencies in the class I peptide complexes present on RMA/S cells. The former interpretation is probably correct based on quantitative but not qualitative differences in allo-specific antigenic peptides recovered from RMA/S and RMA cells.

These findings were rapidly extended to a mutant human cell line, .174, and its derivatives. Line .174 is one of a panel of mutant cells lines produced by DeMars and colleagues (1984) by γ-irradiating Epstein–Barr virus-transformed B lymphoblastoid cell lines and selecting for HLA mutants by resistance to anti-HLA mAb plus complement lysis. One such line, .45.1, selected with an anti-HLA-A8 mAb, has a deletion spanning the entire HLA complex in one copy of chromosome 6. Following re-irradiation, cells were selected with a mAb specific for class II gene products. The .174 cells that resulted from this treatment had a large deletion (roughly one megabase pair) in the HLA class II region of the other copy of chromosome 6 (Erlich *et al.*, 1986). The class I gene products encoded by this chromosome are normal, but their assembly with β_2m and transport to the cell surface occurs less efficiently than in normal cells. On fusion of .174 cells with cells with a normal HLA complex, surface expression of the class I molecules encoded by .174 was restored (DeMars *et al.*, 1985), providing the first clue that trans-acting factors were involved in the assembly and intracellular transport of class I molecules. Similar experiments were per-

[6] The lack of mutation in β_2m is inferred from the observation that the cell surface expression of RMA/S β_2m (which is of the b allotype) was rescued by fusion with L929 cells (which express β_2m of the a allotype) (Ohlen *et al.*, 1990b).

formed using a different fusion partner, the T cell lymphoblastoid line (CEM^R), producing a cell line (termed T1) in which, again, the expression of the class I gene products of .174 was restored. A subclone of T1 cells was isolated (termed T2), in which both copies of chromosome 6 derived from CEM^R were deleted, and class I expression was concomitantly lost, mapping the trans-acting factor to chromosome 6 (Salter *et al.*,1985). Both the T2 and .174 lines were found to present synthetic peptides, but not endogenously synthesized antigens to T_{CD8+} (Cerundolo *et al.*, 1990; Hosken and Bevan, 1990). In contrast to the RMA/S cells, there have been no published reports indicating that the processing defect is less than complete.

Another mutant human cell line that has figured prominently in dissecting antigen processing was selected from γ-irradiated .45.1 cells by treatment with complement plus a mAb specific for HLA-A2 (DeMars *et al.*, 1984). The resulting cells, .134, demonstrated reduced expression of HLA-A and -B molecules at the cell surface. Unlike .174, however, it does not have a detectable deletion in chromosome 6, and the reduced class I cell surface expression appears to result from a point mutation or small deletion (Spies *et al.*, 1990). These cells are similar to RMA/S cells in having a partial rather than absolute defect in presentation of viral antigens (Spies *et al.*, 1992).

In summary, although the underlying defects in human and mouse mutant cells responsible for the low surface expression of class I molecules differ (described in Section II.D), the mutants share a similar phenotype with regard to class I assembly and transport. All synthesize normal amounts of class I α chains and β_2m, but fail to assemble class I molecules and transport them to the surface efficiently.

B. Entry of Proteins into the Class I Processing Pathway

1. Peptons or Proteins?

Immunization with protein antigens only rarely induces antigen-specific T_{CD8+} responses. Generally, noninfectious forms of viral antigens are also poorly immunogenic for T_{CD8+} responses, and do not efficiently sensitize target cells for recognition by T_{CD8+}. These finding demonstrate that extracellular proteins ("exogenous proteins") are not efficiently processed by cells for association with class I molecules or, in other words, do not efficiently enter the class I processing pathway. Biosynthesized proteins ("endogenous proteins"), on the other hand, have ready access to the processing pathway, as proteins produced during a virus infection efficiently elicit T_{CD8+} responses. There are potentially two general explanations for the greatly enhanced ability of endogenous proteins to enter the class I processing

pathway: (1) the location of newly synthesized proteins in the cytosol where protein synthesis occurs, and (2) the production of truncated proteins as a by-product of protein or RNA synthesis.

The latter explanation is consistent with the curious ability of promoterless genes encoding tumor-specific antigens to sensitize target cells for lysis by tumor-specific T_{CD8+} following transfection of the genes into the cell (De Plaen *et al.*, 1988; Sibille *et al.*, 1990; Szikora *et al.*, 1990). Remarkably, the intentional introduction of stop codons or frame alterations upstream of sequences encoding tumor peptides did not abrogate antigen presentation. These observations led to the "pepton hypothesis," which suggested that RNA polymerases not requiring a typical promoter region produce short transcripts that are exported from the nucleus and translated into short peptides (Van Pel and Boon, 1989).

Related findings have been made regarding the recognition of cells transduced with defective retroviruses containing the influenza virus nucleoprotein gene (Fetten *et al.*, 1991). Introduction of a frameshift mutation at the region of the gene encoding the amino terminus of nucleoprotein did not affect recognition of the cells by nucleoprotein-specific T_{CD8+}, despite the failure of cells to produce amounts of nucleoprotein detectable by immunoprecipitation. In contrast, cell lines transduced with retroviruses in which nucleoprotein transcription was controlled by a weaker promoter produced detectable levels of protein, but were not recognized by nucleoprotein-specific T_{CD8+}. This finding argues strongly against the possibility that presentation of frameshifted nucleoprotein is due to residual synthesis of low levels of full-length molecules, and supports the idea that peptides are not derived from full length nucleoprotein. As presentation was related to levels of mRNA, this is inconsistent with the pure pepton hypothesis, suggesting instead that peptons are produced from mRNA and not DNA.

If peptons are a major route of entry of determinants to the class I processing pathway, then some determinants should be derived from out-of-frame sequences. Of the 11 self peptides identified from HLA-B27 molecules, 7 could be identified from known proteins (Jardetzky *et al.*, 1991). All of the tumor-specific peptides identified by Boon and colleagues are coded by in frame sequences. Moreover, there have not been any reports of T_{CD8+} specific for a defined viral gene product whose specificity could not be mapped to an in-frame peptide.[7] The

[7] It is possible that T_{CD8+} recognizing such peptides have been found but have not been reported, their failure to recognize peptides from the appropriate region of the protein being attributed to technical difficulties.

absence of evidence for out-of-frame peptides suggests either that peptons: (1) do not exist or, at least, are never created at levels sufficient to trigger T_{CD8+} recognition; (2) only reach such levels when produced from transfected DNA; and (3) represent prematurely terminated translation products.

In contrast to the absence of direct experimental evidence supporting the pepton hypothesis or similar schemes, there is considerable evidence that the cytosolic location of newly synthesized proteins is critical to their entry into the class I processing pathway. The first experiments to address this issue directly were performed with influenza virus. Chemical or thermal inactivation of viral neuraminidase activity enabled a preparation of influenza virus, whose infectivity was greatly reduced by irradiation with ultraviolet light, to sensitize target cells for recognition by T_{CD8+} specific for various influenza virus proteins (Hosaka *et al.*, 1985, 1988; Yewdell *et al.*, 1988). As it is virtually impossible to completely eradicate the ability of viruses to direct synthesis of their proteins without destroying the virion, it is critical in these types of experiments to eliminate the possibility that sensitization is due to residual viral protein synthesis, particularly as T_{CD8+} are perhaps a more sensitive measure of protein synthesis than any biochemical or antibody-based technique. To minimize this possibility, cells were continuously incubated in protein synthesis inhibitors. Better yet, however, was the finding that two determinants presented by virus-infected cells were not presented by cells sensitized with neuraminidase-inactivated virus. One of these determinants was derived from a viral protein synthesized by virus infected cells but excluded from virions (a "nonstructural" viral protein). This control was crucial in these experiments, as the amount of cell-associated virus is greatly enhanced by inactivating the neuraminidase, which normally functions to destroy virus receptors (terminal sialic acids on glycolipids and glycoproteins) and thereby release virus from the cell surface. Delivery of proteins to the class I pathway required the fusion of viral and cellular membranes, as a number of independent manipulations that prevented virus–cell fusion also prevented sensitization. The delivery of proteins to the cytosol was directly demonstrated by the immunofluorescence localization of viral structural proteins in the nucleus. Subsequently it was demonstrated that presentation of exogenous proteins, like endogenous proteins, was inhibited by the drug brefeldin A (BFA), which, as discussed below (Section II.E.1.a) blocks presentation of processed proteins but not antigenic peptides to T_{CD8+} (Yewdell and Bennink, 1989). Among the viral proteins delivered to the class I pathway was hemagglutinin, although only one of the two determinants presented to T_{CD8+} from endogenous hemagglutinin

was presented from exogenous hemagglutinin. Presentation of hemagglutinin is curious, because as an external viral membrane glycoprotein, it should not be delivered to the cytosol during viral penetration. The presentation of hemagglutinin may mean that the fusion of viral and cellular membranes is not a seamless process, and pores are created that allow for the entry of soluble proteins.[8]

The findings with influenza virus explained much earlier findings obtained with Sendai virus (Gething *et al.*, 1978; Koszinowski *et al.*, 1977), and later with other viruses, that noninfectious virus preparations or subviral preparations (e.g., purified viral glycoproteins in liposomes) could sensitize target cells for T_{CD8+} lysis if they maintained their membrane fusion activity. Ironically, these experiments were among the most persuasive in tilting the field toward the idea that T_{CD8+} recognize intact proteins, as it was believed that viral glycoproteins incorporated into the plasma membrane were associating in native form with class I molecules. The identity of the viral proteins recognized in these circumstances have yet to be defined, and it will be of interest to determine if as with influenza virus, viral glycoproteins are gaining access to the class I processing pathway.

More recently it was found that proteins from human cytomegalovirus virions enter the class I pathway (Riddell *et al.*, 1991). This appeared to represent bona fide presentation of exogenous proteins because presentation was inhibited by BFA, and was insensitive to blocking viral RNA synthesis to an extent that blocked presentation of an endogenously synthesized viral protein. Unlike influenza virus, sensitization with cytomegalovirus was achieved at a low multiplicity of infection, which suggests that the presentation of exogenous proteins from cytomegalovirus might be relevant in the immune response to natural cytomegalovirus infection. This contention is supported by the observation that a healthy portion of the cytomegalovirus-specific T_{CD8+} response is directed to determinants that can be processed from virions.

Another instance in which the entry of exogenous viral protein to the class I pathway might be of physiological relevance occurs with hepatitis B virus surface antigen, which was found to sensitize cells for lysis by hepatitis B virus-specific T_{CD8+}(Jin *et al.*, 1988). These findings were subsequently confirmed (Barnaba *et al.*, 1990), and importantly,

[8] Soluble hemagglutinin may be present in the virus preparation or it might be produced during the internalization process. Possibly germane to this issue, although both hemagglutinin determinants are located in the extracellular domain (Gould *et al.*, 1991), the determinant presented from exogenous virus is the one more distant from the membrane anchor domain and, therefore, more likely to be liberated from the virus by proteolysis.

it was shown that B cells expressing antibodies specific for hepatitis B virus surface antigen were sensitized for T_{CD8+} recognition 10,000-fold more efficiently than B cells expressing other antibodies. This effect is due presumably to the more efficient binding of antigen to cells. It was suggested that T_{CD8+} destruction of B cells expressing anti-hepatitis virus surface antigen antibodies could account for the failure of patients with chronic hepatitis to produce antibodies to surface antigen despite the presence of large amounts of surface antigen in their sera. Although limited evidence was presented that favors the idea that surface antigen was entering the class I processing pathway, and not simply acting as an exogenous peptide antigen (Barnaba *et al.*, 1990), this was not established conclusively. If surface antigen is entering the class I processing pathway, the mechanism needs to be better defined, particularly with regard to the site at which penetration of surface antigen to the cytosol occurs.

The entry of proteins into the class I pathway has also been studied using a soluble protein. Bevan and colleagues expressed ovalbumin in mouse cells by DNA-mediated transfection and used these cells to stimulate K^b-restricted, ovalbumin-specific T_{CD8+} (Moore *et al.*, 1988). They then demonstrated that cells incubated with ovalbumin were not sensitized for lysis by these T_{CD8+}. If, however, cells were incubated with ovalbumin in hypertonic medium, and then briefly shifted to hypotonic medium, the cells were sensitized for lysis. This treatment had been previously shown to deliver molecules internalized by fluid phase pinocytosis to the cytosol via osmotic lysis of endosomal membranes (Okada and Rechsteiner, 1982). Cells treated with 0.05% glutaraldehyde for 15 seconds were unable to present osmotically loaded ovalbumin, but maintained the ability to present a synthetic peptide containing the determinant naturally processed from ovalbumin, indicating that active metabolic processes were required to process ovalbumin (Hosken *et al.*, 1989). When this treatment was used to stop processing, it was determined that ovalbumin bypassed the glutaraldehyde blockade between 30 and 45 minutes after osmotic loading.[9] The antigen presentation-deficient cell line T2 (described in

[9] In the same study it was found via glutaraldehyde fixation that influenza virus-infected cells first present antigen between 90 and 180 minutes after infection. This difference between ovalbumin and influenza virus proteins is probably due to the additional time required to synthesize viral proteins. When noninfectious influenza virus is used, antigens are processed past the BFA blockade between 45 and 90 minutes after addition of virus (J. W. Yewdell and J. R. Bennink, unpublished findings). Taken together, these findings suggest that it requires as little as 45 minutes for peptides to be generated from proteins, transported to class I molecules, and delivered to the plasma membrane.

Section II.A) transfected with the K^b gene did not present ovalbumin after osmotic loading (Hosken and Bevan, 1990). By contrast, T2-K^b cells presented the synthetic ovalbumin peptide, and a control K^b-transfected human lymphoblastoid line efficiently presented osmotically loaded ovalbumin. The failure of T2 cells to present osmotically loaded ovalbumin is an important finding, because it provides genetic evidence that exogenous and biosynthesized proteins are processed in a similar manner. At the same time, it must be recognized that the one megabase pair deletion in T2 cells potentially encodes for a number of gene products required for efficient antigen processing and presentation. Other mutants expressing a deficit in one of these gene products might demonstrate a selective loss in the processing of endogenous or exogenous antigens.

The findings with viral proteins and ovalbumin indicate that intact exogenous proteins can enter the class I processing pathway. Although the data strongly suggest that the cytosol is the portal to this pathway, this remains to be established more conclusively. This is eminently feasible as the model makes a number of verifiable predictions. First, cytosolic delivery methods in general (e.g., electroporation) should deliver exogenous proteins to the class I processing pathway. Second, proteins with membrane translocating activity (many plant and bacterial toxins and several viral transactivating transcription factors have been reported to have this activity) should spontaneously enter the class I processing pathway.

Additionally, a number of satellite phenomena beg the issue of how proteins gain access to the class I processing pathway. It was observed many years ago that large tumor antigen (T antigen) from simian virus 40, or even the carboxy-terminal one-third of the molecule, induces an *in vivo* T_{CD8+} response (Chang *et al.*, 1979; Jay *et al.*, 1978; Tevethia *et al.*, 1980). Immune stimulating complexes (ISCOMS), which are composed of cholesterol, saponin, and lipids, in addition to protein antigens, have been found to induce vigorous T_{CD8+} responses (Heeg *et al.*, 1991; Takahashi *et al.*, 1990). Cells biosynthesizing minor histocompatibility antigens or viral antigens are able to induce T_{CD8+} responses *in vivo* in a non-MHC-restricted manner (Bevan, 1976; Gooding and Edwards, 1980). Splenocytes incubated *in vitro* with ovalbumin induce ovalbumin-specific T_{CD8+}responses *in vivo* (Carbone and Bevan, 1990), or stimulate T_{CD8+} hybridomas *in vitro* (Rock *et al.*, 1990a). In the latter case, presentation was eliminated by treating splenocytes with anti-class II mAbs plus complement, suggesting a specialized class II expressing subset of cells is able to process soluble proteins for association with class I molecules. Processing of soluble

proteins by these cells is energy dependent and apparently requires protein-synthesis, as it is blocked by treating splenocytes with azide or ricin. A population of MHC class II+, Fc receptor-expressing cells present in the thymus have similar properties (Grant and Rock, 1992). The extent to which these phenomena reflect the cytosolic delivery of antigens to antigen presenting cells, versus delivery to another cellular compartment, or extracellular processing to antigenic peptides remains to be established.

2. *Targeting of Biosynthesized Proteins to the Class I Processing Pathway*

The role of the cytosol as the portal to the class I processing pathway is supported by a number of findings made with biosynthesized proteins. The first indication that cytosolic proteins efficiently entered the class I processing pathway came from studies with T antigen, where it was found that cells expressing T antigen following DNA-mediated transfection were recognized by T_{CD8+} induced by immunization with simian virus 40 transformed cells (Gooding and O'Connell, 1983; Tevethia *et al.*, 1983). T antigen contains a nuclear localization sequence and partitions largely into the nucleus of cells. Nuclear localization, or for that matter, even the native structure of T antigen, is not required for entry into the class I processing pathway, as cells expressing carboxy-terminal fragments of T antigen without nuclear localization sequences were recognized by T_{CD8+}.

At the same time, the first evidence was uncovered regarding the identity of gene products recognized by T_{CD8+} specific for cells infected with a lytic virus. By the use of influenza viruses containing gene segments from different serotypes, it was found that the ability of viruses to sensitize cells for recognition by a serotype specific T_{CD8+} clone segregated with one of the viral polymerases (Bennink *et al.*, 1982). A subsequent study demonstrated that another T_{CD8+} clone required the gene encoding the viral nucleoprotein (Townsend and Skehel, 1984). T_{CD8+} recognition of nucleoprotein was directly demonstrated using cells transfected with nucleoprotein DNA (Townsend *et al.*, 1984) and with a recombinant vaccinia virus expressing the nucleoprotein gene (Yewdell *et al.*, 1985). Like T antigen, both PB2 and nucleoprotein have nuclear localization sequences. This was shown to be unnecessary for nucleoprotein, however, as cells expressing fragments of nucleoprotein missing the sequence were recognized by T_{CD8+} (Townsend *et al.*, 1985). Furthermore, vesicular stomatitis virus nucleocapsid protein, which is not targeted to the nucleus, was also shown to be efficiently presented to T_{CD8+} (Pud-

dington *et al.*, 1986; Yewdell *et al.*, 1986). Some of the influenza nucleoprotein fragments presented to T_{CD8+} were degraded rapidly after their synthesis [as was one of the T antigen fragments that efficiently entered the class I processing pathway (Gooding and O'Connell, 1983; Tevethia *et al.*, 1983)] and were difficult to detect by means other than T_{CD8+} recognition. These findings prompted Townsend *et al.*, (1985) to propose that nucleoprotein was degraded to peptides in the cytosol, and that these peptides were somehow transported to class I molecules. This paper incited a small revolution in the immunological community, as prior to its publication, data were almost uniformly interpreted with the idea that intact proteins must first find their way to the cell surface independent of class I molecules for them to be recognized by T_{CD8+}. Although both nucleoprotein and T antigen (and other viral nucleic acid binding proteins) appear to be transported to the cell surface in intact form by a yet to be defined pathway (Lanford and Butel, 1979; Sharma *et al.*, 1985; Virelizier *et al.*, 1977; Yewdell *et al.*, 1981), this pathway is almost certainly unrelated to their processing for class I association.

In the wake of Townsend and colleagues' revelation, numerous examples have accumulated that indicate that the targeting of proteins to various cellular locales is generally irrelevant to their entry into the class I processing pathway. In the case of influenza virus, most if not all of the various types of proteins are presented to T_{CD8+} (reviewed in Yewdell and Hackett, 1988). This includes, in addition to nucleoprotein, the hemagglutinin (Bennink *et al.*, 1984, 1986; Braciale *et al.*, 1984; Townsend *et al.*, 1985) and neuraminidase (Wysoka and Hackett, 1990) integral membrane glycoproteins, viral polymerases, nonstructural proteins involved in transcription (Bennink *et al.*, 1987), and matrix protein, which forms a shell under the virion membrane (Gotch *et al.*, 1987). Similar findings have been made for other viruses. The recent characterization of self-derived peptides isolated from class I molecules indicates that all of the peptides identified are derived from proteins located in the cytosol or nucleus (Jardetzky *et al.*, 1991). Furthermore, expression of determinants in chimeric proteins that alter the targeting of the protein uniformly fails to abrogate presentation of the determinant (del Val *et al.*, 1991a; Kahn-Perles *et al.*, 1989; Rammensee *et al.*, 1989).

Even for determinants present on proteins normally exported into the endoplasmic reticulum by virtue of amino-terminal targeting sequences, it appears that the exported form of the protein is irrelevant for entry into the class I processing pathway. Removal of the ER insertion sequence of hemagglutinin only increased its presentation to T_{CD8+} (Townsend *et al.*, 1986b, 1988). Of the two determinants of this

hemagglutinin presented in association with K^k (Gould *et al.*, 1987, 1991), both are efficiently processed from leaderless hemagglutinin (J. Bennink and J. Yewdell, unpublished observations). Hemagglutinin determinants expressed in smaller fragments consisting of the determinant and some of the natural flanking sequences efficiently enter the class I processing pathway (Sweetser *et al.*, 1988, 1989). Cells with genes consisting of a fragment of nucleoprotein with an internal insert corresponding to 13 residues from HLA molecules were efficiently lysed by mouse K^d-restricted T_{CD8+} specific for the given HLA molecule (Chimini *et al.*, 1989). The identical presentation of ER insertion leader sequence containing and leaderless forms of proteins has two interpretations: (1) some of the exported protein finds its way back into the cytosol from the exocytic pathway; (2) some of the protein produced from leader sequence encoding genes fails to enter the ER, either because of imperfect export of leader-containing proteins or because of the generation of small amounts of leaderless protein as a result of errors in transcription or protein synthesis.

The second explanation is favored by several findings. First, the removal of the membrane anchor of hemagglutinin or its replacement with membrane anchors from other viral glycoproteins had no effect on presentation of a determinant in the luminal domain (Braciale *et al.*, 1987), indicating that there is no essential targeting information contained in the transmembrane or cytosolic region. Analogous findings were made regarding processing of a luminal determinant in HLA class I molecules for recognition in association with K^d (Kahn-Perles *et al.*, 1989). Second, retention of leader-containing hemagglutinin in the ER by addition of an ER retention sequence derived from the adenovirus E3/19K glycoprotein (see Section II.E.1.b) to its carboxy terminus does not diminish its ability to enter the class I processing pathway (L. Eisenlohr, J. Yewdell, and J. Bennink, unpublished observations), which suggests that exported hemagglutinin does not enter the class I processing pathway from the GC or post-GC portion of the exocytic pathway.

Thus, the evidence very strongly favors the idea that all types of biosynthesized viral and cellular proteins enter the class I processing pathway directly from the cytosol. The economy of this solution is obvious, as with the exception of the very limited number of proteins synthesized within mitochondria, all proteins begin their existence in the cytosol, and the class I processing pathway is potentially able to monitor all the types of proteins a virus (or tumor) might synthesize.[10]

[10] Even peptides derived from mitochondrial gene products have access to class I molecules, probably by being exported to the cytosol (discussed in Section IV.B.3).

C. Unfolding and Proteolysis

1. Unfolding

Ultimately, class I molecules present peptides to T_{CD8+}. Although the pepton hypothesis cannot be completely eliminated as accounting for the presentation of biosynthesized proteins, the ability of exogenous proteins to enter the class I processing pathway indicates that cells have the means of generating peptides from intact proteins. This would seem to entail two processes: protein unfolding, to expose the peptide cleavage site, and the actual cleavage events themselves.

In the past few years it has become apparent that cells have the capacity to specifically unfold (and refold) proteins (reviewed in Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Rothman, 1989). The best characterized system in eukaryotic cells is probably the import of proteins into the mitochondria in which members of the heat shock family of proteins (HSP) participate in the unfolding of cytosolic proteins, which are apparently more easily transported across mitochondrial membranes in unfolded form. Once inside the mitochondrion, HSP are involved in the refolding of the protein (reviewed in Glick and Schatz, 1991; Pfanner and Neupert, 1990).

Although cells have evolved a sophisticated system to regulate the conformation of cytosolic proteins, it might not be necessary to specifically unfold proteins for antigen processing. In the case of mitochondrial proteins, this system is employed to allow folding after synthesis (to perform some function or to avoid degradation or adverse interactions with cytosolic components), and then to unfold the protein to transport it across the membrane (perhaps to minimize the energy required for transport, perhaps to avoid having a separate transporter for each protein). For antigen processing, however, the efficiency of unfolding might not be so critical. Enough protein could be sufficiently unfolded at any time just from its normal conformational gyrations, for cellular proteases (or protease-targeting mechanisms like ubiquitin conjugation) to begin the degradation process.

As might be garnered from the general nature of this discourse, no published studies have addressed the contribution of protein unfolding devices in antigen processing. This is not terribly surprising, as protein unfolding is just now attracting wide attention. Furthermore, this is a difficult problem to approach experimentally, particularly if directed unfolding is not a part of antigen processing.

2. Proteolysis

a. Background Information

Although 5 years has elapsed since it was first appreciated that

T_{CD8+} recognize peptides, the proteolytic mechanisms that function in antigen processing have not been defined. This situation will no doubt be rectified in the near future, as major progress has been made in understanding protein degradation in the cytosol. As detailed in Section II.B.2, the cytosol represents the portal to the antigen processing pathway, and it is likely that at least some, and possibly all, proteolysis occurs in this compartment. In any case, as the role of cytosolic proteolysis in antigen processing will be intensively studied in the next few years, it is useful to briefly summarize the salient features of cytosolic proteolysis (for reviews see Finley and Chau, 1991; Hershko and Ciechanover, 1992; Rechsteiner, 1991; Rivett, 1989).

The existence of a cytosolic degradation pathway was first inferred from the inability of agents that inhibit lysosomal proteolysis to completely inhibit protein turnover in cells. Cytosolic proteolysis is believed to be responsible for much of the fine regulation of gene expression and the disposal of damaged, nonfunctional proteins. Through painstaking biochemical work, an ATP-dependent cytosolic proteolytic system has been reconstituted from cytosolic extracts. This system uses ubiquitin to target proteins to cytosolic proteases. Ubiquitin is a 76-residue protein that appears to be expressed by all eukaryotic cells. It is among the most highly conserved proteins among eukaryotes; yeast and human ubiquitin differ in only three residues. Ubiquitin is attached to proteins via a unique peptide bond (termed an isopeptide bond) between its carboxy terminus and the ε-amino group of lysine. Conjugation is mediated by three enzymes. Ubiquitin is initially coupled to the first enzyme in the pathway (E1) via a high-energy bond using energy donated by ATP. Ubiquitin is then transferred to one member of a family of enzymes (termed E2) that has the capacity to transfer ubiquitin to the target protein independently, or with the cooperation of a member of a third enzyme family (E3). E2 and E3 appear to confer specificity to the process by selecting the target protein for ubiquitination. For a protein to be targeted for proteolysis, multiple ubiquitin molecules must be added in treelike structure, in which ubiquitin molecules are conjugated to each other via an isopeptide bond to an internal lysine. By contrast, when added as a single molecule, ubiquitin appears to play an essential role in altering the function of certain proteins (histones, for example). Proteins with ubiquitin trees are degraded by a complex protease in an ATP-dependent manner. As discussed in Section II.C.2.c, this protease has been implicated in the class I processing pathway. If this is true, then polyubiquitination of proteins might well be a critical factor in antigen processing.

Choosing proteins for ubiquitination appears to be governed by a

number of rules. Experiments with chimeric proteins in yeast revealed the "N-end rule": certain residues at the amino terminus target the protein to be polyubiquitinated and degraded (Bachmair *et al.*, 1986). This rule appears to be influenced by other factors, including the presence of lysine residues near the amino terminus, and is clearly not the sole determinant of targeting for polyubiquitination. Many cytosolic proteins have amino terminal residues whose amino groups are covalently modified, and a different recognition system appears to be involved in this circumstance. Moreover, prematurely terminated proteins (induced by puromycin treatment) and misfolded proteins (induced by amino acid analogs or heat shocking the cells) are rapidly polyubiquitinated, and a separate recognition system likely exists that selects abnormally folded proteins (based possibly on the exposure of normally buried hydrophobic residues). Given the great number of proteins that can be processed for T_{CD8+} recognition (many of which have "N-end rule" stabilizing residues at their amino termini), it seems most likely that the pool of truncated or misfolded proteins would be the major source of proteins for polyubiquitination.

b. Functional Evidence for Proteolysis in Antigen Processing

The role of cytosolic proteolysis in antigen processing has been addressed in only a single study, which examined the ability of genetically altered influenza virus proteins to evade a block in antigen processing associated with vaccinia virus infection (Townsend *et al.*, 1988). As first described (Coupar *et al.*, 1986), hemagglutinin expressed under the control of a late vaccinia promoter (the protein is expressed only after viral DNA replication has commenced, several hours into the infectious cycle) is not efficiently presented to T_{CD8+} despite the expression of large quantities of protein. Later it was shown that a similar block existed for nucleoprotein expressed under a late promoter, and also to some extent when expressed under an early promoter (Townsend *et al.*, 1988). For both hemagglutinin and nucleoprotein, presentation was enhanced by expressing forms of the protein that were degraded more rapidly than the native protein (hemagglutinin missing its ER insertion sequence, nucleoprotein truncated at its amino terminus). To further examine the possible involvement of ubiquitin-targeted proteolysis in this phenomenon, chimeric proteins were genetically engineered to consist of ubiquitin attached to the natural amino terminus of nucleoprotein, or nucleoprotein in which arginine was substituted for the natural amino-terminal methionine. The rationale for this experiment came from previous work in yeast in which it had been found that ubiquitin added in this manner to a

reporter protein was rapidly removed post-translationally, and the presence of methionine versus arginine at the junction resulted in stable and rapidly degraded proteins, respectively (Bachmair *et al.*, 1986). Similarly, ubiquitin was rapidly removed from both nucleoprotein constructs, and only the arginine-terminating protein was rapidly degraded. When assessed for antigen presentation following expression under early or late vaccinia virus promoters, the methionine fusion protein behaved like native nucleoprotein, while the arginine fusion protein behaved like the rapidly degraded carboxy terminal nucleoprotein fragments.

The nature of vaccinia virus antigen presentation blockade has not been defined. Two possibilities are offered (Townsend *et al.*, 1988): (1) a blockade in proteolysis of long lived proteins by one or several of the vaccinia virus proteins that are closely related to a family of proteins that inhibit serine type proteases (termed serpins) (Kotwal and Moss, 1989; Pickup *et al.*, 1986; Smith *et al.*, 1989); (2) a partial blockade in class I molecule synthesis or transport to the cell surface such that more peptides are required to load sufficient class I molecules to enable T_{CD8+} recognition.

In the sole additional study to address the vaccinia virus blockade, it was found that neither the inactivation of one of the viral serpins nor the coexpression of K^d by vaccinia virus allowed the recognition of a K^d-restricted determinant from a human papillomavirus antigen (Zhou *et al.*, 1991). These findings are difficult to interpret as vaccinia virus encodes at least two other serpins, and the K^d gene inserted into vaccinia virus may not function to present the papilloma determinant as it contains a substitution in the peptide binding site (P. Kourilsky, personal communication) that diminishes its ability to present some antigens (J. Bennink and J. Yewdell, unpublished observations).

Although the nature of the vaccinia block is not known (and could occur at any stage of antigen processing), the observation that enhanced proteolysis bypasses the blockade is an important finding as it represents the sole evidence to date relating antigen presentation to protein degradation and, further, suggests that ubiquitin-targeted proteolysis contributes to the production of antigenic peptides.

c. Proteasomes in the Pathway?

In vitro reconstitution of ubiquitin-targeted proteolysis revealed that proteolysis catalyzed by a large (1300 kDa) ATP-dependent protease with a sedimentation coefficient of 26 S. It appears that a portion of the 26 S protease is composed of a 20 S ATP-independent protease (M_r 650 kDa) that had previously been discovered first in

pituitary cells (termed *multicatalytic protease complex*), and later in erythrocytes (termed *macropain*). The 20 S protease, also known as the proteasome, comprises 15 to 20 proteins ranging in M_r from 21 to 35 kDa that associate noncovalently to form a cylinder composed of four stacked rings. The proteasome is present in both the cytosol and the nucleus. It appears that the proteasome combines with two regulatory subunits (M_r 600 kDa and 250 kDa) to create the 26 S protease. It is uncertain whether the proteasome always functions as part of the 26 S complex or also leads an independent existence. *In vitro*, the proteasome has been found to produce peptides from insulin B chain, one of which was nine residues long.[11] It appears that the proteasome has multiple specificities.

The link between the proteasome and the MHC was discovered by Monaco and McDevitt (1982, 1984, 1986), who serologically detected two polymorphic genes that could be mapped using congenic mice to the class II region of H-2. The sera immunoprecipitated a complex that they termed the low-molecular-mass polypeptide complex (LMP). The LMP consists of 16 different polypeptides with M_r ranging from 20 to 30 kDa that are noncovalently associated into a 580-kDa complex. The LMP was detected in all human and mouse tissues examined, and its expression was increased by treatment with interferon-γ. Recently the LMP and proteasome were shown to be highly similar by two-dimensional polyacrylamide gel electrophoresis (PAGE) following immunoprecipitation using LMP- or proteasome-specific sera (Brown *et al.*, 1991). Sixteen LMP subunits, including the polymorphic H-2 gene products, comigrated with proteasome subunits. Preclearing extracts with anti-proteasome antiserum depleted all LMP subunits, but clearing with anti-LMP antiserum did not remove proteasomes. That proteasomes and LMP might not be completely identical is further suggested by the presence of four electrophoretic species precipitated with the anti-proteasome sera that were absent from anti-LMP immunoprecipitates. It is uncertain whether these proteins are truly constituents of a subset of proteasomes distinct from LMP or are serologically cross-reactive proteins not present in proteasomes.

The relationship between proteasomes and the LMP is supported by further observations that a polymorphic protein (M_r 23 kDa), present in proteasomes precipitated by an antiproteasome antibody and analyzed by two-dimensional PAGE , maps to the H-2 class II region using congenic mice (Ortiz-Navarrette *et al.*, 1991). When the same anti-

[11] For skeptics, that this is the perfect size for high-affinity binding to class I would be considered to be too good to be true.

serum was used to precipitate human proteasomes, it was found that the protein is present in normal human cells but not the T2 antigen processing deficient cell line (described in Section II.A). The expression of the polymorphic proteasome protein and several additional proteasome proteins was enhanced by interferon-γ. That the polymorphic protein appeared to be a prominent part of the proteasome is at odds with the inference from the anti-LMP experiments described in the preceding paragraph. Perhaps this reflects a difference in the specificity of the anti-proteasome antisera used; the antiserum used in the second study might preferentially bind to LMP-containing proteasomes.

One of the mouse LMP genes (LMP-2) located in the class II region has been cloned and sequenced (Martinez and Monaco, 1991). LMP-2 encodes a 219-residue protein whose amino sequence between positions 21 and 41 is identical in 20 and 14 positions to the amino termini of proteins purified from rat and human proteasomes, respectively.[12] Expression of the H-2^d allele of this gene in a H-2^b cell line enabled an H-2^d-specific, anti-LMP antiserum to precipitate the LMP. A homologous gene in humans has also been localized to the HLA class II region (Kelly *et al.*, 1991). This gene, termed *RING12*, encodes a 219-residue protein identical at all but 25 residues with LMP-2. Another gene in this region, RING10, encodes a protein of 208 residues homologous to proteasome components, and could represent the human equivalent of LMP-1 (Glynne *et al.*, 1991). LMP-2 and RING12 occupy the same position in the class II region; both are immediately centromeric to genes believed to encode proteins involved in transporting determinants from the cytosol into a secretory component (described in Section II.D). RING10 is also immediately centromeric to a second putative transporter gene. Transcription from either RING10 or RING12 is enhance by interferon-γ.

On the basis of these findings it is clear that the LMP is related to the proteasome, and probably represents a subset of proteasomes that contain one or both of the newly described MHC gene products. The highly suspicious location of the LMPs genes adjacent to the putative peptide transporter genes and their induction by interferon-γ suggest that they play a role in antigen processing. As noted (Martinez and Monaco, 1991), the proteasomes are present even in single cell organisms, and clearly did not evolve as part of the antigen processing machinery. Rather, it was suggested that the LMPs function to en-

[12] As noted by the authors, this could mean that (1) the amino termini are cleaved from the proteasome proteins by the cell or as an artifact of purification, (2) the mouse gene has a 20-residue amino-terminal extension, or (3) LMP-2 has at least one highly homologous relative.

hance the efficiency of the proteolytic machinery to deliver antigens to class I molecules, perhaps by physically linking the 26 S complex to the transporter protein. This would serve to enhance greatly the transport of peptides to class I molecules, and would be consistent with the tight linkage of the LMP and transporter genes, which would be advantageous if the various alleles of LMP and transporters had different binding affinities for each other. An alternative but not mutually exclusive possibility is that the LMPs (and other interferon-γ-inducible proteasome subunits) alter the specificity of the 26 S protease, perhaps shifting the proteolytic activity from production of amino acids or short peptides to the production of octamers and nonamers that class I molecules prefer.

d. Functional Evidence for Peptide Production in the Cytosol

If antigenic peptides capable of binding class I molecules are produced in the cytosol, then cells must have the capacity to transport such peptides from the cytosol to class I molecules. This has been tested by using recombinant vaccinia viruses to transiently express peptides in the cytosol from "minigenes" (Sweetser *et al.*, 1988; Whitton and Oldstone, 1989). In the first report in which a peptide of less than 30 residues was expressed in cells, it was found that influenza virus nucleoprotein-specific T_{CD8+} recognized cells infected with a vaccinia virus expressing a 16-residue peptide corresponding to an initiating methionine plus 15 residues from influenza nucleoprotein (Gould *et al.*, 1989). Within the 15 residues was the "naturally processed" nonamer recognized in association with D^b. Sensitization required protein synthesis and was not transferred to uninfected cells coincubated with infected cells, which suggests that the peptides were processed like cytosolic proteins and were not binding to cell surface D^b as would exogenously added synthetic peptides. Subsequently, it was found that a recombinant vaccinia virus expressing a peptide corresponding to the "natural" K^d-restricted influenza nucleoprotein nonamer (residues 147–155) and an initiating methionine sensitized cells for lysis by nucleoprotein-specific T_{CD8+} (Eisenlohr *et al.*, 1992). Sensitization was blocked by BFA, which indicates that the peptide was processed like cytosolic proteins.[13] Further, the vaccinia virus

[13] A potential complication of minigene experiments is the production of unexpected peptides arising from errors in transcription or translation. This is known to occur with vaccinia virus under normal assay conditions, as antigenic sites downstream of frameshift (Hahn *et al.*, 1991), or single termination codons (L. Eisenlohr, J. Yewdell, and J. Bennink, unpublished observations) have been found to be presented to T_{CD8+}. To minimize this possibility, in each of the studies nucleoprotein minigenes were con-

recombinant efficiently induced T_{CD8+} responses *in vivo*, demonstrating that natural antigen presenting cells can present natural ligands expressed in the cytosol.

It has also been found that HLA-A2-restricted T_{CD8+} recognize cells constitutively expressing a 12-residue peptide consisting of methionine and 11 residues from influenza virus matrix protein containing an antigenic peptide (Anderson *et al.*, 1991). Although it is more difficult with constitutive expression systems to exclude the possibility that peptides are processed as cytosolic and not exogenous antigens, this is likely as control HLA-A2 expressing cells cocultured for 1 week with peptide synthesizing cells were not sensitized for lysis by matrix-specific T_{CD8+}.

Taken together, these findings indicate that cells are able to present to T_{CD8+}, peptides present in the cytosol, including a peptide corresponding nearly exactly to a natural class I ligand. This is consistent with models in which most or all proteolysis occurs in the cytosol, the resulting peptides being transported from the cytosol to meet class I molecules. By no means, however, do these findings conclusively demonstrate that proteolysis occurs exclusively within the cytosol. Indeed, as discussed in Section II.C.2.f, it was recently found that expression of a carboxypeptidase in the exocytic compartment can greatly enhance the presentation of a determinant from influenza virus nucleoprotein in certain circumstances, which indicates that post-cytosolic proteolysis can influence peptide production.

e. Role of Class I Molecules in Proteolysis

In recovering antigen peptides from acid extracts of cells, Rammensee and colleagues made the remarkable discovery that in most cases, peptides are recovered only from cells expressing a class I molecule that binds the peptide with high affinity (Falk *et al.*, 1990; Rötzshke *et al.*, 1990b). There are three factors that could contribute to the MHC dependence of peptide recovery:

1. Until they are bound to class I molecules, antigenic peptides are largely sequestered in the cellular compartment not disrupted by the

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structed with double-stop codons. Additionally, the M147–155 nucleoprotein–vaccinia virus was found to sensitize target cells as efficiently as a vaccinia virus expressing the full-length gene, which suggests that sensitization was not due to the synthesis of alternative peptides, which would be expected to occur at much lower efficiency than synthesis of the desired peptide (L. Eisenlohr, J. Yewdell, and J. Bennink, unpublished observations).

homogenization conditions, or exist in an acid-resistant complex with some other material.

2. Peptides are rapidly destroyed by cells unless protected by binding to MHC molecules (Falk *et al.*, 1990).

3. The MHC plays an active role in directing the formation of peptides, either by cleaving the peptide itself or by holding the peptide while cellular proteases trim the peptide to the proper size (Falk *et al.*, 1990).

The first possibility is somewhat unattractive, perhaps most of all because it would be difficult to disprove completely. At present, the most persuasive evidence against this possibility is the observation that some peptides are extracted from cells that do not express the corresponding class I allomorph, and that in at least one case, this peptide was not recovered from the two polymorphic class I molecules expressed by the cell (K^b or D^b) (Rötzschke *et al.*, 1991).[14]

Although the latter two possibilities are attractive on theoretical grounds, supporting experimental evidence is lacking. Two findings suggest that class I molecules do not have intrinsic protease activity. First, class I complexes present in detergent extracts containing longer peptides are less stable than those containing optimally sized peptides, suggesting that the longer peptides are not converted to the optimal peptide once bound (Cerundolo *et al.*, 1991). Second, when class I molecules were tested for their ability to bind a mixture of radioiodinated peptides of various lengths, only the optimally sized peptide was bound (Schumacher *et al.*, 1991).

Further experiments are required to determine whether peptides are trimmed by cellular proteases following association with class I molecules. Such proteases could be located in any of the exocytic compartments traversed by class I molecules, and could play a critical role in producing stable class I molecules.

f. Does the Proteolytic Machinery Demonstrate Selectivity?

One of the more striking features of T_{CD8+} recognition of foreign antigens is that very few determinants (often none or one) can be found to be recognized in association with any given class I allomorph (Ben-

[14] These peptides could, however, owe their existence to their binding to the nonpolymorphic class Ib molecules expressed in many cells (discussed in Section IV). This possibility would be eliminated if the peptides could be recovered from cells deficient in β_2m synthesis, as binding to all class I molecules should be compromised. If the existence of these peptides is truly independent of class I molecules, it will be important to determine their location in cells.

nink and Yewdell, 1988; Pala and Askonas, 1986). This seems unlikely to be caused solely by the absence of peptides within a protein capable of binding with high affinity to class I molecules, as the requirements for such binding do not appear to be so stringent. Additional factors, then, are likely to play a role in restricting the number of peptides recognized. Four possible factors are: (1) lack of T_{CD8+} precursors with an appropriate T cell antigen receptor as a result of genetic deficiency or thymic deletion, (2) failure to stimulate existing T_{CD8+} with the appropriate receptor as a result of immunoregulatory phenomena, (3) failure to deliver antigens from the cytosol to class I molecules,[15] and (4) inability of proteases to liberate antigenic peptides from a given protein or protein fragment. Although the first factor must contribute to the paucity of determinants recognized by T_{CD8+}, experimental evidence is lacking. The third factor is a less certain contributor, and here again, evidence is lacking. The second factor clearly contributes in some circumstances, as it has been shown that T_{CD8+} specific for determinants in influenza virus neuraminidase are detected only if special immunization protocols are employed (Hackett *et al.*, 1991). Similarly, T_{CD8+} specific for some determinants in simian virus 40 T antigen (Tanaka and Tevethia, 1988a; Tanaka *et al.*, 1989) are recovered only if other "immunodominant" determinants are removed from T antigen.[16]

Regarding the last possibility, initial findings suggested that the context of antigenic peptides in a protein was not likely to influence their presentation to T_{CD8+}. Expression of a 13-residue segment (with the likely natural determinant starting at the amino terminus) from a HLA class I molecule in the middle of influenza virus nucleoprotein did not prevent its presentation to K^d-restricted, HLA-specific T_{CD8+} (Chimini *et al.*, 1989). Similarly, when vaccinia virus recombinants were used to express altered influenza hemagglutinin molecules, the position of an antigenic determinant within the same protein was not found to be critical for its presentation to T_{CD8+} (Hahn *et al.*, 1991). In this experiment, a 20-residue sequence (containing a likely natural

[15] This in turn could have a number of causes, including sequestration of peptides on peptide binding proteins in the cytosol or exocytic compartment, lack of peptide transport from the cytosol to the exocytic compartment, or failure of peptide intermediates to associate with accessory molecules that might function to deliver peptides to class I molecules in the exocytic compartment.

[16] In either case, this is unlikely to be the result of competition from other viral peptides for class I molecules, as the phenomenon is observed only at the effector level; target cells infected with influenza virus or expressing intact T antigen present the "weak" determinants indistinguishably from the more immunogenic determinants.

antigenic determinant starting at position 3) was removed from its normal site in the hemagglutinin and placed in six different locations. In every case the determinant was presented in association with K^d.

In contrast, in other studies it has been found that the context of a determinant can greatly influence its presentation. A 9-residue peptide corresponding to the natural L^d-restricted determinant from a murine cytomegalovirus protein (pp89) or an 18-residue peptide consisting of the nonamer and the natural flanking sequences of pp89 was inserted near either the carboxy terminus or the amino terminus of hepatitis B virus core antigen and expressed in target cells using a recombinant vaccinia virus (del Val *et al.*, 1991a). The 18-mer was presented to T_{CD8+} in either context; however, the nonamer was not presented in the context of the amino terminus. This could not be correlated with either the amount of protein produced or its metabolic stability. Moreover, the poor presentation was not caused by vaccinia virus infection as cells constitutively expressing the chimeric proteins from transfected genes demonstrated an identical pattern of presentation. These findings do not reflect some peculiarity of the target cells used, as the ability of the vaccinia virus recombinants to induce protective immunity to murine cytomegalovirus [previously shown to be correlated with induction of T_{CD8+} (del Val *et al.*, 1991b)] correlated with the *in vitro* results. The poor presentation of the amino-terminal nonamer was not due simply to its proximity to the amino terminus, as addition of ten amino acids on the amino side of the peptide did not restore presentation. On the other hand, the insertion of five alanine residues on each side of the determinant rescued presentation. Further supporting that it is the immediate context of the determinant and not its position in the protein that governs its processing, relocation of the nonamer toward the carboxy terminus of a deletion mutant of pp89 did not block its presentation *in vitro* or *in vivo* (del Val *et al.*, 1991b). Finally, the nature of the L^d-associated peptides derived from pp89 or the hepatitis B virus core antigen–peptide chimeras was determined by HPLC analysis of acid extracts of cells. This revealed that the identical nonamer was produced from each protein, but in different amounts that correlated with the degree of recognition (del Val *et al.*, 1991a).

Flanking sequences have also been found to greatly influence the presentation of oligopeptides (Eisenlohr *et al.*, 1992). Using vaccinia virus recombinants to express oligopeptides, extension of the Met 147–155 determinant from influenza virus nucleoprotein (see Section II.C.2.d) by two amino acids (corresponding to nucleoprotein residues 157 and 158; this recombinant is termed Met 147–155, 157, 158) was

found to greatly diminish its antigenicity *in vitro* and *in vivo*. Presentation was not restored by adding ten residues to the amino and/or carboxy termini of this peptide, corresponding to the natural flanking residues in nucleoprotein. A full-length nucleoprotein with residue 156 deleted was presented, however, as was a 147–155, 157, 158 construct with a carboxy terminus consisting of random residues encoded by plasmid DNA as a result of the inadvertent introduction of a frameshift mutation prior to the stop codons. These findings demonstrate that the negative influence of flanking sequences can be overcome by adding more residues to the peptide. The failure of the Met 147–155, 157, 158 vaccinia virus recombinant to sensitize cells is particularly surprising, as it was reported that a synthetic peptide corresponding to the sequence was highly efficient at sensitizing cells for lysis by nucleoprotein-specific T_{CD8+} (34).[17] Recently, however, it was found that the ability of this peptide to sensitize cells is dependent on its conversion to the 147–155 peptide by angiotensin converting enzyme or a similar activity present in fetal bovine serum (Sherman *et al.*, 1992) (these findings are discussed in Section III.A.3). Thus, the poor presentation of the endogenously synthesized Met 147–155, 157, 158 peptide does not necessarily indicate a failure in transporting the peptide, as the lack of presentation could also result from a failure of cells to trim the peptide properly before or after transport. Indeed, recent findings indicate that coexpression of angiotensin converting enzyme with the Met 147–155, 157, 158 peptide greatly enhances its presentation to nucleoprotein-specific T_{CD8+} (L. Eisenlohr, J. Bennink, and J. Yewdell, unpublished observations). This could not be attributed to secretion of angiotensin converting enzyme into the culture medium by infected cells since presentation of the biosynthesized peptide was not enhanced either by adding angiotensin converting enzyme to culture medium or by coincubating cells expressing angiotensin converting enzyme with cells expressing Met 147–155, 157, 158. Angiotensin converting enzyme is a secretory protein and would not be expected to be present in active form in the cytosol. Supporting this conclusion, enzyme activity is not recovered from cells expressing a form of angiotensin converting enzyme without its ER insertion sequence (L. Eisenlohr, J. Bennink, and J. Yewdell, unpublished observations). Thus, in this case, it is clear that flanking sequences inhibit presentation not by preventing peptide delivery to the

[17] This peptide has been a work horse for a number of laboratories because it was serendipitously found to be presented extremely efficiently to T_{CD8+} (Bodmer *et al.*, 1988). In fact, this peptide held the record for most efficient target cell sensitizer prior to the discovery of the "natural" peptides.

exocytic compartment, but by preventing the proper proteolytic events from occurring.

The findings with nucleoprotein and cytomegalovirus pp89 indicate that flanking sequences can influence the liberation of antigenic peptides and, thus, likely play an important role in limiting the number of antigenic peptides present in proteins. It is striking that the negative influences of flanking sequences were observed only when altering the context of nonameric or decameric peptides. Although a greater sample size must be accumulated, this suggests that the important information for liberating determinants is contained in the local environment of the peptide, perhaps within the five residues on either side of the peptide.

g. Are There Additional Factors in Selectivity?

One of the mysteries of antigen processing is how seemingly minor amounts of foreign proteins can be presented by cells in the presence of what would seem to be overwhelming amount of cellular structural proteins. As an upper limit, cells express 10^6 copies of class I molecules at their surface. As a lower limit, 100 molecules containing a given peptide sensitize cells for T_{CD8+} recognition (Christinck *et al.*, 1991). If it is assumed that native proteins randomly enter the class I pathway and antigenic peptides that can be liberated by the proteolytic machinery are distributed randomly among foreign and self proteins, a protein would have to present at an abundance of 0.01% to be recognized. If cells have on average 10^9 copies of proteins of 50 kDa (this is based on the average protein content of cells), then a viral protein of this size would have to reach 10^5 copies to be presented. It seems that for some viral proteins much smaller amounts are required to sensitize cells.[18] As it is difficult to imagine that self proteins have been selected for their lack of antigen peptide, this suggests that some features of proteins favor (or disfavor) their proteolytic processing. With time, as more peptides from viral and self proteins are isolated and characterized, factors that favor the generation of determinants should become apparent.

An observation that may be pertinent to this discussion is that viral

[18] This conclusion is based on two findings. First, using neuraminidase-inactivated influenza virus, presentation of one of the polymerases present at a few copies per virion is observed after exposing cells to roughly 1000 virions per cell (J. Yewdell and J. Bennink, unpublished results). Second, presentation of vesicular stomatitis virus nucleocapsid proteins is observed within 45 minutes of adding virus to cells, which seems too short to produce enough mRNA and protein to reach 10^5 copies (Esquivel *et al.*, 1992).

nucleoproteins seem to be overrepresesented among viral proteins recognized by T_{CD8+}. This might simply reflect that these proteins are often the first proteins synthesized during a virus infection, and that presentation of later antigens is inhibited by processes related to the viral infection. Inhibition of presentation of proteins expressed relatively late in the viral infectious cycle has been observed for both vaccinia virus (discussed in Section II.C.2.b) and murine cytomegalovirus (del Val *et al.*, 1989).[19] Consistent with this possibility is the observation that expression of either peptide determinants from influenza virus nucleoprotein or determinants from cytomegalovirus pp89 in chimeric proteins under the control of an early vaccinia virus promoter still elicits T_{CD8+} responses *in vivo* (del Val *et al.*, 1991b; Eisenlohr *et al.*, 1992). This would suggest that the physical properties of nucleoproteins are not related to their efficiency of presentation. On the other hand, presentation in these circumstances might be facilitated by the peptide nature of the minigene product or the abnormal folding of the chimeric protein. If presentation of nucleoproteins is favored by their structure or function, perhaps the critical property these proteins share is their capacity to bind nucleic acids.[20] It is noteworthy that of the self peptides recovered from HLA-B27, most are derived from proteins that bind directly or indirectly to DNA or RNA (Jardetzky *et al.*, 1991). Could nucleic acid binding favor the production of antigenic peptides from proteins by enhancing proteolysis of intact proteins or by limiting the extent of proteolysis?

D. Translocation

1. MHC ABC Protein Genes

Although the evolutionary wisdom of using the cytosolic protein pool as a source of antigen to display on the cell surface can be discerned, this imposed a topological hurdle into the process, as the peptide must be transported across a membrane to reach the plasma membrane. In broad terms this problem has four potential solutions. First, class I molecules could carry the peptides across the membranes during their own translocation. Second, cells could have evolved a

[19] In contrast to vaccinia virus, it did not appear that the antigen processing blockade by mouse cytomegalovirus was related to protein degradation. The block also could not be explained by a competition for binding to the class I presenting molecule [L^d] or general inhibition of L^d transport to the cell surface, as another protein expressed at the same time was presented in association with L^d.

[20] Although some of these proteins contain nuclear localization sequences, others do not, so nuclear location per se is unlikely to be a factor in enhanced presentation.

specific mechanism to transport antigenic peptides. Third, peptides could cross the membrane using channels whose primary function lies elsewhere. Finally, the peptides could diffuse across the membrane in a nonfacilitated fashion.

Although the first solution would almost certainly be the most efficient means of capturing peptides, this is apparently incompatible with the machinery used to translocate proteins into the exocytic compartment. This pore is located in the ER and is believed to transport proteins only in an extended conformation, usually as proteins are being produced by the ribosome. In support of this conclusion, cells maintain the capacity to present influenza virus proteins delivered to the cytosol up to 32 hours after class I molecule synthesis has been blocked by the addition of protein synthesis inhibitors (J.Yewdell and J.Bennink, unpublished observations).

Recent evidence for the second solution has been provided from studies of antigen processing-deficient cells. Indeed, the ability of RMA/S cells to present exogenous peptide antigens but not cytosolic antigens first prompted speculation that the cells were deficient in a specific mechanism for translocating peptides from the cytosol (Townsend *et al.*, 1989). Sixteen months later, four reports appearing simultaneously proclaimed the existence of two genes in the class II region of human (Spies *et al.*, 1990; Trowsdale *et al.*, 1990), mouse (Monaco *et al.*, 1990), and rat (Deverson *et al.*, 1990) MHCs encoding proteins highly homologous to a family of transport proteins known as the "ABC" superfamily, named after a highly homologous region believed to bind ATP (the ATP Binding Casette) that is present in all members. This family contains more than 30 members from prokaryotes and eukaryotes that transport ions, saccharides, amino acids, peptides, and proteins of greater than 100 kDa (for review see Juranka *et al.*, 1989). All of the eukaryotic non-MHC members of the ABC superfamily characterized to date consist of a large protein composed of homologous halves, each consisting of an ABC domain toward the carboxy end preceded by a region that has six hydrophobic domains proposed to act as membrane spanning regions. Some of the bacterial members of the family consist of two separate polypeptides, each with the characteristic of half of a transporter. It was recently reported that one of the eukaryotic transporters (the yeast STE6 gene product which transports a lipid-linked peptide mating factor) functions when the two halves of the protein are coexpressed as separate proteins (Berkower and Michaelis, 1991). This indicates that the MHC-encoded ABC proteins might function as homodimers, heterodimers, or a mixture of homo- and heterodimers.

In mouse the putative transporters were named HAM1 and HAM2 [for histocompatibility antigen modifier] (Monaco *et al.*, 1990). The predicted HAM1 gene product is a basic protein of 577 residues with a M_r of 63 kDa. The protein contains consensus N-linked glycosylation sequences, but based on the predicted topology of the protein, all would be expected to be in the cytosol.[21] A partial sequence of the HAM2 gene (located approximately 15 kb telomeric to HAM1), revealed a high homology (77% at the amino acid level) with HAM1. Low-stringency Southern blotting of cosmid DNAs from surrounding regions of the H-2 encompassing roughly 400 kb failed to reveal additional HAM1-like genes.

In rat, the homologous genes were termed *mtp1* and *mtp2* (for MHC-linked transporter protein) (Deverson *et al.*, 1990). *mtp1* is believed to encode a 725-residue protein with an M_r of 79 kDa. This protein might be glycosylated, as two of the three potential N-linked glycosylation sites are predicted to be on luminal domains. The amino terminus of the predicted protein contains roughly 100 amino acids present on single-subunit bacterial transporters, but not bacterial or eukaryotic transporters with tandemly repeated subunits. This sequence is also absent from HAM1. A further difference from the eukaryotic transporters is that the amino-terminal residues are typical of those that function as ER insertion sequences. A second cDNA clone was found to have a deletion resulting in an in-frame deletion of 57 amino acids, suggesting the *mtp1* gene might produce multiple products by alternative splicing. The *mtp2* gene encodes a 703-residue protein whose closest relative among ABC family members is *mtp1* (Powis *et al.*, 1991). Based on their hydrophobicity plots, the structures of *mtp1* and *mtp2* are likely to be very similar.

The genes in humans homologous to HAM1 and *mtp1* have been termed RING4 (Trowsdale *et al.*, 1990) and PSF1 (for peptide supply factor)(Spies *et al.*, 1990). A cDNA believed to represent the complete RING4 DNA has been sequenced. Comparison of this and a partial PSF1 sequence reveals the two to represent the identical gene. The RING4 gene likely encodes a protein of 808 residues that, like *mtp1*, could contain an ER insertion sequence at its amino terminus.[22] There

[21] The value of such topological predictions is uncertain, particularly in view of a recent report suggesting that several of the presumed membrane spanning domains of the mouse multidrug transporter may be located in solvent-accessible portions of the molecules (Berkower and Michaelis, 1991).

[22] The similarities between the human and rat MHC ABC genes suggest that HAM1 represents a partial sequence.

are three potential N-linked glycosylation sites. Northern blotting analysis revealed that RING4 mRNA was present in several cell lines and absent in several others. In two negative cell lines, RING4 message was detected after 2-day treatment with interferon-γ (Trowsdale *et al.*, 1990). This finding links RING4 with the antigen presenting function of the MHC, as interferon-γ treatment has been found to induce class I expression and antigen presentation under a number of conditions.

2. *MHC ABC Protein Function*

More direct evidence for involvement of PSF1 in class I surface expression comes from studies of PSF1 expression (Spies *et al.*, 1990) in the mutants produced by DeMars and colleagues (described in Section II.A). Northern blot analysis revealed that PSF1 was not expressed in .174 cells. This was expected, as these cells have a large deletion that includes the PSF1 region. PSF1 mRNA was also absent in .134 cells, which do not have a detectable deletion. Transfection of .134 cells with plasmids containing cDNA encoding PSF1 and drug resistance markers, followed by drug selection, resulted in the rescue of HLA-A2 and HLA-B5 expression to control levels in approximately half of the drug resistant transfectants, as measured by $\alpha 1\alpha 2$ conformation-specific mAbs (the use of mAbs as probes of class I structure is described in Section II.E.2.a). Based on the size of mRNA hybridizing to a PSF1 probe in Northern blots, this was attributed to expression of the cDNA and not reactivation of the cellular gene. Transcripts of similar size and abundance were detected in approximately half of .174 cells transfected and selected under identical conditions. None of these transfectants, however, demonstrated increased amounts of cell surface class I molecules. In a later report, it was shown that one of the transfected .134 cell lines demonstrating enhanced class I surface expression presented influenza virus matrix proteins to HLA-A2-restricted T_{CD8+} as efficiently as wild type cells, following their infection with a recombinant vaccinia virus containing the matrix gene (Spies *et al.*, 1992). In this report, an antiserum raised to the carboxy-terminal 14 residues of PSF1/RING4 was used to immunoprecipitate PSF1 from detergent extracts. Because of the presence of contaminating [^{35}S]methionine-labeled proteins comigrating with the MHC ABC genes, immunoprecipitates were analyzed by silver staining. Three protein bands specifically immunoprecipitated were detected by this method. One, migrating with a M_r of 68 kDa, was absent in .134 and .174 cells, but present in all normal cells, and .134 successfully transfected with PSF1. This band probably represents PSF1, although it

migrates with an apparent mass that is 13 kDa less than predicted. This could be caused by aberrant behavior in sodium dodecyl sulfate (SDS)–PAGE that is either inherent, acquired following post-translational modification, or the result of proteolytic processing. That this protein is similar in size to the predicted HAM1 gene product suggests, however, that the protein is produced from a spliced message corresponding to the HAM1 sequence. The presence of two other specifically immunoprecipitated bands, migrating with M_r of 76 and 74 kDa, was variably detected between cell lines. Parental 721 cells expressed both bands, whereas .45 cells, missing one copy of chromosome 6, expressed the slower migrating band, and .112, a 721 derivative missing the other copy of chromosome 6, expressed the faster migrating band. Sequencing PSF genes from the different alleles revealed the faster migrating allele to have a substitution of residue 687 by a stop codon. Based on the detection of the ABC gene products by the relatively insensitive method of silver staining, it was estimated that each cell expresses several hundred thousand molecules of each.

There is also direct evidence that the second ABC gene functions in class I assembly and antigen presentation. Stable transfection of RMA/S cells with the rat *mtp2* gene led to the recovery of cells with levels of cell surface K^b and D^b near those exhibited by RMA cells (Powis *et al.*, 1991). Transfected cells also demonstrated a nearly complete recovery in class I assembly and transport from the ER and in their ability to present influenza virus NP to T_{CD8+} after infection with influenza virus. By contrast, transfection with the *mtp1* gene did not rescue class I surface expression. With anti-peptide antisera raised to the carboxy termini of *mtp1* or *mtp2*, there were no gross alterations in either the quantities or mobilities of the proteins detected in RMA and RMA/S cells. This suggests that the defect in *mtp2* function in RMA/S cells results from amino acid substitutions in HAM2. Thus, the residual antigen presentation capacity of RMA/S cells discussed in Section II.A could reflect either the partial functioning of HAM2 or the ability of antigen processing to proceed at a reduced level in the absence of HAM2.

These findings indicate that both MHC ABC genes play an important role in class I surface expression and the presentation of cytosolic antigens. On the basis of the immunoprecipitation data, it appears that PSF1 and PSF2 associate noncovalently to form a functional subunit. Although the antigen processing capacity of .134 cells has to be characterized in more detail, it seems that they, like RMA/S cells, have a partial deficit in their capacity to process antigen. This suggests either that each ABC gene is able to function independently, albeit at lower

efficiency, or that the function of the complex can be performed in another manner by cells, although again, with diminished efficiency. The complete nature of the antigen presentation defect in T2 cells is consistent with either possibility, as other gene products in the one-megabase pair portion of the MHC deleted in these cells might also be required for efficient class I surface expression and antigen presentation. For example, *mtp*1/*mtp*2 might be required to anchor the proteasome to PSF1/PSF2 to enhance the concentration of peptides near the transporter. Alternatively, non-ABC MHC gene products might act after peptides are transported from the cytosol, perhaps shuttling transferred peptides to class I molecules or trimming peptides to the proper length before or after class I binding. Obviously, none of these possibilities are mutually exclusive, and multiple gene products might be missing from .174 cells that are required for efficient antigen presentation. A relatively simple experiment that would greatly help in sorting out these possibilities is to transfect PSF1 and PSF2 genes into .174 cells and to determine the extent to which class I assembly and antigen presentation are restored.

That peptide transport from the cytosol is the primary or sole deficit in .174 cells is suggested by a study in which the T2 derivative of .174 cells was transfected with a gene corresponding either to methionine plus 11 amino acids from influenza matrix protein containing an HLA-A2 restricted determinant, or these 11 residues behind 18 residues corresponding to an ER insertion leader sequence derived from adenovirus E3/19K glycoprotein (Anderson *et al.*, 1991). Although either of the constructs was able to sensitize control cell transfectants (the T1 cell line) for lysis by matrix-specific, A2-restricted T_{CD8+}, only T2 cells transfected with the leader containing peptide presented the matrix peptide. Presentation of peptide did not appear to occur via secretion and binding to class I molecules at the cell surface since 1 week of cocultivation of T2 cells with class I molecule negative transfected cells presumably expressing the exported peptide did not result in sensitization of T2 cells. This would suggest that the antigen presentation deficit in T2 cells is overcome by transporting peptides into the ER. This supports the idea that MHC gene products are required for transport of antigenic peptides from the cytosol, and suggests that the all of missing gene products in T2 cells act prior to the transport step. There are a number of potential difficulties with this interpretation, however, as the sole criterion for assessing production of leaderless and leader-containing peptides was T_{CD8+} recognition of targets, and it is possible that differences in presentation are related not to the

presumed differences in peptide transport but to differences in levels of peptide expression. Even if T2 and T1 cells express equivalent levels of the two peptides, it is possible the failure of T2 cells to present leaderless peptide is related to deficiencies in proteolysis and not transport.

That T2 cells might not be deficient in peptide transport is supported by the findings of Kvist and colleagues. Using microsomes prepared from human lymphoid cells, they established an *in vitro* translation system for HLA-B27 α chains and β_2m, and found that addition of a B27-restricted influenza virus nucleoprotein peptide at 10 μM stimulated class I assembly (class I assembly is discussed in detail in Section II.E.2)(Kvist and Hamann, 1990). A biotinylated derivative of the peptide was then used to show directly peptide association with α chains and β_2m (Lévy *et al.*, 1991b). Class I molecules assembled under these conditions were resistant to protease digestion (except for its cytosolic tail), verifying the integrity of the microsomes and indicating that the peptides must be transported across the microsomal membrane. Peptide binding to class I molecules was observed as quickly as 15 seconds after addition to microsomes, and reached maximal values by 2 minutes. Three findings indicated that peptide transport in this system occurred independently of MHC-encoded proteins. First, peptide binding to class I molecules was not affected by treating microsomes with protease prior to the addition of peptides, which might be expected to destroy peptide transporters consisting of proteins. Second, using peptide binding to immunoglobulin binding protein (BiP, also known as GRP78; a member of the HSP family; present in high concentrations in the ER that binds unfolded proteins and peptides) as a measure of peptide translocation, they found that transport did not require ATP. As the transporters contain an ATP binding site, and transport mediated by other members of the ABC transporter family is ATP dependent, it is to be expected that their function requires ATP. Finally, and most directly, no difference was found between peptide transport in T2 and T1 cells as measured by BiP binding. Most intriguingly, although peptide was transported in T2 cells, it could not induce class I assembly (this is discussed further in Section II.E.2.b).

These findings demonstrate that peptide transport in a microsomal system occurs in the absence of the MHC ABC gene products and other proteins encoded by the one megabase pair portion of HLA deleted in T2 cells. Further, transport either occurs in the absence of a protein channel or, less likely, is accomplished by one extremely resistant to proteolysis. The critical issue in interpreting these experiments

is the relevance of the microsomal system to transport in cells. It is inconceivable that peptide concentration in cells would ever even approach 10 μM (this corresponds to 10^6 to 10^7 copies of peptide in an average-sized cell), and it is possible that the normal transport system was destroyed in producing the microsomes, or that peptide transport (and presumably class I molecule assembly) normally occurs in a specialized region of the ER or a post-ER compartment that constitutes a minority of the microsomes. On the other hand, the peptide used in these studies was 11 residues in length, and probably binds to B27 with a much lower affinity than the natural peptide. If the microsomes are incapable of trimming peptides as might normally occur, or if nonapeptides are normally produced in the cytosol, this would explain the requirement for superphysiological levels of peptides. Further studies using high-affinity peptides are required to sort out these possibilities.

E. Assembly of Class I Molecules

1. Subcellular Location of Class I Assembly

a. Effects of Brefeldin A on Antigen Processing and Presentation

As discussed above, it is extremely unlikely that class I molecules bind antigens in the cytosol and carry them into the exocytic pathway. Thus, the union of class I molecules with antigen must occur either during or after the transport of class I molecules to the cell surface from the ER. The first studies to examine this question made use of the drug BFA. BFA is an antibiotic independently isolated from fungi at least five times and given a number of additional names (decumbin, cyanein, ascotoxin, and synergisidin). Because of its broad range of antifungal and antiviral activities, there has been lively interest in BFA for many years, and its complete chemical synthesis has been described by several prominent organic chemists. BFA began to attract the attention of cell biologists when Takatsuki and colleagues reported that BFA specifically blocked the export of the vesicular stomatitis virus glycoprotein to the cell surface (Takatsuki and Tamura, 1985) and protein secretion by hepatocytes (Misumi *et al.*, 1986). Based on biochemical characterization of proteins biosynthesized in the presence of BFA and examination of thin sections of BFA-treated cells by electron microscopy, it was proposed that BFA blocked transport at the endoplasmic reticulum by destroying the GC (Fugiwara *et al.*, 1988). Subsequently it was found that as the GC disappears, some of its proteins (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989) and

lipids (Young *et al.*, 1990) are returned to the ER, providing additional evidence for a GC-to-ER recycling pathway proposed to exist on theoretical grounds, but that proved to be very slippery to demonstrate experimentally (Pelham, 1989, 1991a). It now appears that remnants of the GC persist in BFA-treated cells as smaller vesicular structures, and that some proteins might continuously recycle between the ER and this compartment (Russ *et al.*, 1991; Ulmer and Palade, 1991; Yamashina *et al.*, 1990). Initially it was thought that the trans-GC was spared the effects of BFA (Lippincott-Schwartz *et al.*, 1989). Subsequently, it was shown that elements of the trans-GC are returned to ER, whereas the next compartment in the pathway, the trans-GC network, is not connected with the ER (Chege and Pfeffer, 1990). A spate of recent reports indicate that the trans-GC network is not totally spared from BFA action; it appears to form tubular connections with early endosomes, although bidirectional transport between the plasma membrane and the trans-GC network appears to be largely unaffected, as does transport of material from early endosomes to later endosomes and the endosomal sorting compartment that bypasses the trans-GC network (Damke *et al.*, 1991; Hunziker *et al.*, 1991; Lippincott-Schwartz *et al.*, 1991; Pelham, 1991b; Wood *et al.*, 1991). BFA appears to cause these myriad effects by removing the proteins that normally coat the cytosolic surface of transport vesicles (Donaldson *et al.*, 1990; Orci *et al.*, 1991). In the absence of BFA, these proteins seem to function to induce vesicle formation, and then to guide vesicles to the proper destination.

Appreciation of the blocking effect of BFA on exocytosis occurred at a time when the intellectual climate was ripe for asking whether antigens processed from the cytosol associated with class I molecules prior to or after their arrival at the cell surface. BFA was found to block the presentation of influenza virus matrix protein to human T_{CD8+} (Nuchtern *et al.*, 1989), and influenza nucleoprotein, hemagglutinin, non-structural 1 and basic polymerase 1 to mouse (Yewdell and Bennink, 1989) T_{CD8+}, respectively. The blocking effect of BFA was not related to blocking virus infection, as viral proteins appeared to be synthesized in normal amounts, and removal of BFA from cells resulted in their sensitization, even if protein synthesis inhibitors were added to prevent the synthesis of additional viral proteins. Furthermore, BFA also blocked the presentation of structural viral proteins to mouse T_{CD8+} following their introduction into the cytosol using neuraminidase-inactivated virus (Yewdell and Bennink, 1989). BFA had to be added to cells within a few hours of the addition of infectious

or noninfectious virus to block presentation, and had to present throughout the period prior to the chromium-51 release assay. Moreover, it had no effect on the presentation of hemagglutinin from mouse cells constitutively synthesizing hemagglutinin. Thus, BFA did not act by disrupting preformed class I–antigen complexes or by interfering with the process of T_{CD8+} recognition. Supporting this conclusion, BFA failed to block presentation of synthetic peptides containing determinants recognized by matrix- or nucleoprotein-specific T_{CD8+}. Cells treated with BFA for 5 hours or longer still presented the nucleoprotein peptide. As this is far in excess of the time required for class I molecules to reach the plasma membrane from the trans-GC, this provides very strong evidence that exogenously added synthetic peptides associate with class I molecules that have already reached the cell surface (further evidence for this conclusion is presented in Section III.A.1). On the other hand, the BFA blockade of cytosolic antigens suggests that these antigens associate with class I molecules at some time during the transport process.

The sole finding in these original studies linking the BFA blockade of antigen presentation to its effect on exocytosis was that cells had to be incubated at temperatures greater than 20°C to reverse either effect when BFA was removed (Yewdell and Bennink, 1989). Given the pleiotropic effects of BFA on cells, it was entirely possible that BFA also interfered with proteolysis or peptide transport. As mentioned in Section II.C.2.d, however, it was recently found that BFA blocks the presentation of a biosynthesized decameric peptide consisting of the natural K^d-restricted influenza virus nucleoprotein nonamer plus an initiating methionine (Eisenlohr *et al.*, 1992). This indicates that the action of BFA occurs independently of whatever effects it might have on proteolysis. Most recently, it was found that peptides derived from cytosolic influenza virus proteins can be isolated from BFA treated, influenza virus infected cells following acid extraction and HPLC analysis (C. Lapham, J. Bennink, and J. Yewdell, unpublished observations). As described for non-BFA treated cells, antigenic peptides were detected only when the appropriate restriction element was expressed by the cells. This provides the most direct demonstration that peptides from cytosolic proteins associate with class I molecules in an intracellular compartment, either the ER or GC.

b. Effects of Adenovirus E3/19K Glycoprotein on Antigen Processing and Presentation

Information regarding the intracellular location of antigen binding to class I molecules has also come from studies of the adenovirus

E3/19K glycoprotein. The interaction of this protein with class I molecules has a rich and checkered history. Following Zinkernagel and Doherty's discovery of MHC restriction, there was a tremendous effort to demonstrate physical interaction between class I molecules and viral glycoproteins. Although there were numerous reports of such interactions, only one example withstood the test of time. This protein, E3/19K, is an integral membrane glycoprotein expressed early in the infectious cycle of a number of adenovirus serotypes. It is a nonstructural protein, and its deletion from the adenovirus genome does not reduce adenovirus infection *in vitro*. Given the intellectual climate of the time, it is hardly surprising that it was believed to represent the first bona fide target antigen for T_{CD8+} (these early findings are reviewed in Pääbo *et al.*, 1985).

Whether or not E3/19K has determinants that are recognized by T_{CD8+} remains to be established. It is clear, however, that the interaction of E3/19K with class I molecules is hardly fortuitous, although in a delicious irony, the relevance of this interaction is opposite to the original vision—E3/19K functions to prevent T_{CD8+} recognition of other adenovirus antigens. The initial blow to the original model came from more careful studies of E3/19K trafficking in cells. Instead of being expressed on the cell surface as first believed, E3/19K was found to be retained in the ER of cells (Andersson *et al.*, 1985; Burgert and Kvist, 1985). ER retention is conferred by the cytosolic carboxy-terminal residues of E3/19K, and can be abrogated by removing the six terminal residues (Gabathuler and Kvist, 1990; Jackson *et al.*, 1990; Nilsson *et al.*, 1989). By virtue of its cytosolic domain, E19 functions to retain many human and some rodent class I allomorphs in the ER.

The effect of E3/19K on antigen presentation was first examined using alloreactive T_{CD8+}, where it was found that cells transfected with the E3/19K gene (Burgert *et al.*, 1987), or infected for 10 hours with adenovirus (Andersson *et al.*, 1987), demonstrated reduced recognition that correlated with reduced surface expression of the restricting class I molecule. It could not be determined from these experiments whether E3/19K was blocking presentation by preventing transport of class I–antigen complexes or by simply reducing surface expression of class I molecules. The effect of E3/19K on presentation of simian virus 40 T antigen was indirectly examined by coinfection of cells with simian virus 40 and either wild-type adenovirus or a mutant missing the E3 region [in addition to E3/19K, other proteins that affect the host response to adenovirus are encoded in this region (reviewed in Gooding and Wold, 1990)]. Coinfection with wild-type but not the mutant adenovirus was found to reduce presentation of T antigen in associa-

tion with either D^b or K^b (Tanaka and Tevethia, 1988b).[23] These findings were difficult to interpret because of the coexpression of other E3 region genes, and uncertainties regarding the effect of E3/19K on the transport of K^b and D^b molecules from the ER.

To minimize difficulties with reduction in cell surface level of class I molecules encountered with transfection, the E3/19K gene was inserted into vaccinia virus under the control of an early vaccinia virus promoter, resulting in the rapid expression of large amounts of E3/19K (Cox *et al.*, 1990). Expression of E3/19K prevented the export of K^d and L^d through the GC, but not D^d, K^k, or D^k.[24] E3/19K was also shown to block the surface expression of K^d in cells coinfected with vaccinia viruses expressing E3/19K and K^d as determined by cytoflourography. Under infection conditions in which the cell surface expression of constitutively expressed class I molecules was unchanged, expression of E3/19K was found to block presentation of K^d- and L^d- restricted determinants, but not D^d- or H-2^k-restricted determinants. The effects of E3/19K were more complete when determinants were derived from influenza virus proteins introduced into cells by coinfection with vaccinia virus recombinants containing influenza virus genes than when determinants were derived from vaccinia virus proteins. This might be due to kinetics of determinant expression: the influenza virus antigens are expressed under the control of the same promoter used for E3/19K, whereas the vaccinia virus antigens (which are undefined) could be

[23] Interestingly, cells coinfected with wild-type adenovirus and an E3 adenovirus deletion mutant encoding a truncated form of T antigen containing a K^b-restricted determinant were recognized by K^b restricted-, T antigen-specific T_{CD8+}. Assuming that the adenovirus block in presentation observed in other circumstances is due to inhibition of K^b transport, this may reflect either the relatively trivial explanation that T antigen is expressed earlier from adenovirus than from SV40, or the more interesting explanation that an altered form of the protein bypasses the blockade, perhaps by being more rapidly converted into antigenic peptides.

[24] This was determined by SDS–PAGE analysis of class I molecules immunoprecipitated from cells pulse-labeled with [^{35}S]methionine and chased for up to 4 hours. As a measure of intracellular transport, immunoprecipitates were treated with endo-β-*N*-acetylglucosaminidase H (endo H), which cleaves N-linked oligosaccharides in the simple forms characteristic of the ER and early GC, but not the more complex forms that are often, but not always, generated from the simple forms, as oligosaccharides are modified by enzymes present in the medial and trans-GC (reviewed in Maley *et al.*, 1989). Oligosaccharide cleavage is easily detected as a decrease in apparent M_r in SDS–PAGE. Resistance of N-linked oligosaccharides to endo H digestion is the easiest method of biochemically monitoring glycoprotein transport through the exocytic compartment, and has been used extensively in studies of class I molecule assembly and transport. Proteins whose N-linked oligosaccharides are resistant to digestion will be subsequently referred to as "endo H resistant"; those with N-linked oligosaccharides that are removed by endo H digestion will be referred to as "endo H sensitive."

expressed under the control of an earlier virus promoter or might be introduced into the cells as viral structural proteins.

These findings indicate that class I molecules associate either in the compartments in which E3/19K–class I complexes are retained or in a more distal exocytic compartment that is rapidly depleted of class I molecules once its supply is shut off by E3/19K sequestration. This latter possibility is more than academic. Class I transport to the cell surface following its export from the ER appears to be quite rapid, with a half-time on the order of 10 to 30 minutes (Williams *et al.*, 1985), which means that the early portions of the GC, for example, could be completely depleted within minutes of shutting off class I export from the ER. To resolve this issue unambiguously it will be necessary to determine whether the class I–peptide complex is formed in cells expressing E3/19K. This presupposes of course that E3/19K does not block class I association with antigen.

This would appear to be the case based on a recent study in which an E3/19K gene missing its six carboxy-terminal residues was inserted into vaccinia virus (Cox *et al.*, 1991). This protein was exported to the cell surface. Although it formed a tight complex with K^d, it did not block K^d transport to the cell surface, and it did not affect the presentation of K^d-associated determinants derived from influenza virus proteins. That the lack of inhibition is not simply due to a decreased affinity for K^d is suggested by the ability of the nonretained E3/19K to overcome the antigen presentation blockade effected by wild-type E3/19K when the two are coexpressed in cells.[25] Thus, if the class I molecule–peptide complex is present in E3/19K-expressing cells, it should be possible to isolate antigenic peptides by HPLC analysis of acid extracts. Should this come to pass, it will be critical to determine the mechanism by which E3/19K is retained in the ER, as it has been proposed (Jackson *et al.*, 1990) that the ER retention of E3/19K is due to the retrieval of E3/19K from either the intermediate compartment situated between the ER and GC or the cis-GC. In this case, antigen association could occur in any of these compartments.

c. Assembly Deficient Cells

Information regarding the site of antigen association with class I molecules has also come from studying a number of antigen

[25] Native E3/19K is a homo-oligomeric protein, possibly a dimer, which is how the protein migrates in SDS–PAGE under nonreducing conditions. It is therefore possible that the tailless E3/19K protein blocked the function of wild-type E3/19K by producing nonfunctional oligomers. This possibility appears highly unlikely, however, as such hetero-oligomers were still tightly bound to K^d (Cox *et al.*, 1991).

processing-deficient cell lines. As described in Section II.A, three of these cell lines, RMA/S, .134, and T2, efficiently present synthetic peptide antigens, but not biosynthesized antigens to T_{CD8+}. These cells also demonstrate deficiencies in assembling class I molecules and transporting them to the cell surface. On the basis of these findings, it is believed that these cells, and by extrapolation other cell lines that demonstrate inefficient assembly and transport of class I molecules, lack either a sufficient supply of peptides or the appropriate accessory molecules required to assemble peptides with α chains and β_2m. If this proves to be true, the location of the unassembled class I molecules could well represent the site of peptide binding to class I molecules.[26]

In RMA/S cells, a substantial fraction (80 to 90%) of class I molecules show prolonged sensitivity to digestion with endo H (Ljunggren *et al.*, 1989; Townsend *et al.*, 1989). Similar findings were made regarding the transport of HLA-A2 in T2 cells (Salter and Cresswell, 1986). Class I molecules in RMA/S cells are also resistant to digestion with endo-β-*N*-acetylglucosaminidase D (endo D), which would suggest that they do not reach the more proximal reaches of the GC containing Golgi mannosidase I, whose action renders oligosaccharides sensitive to endo D until acted on by *N*-acetylglucosamine (GLcNAc) transferase I. Such oligosaccharides are not resistant to digestion by endo H, however, until acted on by two more GC enzymes (GlcNAc transferase I and GC α-mannosidase II). This step can occur rapidly, so it is equally plausible that class I molecules rapidly pass through an endo D-sensitive stage and then await further processing in the GC. Further clouding the issue is that, by immunofluorescence, most K^b and D^b molecules are located not in the ER but in a compartment that is closely related, if not identical,to the GC (F. Esquivel, J. Bennink, and J. Yewdell, unpublished observations). Given these ambiguities, at the present time the only conclusion that can be drawn is that class I molecules might reach the GC in cells deficient in the MHC ABC genes, but only the most proximal regions.

Similar findings have been made with spontaneous lung carcinoma and fibrosarcoma cells (CMT 64.5) (Klar and Hämmerling, 1989). Class I genes are expressed at very low levels in these cells, but transcription can be greatly enhanced by treatment with interferon-γ. Transfection of class I genes under the control of heterologous promoters resulted in the synthesis of large amounts of unassembled α chains, which re-

[26] At the same time, an alternative explanation that cannot be eliminated at present is that the cells are deficient in antigen processing because they are deficient in a factor required to transport class I molecules to the appropriate secretory compartment.

mained sensitive to endo H digestion. Assembly was not promoted by cotransfection with β_2m. It was enhanced, however, by treatment with interferon-γ. Assembled class I molecules became resistant to endo H digestion and were transported to the cell surface. One of these cell lines transfected with K^d was the subject of a further study, whose findings suggested the existence of a GC-to-ER recycling pathway for class I molecules (Hsu *et al.*, 1991). In cells cultured at 37°C, class I molecules were localized to the ER by immunofluorescence microscopy. After incubation for 2 hours at 16°C followed by 5 minutes at 37°C,[27] class I molecules were found to colocalize more extensively with a GC-resident protein. A similar shift in distribution was observed after incubating cells with the microtubule inhibitor, nocodozole. These findings were consistent with previous observations that the GC-to-ER transport induced by BFA is partially inhibited by nocodozole or by incubating cells at 16°C (Lippincott-Schwartz *et al.*, 1990). By indirect immunoperoxidase localization of mAb binding to thin sections using the electron microscope, class I molecules were detected primarily in the ER of 37°C incubated cells, and the cis-most cisternae of the GC of 16°C incubated cells. Via cell fractionation, warming cells from 16°C to 37°C was found to alter the location of [^{35}S]methionine-labeled class I molecules from the light fractions characteristic of GC-derived vesicles to the heavier vesicles characteristic of the ER. Despite the apparent presence of K^d in the GC, it did not demonstrate resistance to either endo H or endo D. It is not clear whether this was due to the absence of the appropriate carbohydrate-modifying enzymes in the GC compartments visited by K^d, the lack of access of the enzymes to K^d-associated oligosaccharides, or the inability of the enzymes to function at 16°C. It is also unclear to what extent the proposed recycling pathway extends to other ER proteins or to class I molecules in other cell lines, particularly as the control protein illustrated was expressed in a different cell line. The study does, however, indicate that class I molecules that seem to be located in the ER by normal criteria might actually recycle between the GC and the ER, and further suggests that the protein(s) that controls class I molecules exocytosis might be located in the GC or the intermediate compartment located between the ER and GC. This would be consistent with the localization of class I molecules to the GC of RMA/S cells by immunofluorescence.

d. So, Where Do Class I Molecules Associate with Antigen?

It is frequently written that peptides processed from cytosolic

[27] It is not clear from the paper whether this 5-minute incubation step was necessary to observe the redistribution of class I molecules.

proteins associate with class I molecules in the ER. This an overstatement of the available evidence, which only justifies the conclusion that association occurs in an intracellular exocytic compartment. The apparent post-ER localization of class I molecules in antigen processing deficient cells suggests that class I molecules without peptides can exit the ER. This implies either that peptides associate with class I molecules in a post-ER compartment or that class I export control molecules that monitor class I conformation are present in a post-ER compartment, allowing peptide-containing molecules further passage while returning empty molecules to the ER (the possible existence of such class I inspectors is discussed in Section II.F).

On theoretical grounds it can be argued that peptides should associate with class I molecules in a post-ER compartment. Of the compartments of the exocytic pathway the ER is by far the largest. Although precise measurements are not available, the ER is at least 10-fold and possibly 100-fold more voluminous than post-ER compartments (e.g., the intermediate compartment) through which all class I molecules must pass. It would therefore require much less antigenic peptide to reach the same concentration in a post-ER compartment than in the ER (unless antigenic peptides and class I molecules are confined to a subregion of the ER). Similarly, the concentrations of α chains and β_2m would also be higher in post-ER compartment, further favoring assembly. Two observations lend indirect support to association occurring in a post-ER compartment. First, as discussed in Section II.D.2, the extrapolation of the concentrations of peptide required for *in vitro* assembly to the number of peptides that would be required in the ER do not seem to jibe with the extreme sensitivity of the antigen processing pathway. Second, the failure to detect specific peptide transport in microsomal vesicles containing newly synthesized class I molecules (discussed in Section II.E.2) would be expected if the putative MHC ABC peptide transporters were localized to the post-ER compartment.[28]

2. *Folding and Assembly of Class I Molecules*

a. Antibodies as Probes of Class I Structure

One of the most powerful tools used to study the assembly of α chains with β_2m have been class I molecule-specific mAbs. Most of the hybridomas secreting these antibodies were obtained from mice im-

[28] This might also provide the added benefit of decreasing the chance of peptide binding to BiP or other HSP, if the antigen association compartment was distal to the compartment from which these proteins are retrieved by the KDEL receptor (see Section II.F.1).

munized with viable allogeneic or xenogeneic cells, and consequently, almost all recognize discontinuous determinants on class I molecules.[29] Identification of residues that constitute discontinuous determinants is a difficult process and can be fully accomplished only by co-crystallization of antibody–antigen complexes.[30] In the absence of this, it is necessary to settle for less precise methods. Discontinuous determinants have been located in class I molecules primarily through the use of chimeric molecules, created by genetically shuffling regions from different class I allomorphs. Most often, intact exons have been shuffled between genomic DNA clones, as this has been easiest to accomplish through genetic engineering techniques. In some cases, residues that contribute to antibody binding have been identified through the use of molecules with defined amino acid substitutions (in some cases, mutants with single amino acid substitutions were selected by virtue of their loss of antibody reactivity). It should be kept in mind that neither of these methods directly identifies the residues that constitute the determinant. This is particularly true with class I molecules, where it is known, for example, that substituting β_2m of one species for that of another can result in alterations in the binding of antibodies whose reactivities map to the $\alpha 1\alpha 2$ domains (Ferrier *et al.*, 1985; Jefferies and MacPherson, 1987; Mierau *et al.*, 1987; Nieto *et al.*, 1989; Schmidt, 1988).

Regardless of the exact residues that comprise the determinants, antibody binding clearly serves as some measure of class I molecule conformation. Generally, antibodies whose binding is influenced by polymorphic residues in $\alpha 1$ or $\alpha 2$ domains are the most sensitive measure of class I molecule folding into the native, peptide-containing structure. Antibodies whose reactivities map to the $\alpha 3$ domain also require class I molecules folding, but to a somewhat lesser extent, and often bind α chains in the absence of β_2m. There are only a few mAbs described that detect denatured class I molecules, and all are specific for HLA class I molecules (Schnabl *et al.*, 1990; Stam *et al.*, 1990).

[29] Discontinuous determinants are formed by residues derived from separate portions of the protein that are brought into apposition by folding. This contrasts with the continuous determinants recognized by antibodies elicited by immunization with synthetic peptides corresponding to linear sequences in the protein (or by T cells). Discontinuous determinants are also often recognized by antibodies elicited by immunization with denatured intact proteins.

[30] "Fully accomplished" is admittedly an overstatement. The antibody–antigen complex is not static, and the dynamic aspects of the interaction can be appreciated only by applying additional techniques.

Detection of unfolded mouse class I molecules requires the use of antipeptide antisera. The most useful and most widely used antipeptide serum was raised to a peptide corresponding to residues in exon 8 that are present in H-2K allomorphs (Smith *et al.*, 1986), and appears to react with all forms of class I molecules. It is important to keep in mind that the interpretation of many of the studies that follows hinges on the specificity of the antibodies used and to entertain the possibility that the antibodies used detect only a subset of the class I molecules produced by cells. Furthermore, the effects of detergents on class I structure and antigenicity and on the specificity of antibodies have not been defined, and could influence the results. As a final caveat, it should be recognized that protein conformation is dynamic, and antibodies have the ability to freeze proteins in conformations that optimize the free energy of the antibody–antigen interaction, so that the differential recognition of two antigens by antibodies does not necessarily indicate that the reactive form exists more or less permanently in an antibody binding conformation, but rather that the one antigen is able to adopt that conformation more readily than another.

b. Creation of the Trimolecular Complex

The assembly of class I molecules was first extensively examined in the classic study of Krangel *et al.*, (1979). Using the W6/32 mAb, which recognizes a determinant expressed on most human class I molecules, but apparently only when associated with β_2m,[31] and a rabbit serum raised to denatured α chains that was not reactive with β_2m associated α chains, they established a precursor–product relationship between class I molecules reactive with rabbit serum and those reactive with W6/32. The half-time for conversion from the unfolded to the folded form was approximately 5 minutes. W6/32-reactive class I molecules became resistant to endo H digestion with a half-time of 40 minutes, whereas endo H-resistant free α chains could not be detected. W6/32-reactive class I molecules were resistant to proteolysis, whereas the free α chains were easily digested, indicating that the binding of β_2m was associated with α chains folding into a more compact conformation. The importance of β_2m in this conformational alteration was supported by findings with Daudi cells, a human B cell lymphoma lacking β_2m (Arce-Gomez *et al.*, 1978; Fellous *et al.*, 1977). In Daudi

[31] W6/32 also recognizes some mouse class I molecules when complexed with bovine or human β_2m (Jefferies and MacPherson, 1987; Mierau *et al.*, 1987). This reactivity segregates with the α2 domain in chimeric class I molecules (Jefferies and MacPherson, 1987; Marziarz *et al.*, 1986). It seems likely that the residues that bind this antibody derive, at least in part, from the α2 domain and β_2m.

cells, α chains remained nonreactive with W6/32, sensitive to proteolysis, and sensitive to endo H digestion (Kissonerghis *et al.*, 1980; Ploegh *et al.*, 1979).

Similar experiments have been performed with mouse cells lacking β_2m. For some class I allomorphs, no transport is detected in these cells by the criteria of endo H resistance or surface expression. For others, transport to the cell surface occurs but at a much lower efficiency (Allen *et al.*, 1986; Williams *et al.*, 1989). The conformation of class I molecules expressed by β_2m-deficient cells has been examined using mAb panels. Antibodies specific for the α3 domain[32] (particularly 28-14-8s) have been found to react with α chains present in detergent extracts under these conditions. Although it is generally considered that α3-specific reagents react with all forms of class I molecules, this is unlikely to be the case, as the reactivity of metabolically radiolabeled D^b molecules with 28-14-8s is increased on addition of peptides to detergent lysates (Townsend *et al.*, 1989). This suggests that 28-14-8s and perhaps other α3-specific mAbs recognize class I molecules that partially fold in the absence of β_2m. In support of this conclusion, mAbs specific for the $\alpha 1\alpha 2$ domain have been found to react with D^b or K^b molecules expressed in the absence of β_2m.[33] These findings suggest, therefore, that mouse class I molecules are not completely unfolded in the absence of β_2m. Whether human α chains behave similarly remains to be determined.

The partial folding of α chains in β_2m-deficient cells may reflect the binding of antigenic peptides to α chains. This conclusion is supported by two reports. In the first, purified HLA-A2 α-chains in detergent were found to bind, in the absence of β_2m, a fluoresceinated 14-residue peptide containing an antigenic determinant from influenza virus matrix protein (Dornmair *et al.*, 1991), as determined by the presence of peptide bound to class I molecules in SDS–PAGE. In the second, it was found that addition of antigenic peptides in the range of $4\mu M$ to detergent lysates of β_2m-deficient cells allowed mAbs specific for $\alpha 1$ or

[32] As noted above, "specificity" has been determined by segregation of antigenicity with segments in chimeric class I constructs. It is possible that residues in the $\alpha 1\alpha 2$ domain also contact the antibody binding site.

[33] This was detected by immunofluorescence of fixed and permeabilized β_2m-deficient RIE cells transfected with either K^b or D^b genes (J. Yewdell and J. Bennink, unpublished results). These findings differ from those in the original description of the transfected RIE cells (Allen *et al.*, 1986). This is probably related to the fact that only antibody reactivity with cell surface class I molecules was measured in this study. During or rapidly following transport to the cell surface, class I molecules probably unfold (see Section II.F.4).

$\alpha 2$ domains to bind to [^{35}S]methionine-labeled D^b (Elliot *et al.*, 1991). Importantly, only an octameric peptide that corresponds to the natural peptide recovered from influenza virus-infected cells could mediate this effect. The same peptide was most efficient in driving α chain and β_2m assembly *in vitro* (Townsend *et al.*, 1990), and binds to assembled chains with an affinity 50-fold higher than that of a longer peptide (15 residues) that did not alter the conformation of D^b in the absence of β_2m. The discrepancy between the studies regarding the ability of long peptides to bind class I molecules could result from differences between A2 and D^b, or a difference in the sensitivities of the two assays employed, or might perhaps indicate that, although both long and short peptides bind class I molecules, only short ones induce conformational alterations required for antibody binding.

The assembly of the trimolecular complex has also been studied using antigen processing-deficient cells. In considering these results, it should be appreciated that the absence of peptides in these cells is really only a working hypothesis at this stage. Moreover, as RMA/S cells clearly present antigens, their class I molecules cannot totally lack peptides. Of the cell lines studied, .174 and its derivative T2 are likely to have the most profound deficit in peptide production or transport, because of the size and location of their deletion in the HLA complex.

It was originally reported that metabolically radiolabeled class I molecules from RMA/S cells are less reactive with mAbs specific for $\alpha 1\alpha 2$ domains than for $\alpha 3$ domains (Townsend *et al.*, 1989). In these experiments, detergent extracts were incubated overnight at 4°C prior to the addition of antibodies. If $\alpha 1\alpha 2$-specific antibodies are added immediately to extracts, or if the volume of extracts is minimized, intact class I molecules are precipitated from RMA/S cells (Elliot *et al.*, 1991). Diluting extracts in the presence or absence of antigenic peptides indicated that the stability of the complexes was increased tenfold by including antigenic peptides (half-times of dissociation of 4 hours versus 50 hours). Peptides did not have to be of optimal length to mediate this effect. A similar effect on class I conformation was detected by increasing the concentration of β_2m in the extracts. Thus it appears that β_2m is associated with newly synthesized class I molecules in RMA/S cells, but that this association is weak, and requires peptide binding for stabilization. Consistent with this conclusion is the strong immunofluorescence staining of intracellular class I molecules by $\alpha 1\alpha 2$-specific mAbs in fixed and permeabilized RMA/S cells (F. Esquivel, J. Bennink, and J. Yewdell, unpublished observations).

In T2 cells, immunoprecipitation revealed that some HLA-A2 and

little, if any, HLA-B5 reacted with W6/32 (Salter and Cresswell, 1986). Based on sequential precipitation with an antiserum specific for β_2m, followed by an antiserum believed to react with all forms of α chains, it was estimated that only 10–20 percent of HLA molecules from T2 cells were complexed with β_2m, as opposed to 70 percent in control cells. Subsequently, it was found that either antigenic peptides or β_2m induced conformational alterations in A2, as detected by an α1-specific mAb when detergent lysates were incubated overnight prior to antibody addition (Elliot *et al.*, 1991). These findings are similar to those obtained with RMA/S cells. It is, therefore, somewhat surprising, that it appears that mouse class I molecules assemble normally in T2 cells transfected with mouse class I genes (Alexander *et al.*, 1989). Two-dimensional gel analysis of D^p and K^b immunoprecipitates from T2 and control cells revealed a similar degree of charge heterogeneity and a similar amount of β_2m, which suggests that these molecules are assembled and transported normally. Consistent with this possibility, K^b, D^p, D^d, L^d, and K^b are expressed in normal quantities on the surface of T2 cells, as detected by antibodies specific for the $\alpha 1\alpha 2$ domains (Alexander *et al.*, 1989, 1990; Hosken and Bevan, 1990).

This assembly of mouse class I molecules in T2 cells is puzzling. Based on the more profound antigen presenting deficit in T2 cells, it would be expected that the peptide concentration at the site of class I assembly would be lower than in RMA/S cells. It has been suggested (Townsend *et al.*, 1990) that the differences in class I assembly in the two cells might reflect differences in β_2m: T2 cells might express greater amounts of β_2m, and human β_2m might induce assembly more efficiently than mouse β_2m. To support this latter possibility, it is often stated that mouse α chains have a higher affinity for human β_2m than mouse β_2m, based on the binding of exogenous human β_2m to cell surface class I molecules. Even if this were true, it may not apply to intracellular class I molecules, particularly in view of findings that in cells coexpressing mouse and human α chains and β_2m, α chains preferentially associate with β_2m of the same species (Perarnau *et al.*, 1988).[34]

It is important to mention that similar, though occasionally less dramatic enhancement of class I molecule assembly is observed following addition of antigenic peptides or β_2m to detergent extracts obtained from nonmutant cells (Elliot, 1991). This does not negate the importance or relevance of the findings with mutant cells, but rather

[34] It should be noted, however, that only K^d was examined in this study, and that D^b and K^b might differ in their affinities for β_2m.

suggests that in normal circumstances, class I molecules are not saturated with high-affinity peptides. This conclusion is supported from studies on the assembly of H-2 L^d molecules in cells with normal antigen processing capacity. These studies were greatly facilitated by the availability of mAbs that recognize two distinct forms of L^d, as demonstrated by sequential immunoprecipitations (Lie *et al.*, 1990, 1991; Myers *et al.*, 1989). One form represents the native L^d recognized by T_{CD8+}; the other represents a more unfolded form of L^d. Antigenic peptides induce the conversion of the unfolded form to the folded form in detergent extracts. This might reflect binding to free α chain, as L^d has a low affinity for β_2m, which is often not coprecipitated by antibodies specific for the folded form, particularly once L^d acquires complex oligosaccharides in the GC. Additional experiments are required, however, to determine whether β_2m dissociates from α chains during the immunoprecipitation process (as a result of conformational alterations induced by detergent or even antibody binding).

Class I assembly has also been studied by *in vitro* translation of mRNA for class I and β_2m in the presence of microsomes derived from mutant or wild-type cells (Kvist and Hamann, 1990; Lévy *et al.*, 1991a,b). Assembly of HLA-B27 was first detected by addition of an 11-residue peptide at 1 μM, and was maximized at 14 μM (Kvist and Hamann, 1990). This explained an early report that assembly does not occur in microsomes without adding peptides (Ploegh *et al.*, 1979). Using biotinylated peptide, binding to assembled complexes could be directly demonstrated (Lévy *et al.*, 1991b). In this report, however, the requirement for peptide in class I assembly was not absolute. This presumably reflects some variability between microsomal preparations, as microsomes were prepared from the same cells as in the first report. Class I molecules assembled to the same or greater extent at 26°C in the absence of peptides as at 37°C in the presence of peptides. Molecules assembled at 26°C were unstable at 37°C unless peptides were present. Using microsomes prepared from Daudi cells,[35] peptide-containing or W6/32-reactive class I molecules were not detected at either 26 or 37°C. Note, however, that the peptide used is three residues longer than the highest-affinity peptides, which were not tested in this study.

Finally, class I assembly has been studied biochemically using purified α chains and β_2m obtained via gel filtration following dena-

[35] It was necessary to use Daudi cells as microsomes prepared from normal cells contain large amounts of previously synthesized β_2m.

turation of purified class I molecules (Silver *et al.*, 1991). Separated chains were combined in a chaotropic buffer with or without peptides, and renaturation was assessed by gel filtration following dialysis to remove the denaturant. In the presence of peptides, more than 40 percent of class I complexes were obtained. Remarkably, even under these harsh conditions, approximately 10 percent of complexes assembled in the absence of added peptides. This confirmed an earlier observation that purified papain cleaved K^b could associate with β_2m in the absence of other factors (Yokoyama *et al.*, 1985).

Taken together, these findings from diverse systems are remarkably consistent in indicating that assembly of α chains with β_2m can occur in the absence of peptides, and that this interaction is stabilized by antigenic peptides. It also appears that α chains could interact with peptides of the optimal length prior to their binding β_2m. Although the present evidence does not allow for definitive conclusions regarding the relative contributions of these pathways to class I assembly *in vivo*, it appears more likely that the first pathway is used in most circumstances. At the very least, it seems that low-affinity peptides would have to associate with preformed α chain–β_2m complexes. That a considerable portion of class I molecules dissociate on arrival to the cell surface even in normal cells (discussed in Section III.B) argues that many class I molecules contain such low-affinity peptides. Regarding the association of high-affinity peptides, it is notable that the concentration of natural peptides required to induce conformational changes in D^b molecules in detergent extracts from β_2m-deficient cells, 5 μM, would correspond to approximately 10^6 molecules in a typically sized ER. As it seems unlikely that this concentration would ever be reached in cells, this either (1) calls into question the relevance of class I assembly in detergent extracts to assembly *in vivo*, (2) casts doubt on the possible binding of peptides to α chains prior to β_2m association, or (3) indicates that class I assembly occurs in a much smaller compartment (an ER subcompartment or post-ER compartment) into which peptides are specifically transported and can reach micromolar concentrations (a possibility discussed in Section II.E.1.d).

A critical missing piece of information that would greatly help in deciding among these possibilities is the concentrations of various-length peptides required to stabilize the initial β_2m–α chain complex detected in concentrated, non-precleared RMA/S lysates. If peptide concentrations in the nanomolar range suffice, this would strongly suggest that in most circumstances such peptides bind to preformed complexes. If, on the other hand, micromolar concentrations are re-

quired, this would suggest that binding of these peptides is facilitated *in vivo* by a mechanism not operative in detergent lysates.

That such a mechanism exists is suggested by the characterization of class I assembly in microsomes (also discussed in Section II.D.2), which revealed that peptide-dependent assembly of HLA-B27 was ATP dependent (Lévy *et al.*, 1991a). Because of the requirement for ATP for *in vitro* translations, it was not possible to determine whether peptide-independent assembly was also ATP dependent. Curiously, however, depletion of ATP during a 15-minute incubation following a 45-minute translation period resulted in the disappearance of W6/32-reactive class I molecules, suggesting that stability of this complex, if not its assembly, requires ATP. It was not determined whether depletion of ATP following the addition of exogenous peptide had a similar effect on complexes containing this peptide. As mentioned above, these effects are unlikely to be attributed to ATP-dependent transport of peptides into the microsomes, as depletion of ATP did not affect peptide transport as determined by the binding of peptide to BiP. Further supporting that assembly is a facilitated process, less assembly of B27 molecules was observed in microsomes from T2 cells in both the presence and the absence of exogenous peptide, which did enhance assembly to some extent. Again, differences between T2 and control cells could not be attributed to differences in peptide transport into microsomes.

The diminished assembly in T2 cells suggests that in addition to any transport deficiency they might possess, they also lack a factor that enhances class I assembly. Additional experiments are necessary to determine whether this factor is encoded by one or more of the ABC transporter- or proteasome-associated gene products, or other gene products encoded within the one-megabase-pair region deleted in T2 cells. This factor might function independently, or in conjunction with other gene products, either to alter the conformation of α chains or β_2m, to deliver peptides to the complex, or to trim peptides to the proper length. Further experiments using microsomes derived from .134 cells or from T2 cells transfected with MHC-encoded antigen processing genes in conjunction with peptides of assorted sizes are required to resolve these myriad possibilities. It will also be of interest to examine the properties of microsomes derived from RMA/S cells and other cell lines that exhibit diminished class I assembly (Bikoff *et al.*, 1991b; Klar and Hämmerling, 1989).

The ATP dependence of class I assembly has three general interpretations. First, ATP might be required for the function of molecules that facilitate class I assembly. These molecules might represent either

MHC or non-MHC gene products. The observation that assembly in T2 cells is also dependent on ATP would suggest that if these molecules do exist, at least some are not encoded in its 1-megabase-pair HLA deletion. One potential molecule that could fulfill this function is the 88-kDa protein that binds newly synthesized class I molecules (Degen and Williams, 1991) (described in more detail in Section II.F.3). Second, if class I association normally occurs in a post-ER compartment, ATP might be required for fusion of ER-derived microsomes with microsomes derived from the post-ER compartment. Third, ATP might simply be required to decrease the binding of peptides to BiP (or a BiP-like molecule), which is present in very high concentrations in the ER. Consistent with this third explanation, depletion of ATP actually increased peptide binding to BiP, in agreement with prior observations of BiP binding to its ligands (Flynn *et al.*, 1989). This could also explain the ATP dependence for stable class I complexes, as the longer peptide used probably dissociates fairly rapidly from class I complexes, and in the absence of ATP, peptide could be trapped by BiP. It will, no doubt, be interesting in future experiments to explore the behavior of other peptides, including those of optimal size and those that bind class I but not BiP.

Regarding this latter property, it is possible that peptide binding to molecular chaperones like BiP plays a critical role in determinant selection. In intact cells, this could have either a negative or a positive influence on antigenicity: in the former case by sequestering proteins, in the latter by protecting them from transport or proteolysis. In a recent study, the specificity of BiP for peptides was examined using a pool of peptides of 4 to 12 residues containing random amino acids at each position (Flynn *et al.*, 1991). This revealed that binding of peptides to BiP increased with length up to 7 or 8 residues and plateaued. Sequencing of heptamers bound to BiP revealed a preference of hydrophobic amino acids at each position, particularly alanine, leucine, and methionine. As the motifs for peptide binding to four class I allomorphs include at least one these residues (Falk *et al.*, 1991b), this suggests that BiP binding may favor peptide binding to class I molecules.

c. Role of Post-Translational Modifications in Class I Assembly and Transport

i. Glycosylation. Most, if not all, class I allomorphs contain one to three N-linked oligosaccharides. As with other glycoproteins, oligosaccharides are rapidly added to most α chains following synthesis, possibly during the process of translocation into the ER. The requirement

for glycosylation in the assembly and transport of class I molecules has been examined by treating Epstein–Barr virus-transformed human B cells with tunicamycin, a specific inhibitor of N-linked glycosylation (Neefjes and Ploegh, 1988). The effect of tunicamycin was highly variable on the 27 allomorphs examined. Nine of eleven HLA-A locus allomorphs assembled with β_2m and were transported to the cell surface with normal kinetics. Assembly of the other two A locus allomorphs and all 16 B locus allomorphs was inhibited to various degrees by tunicamycin, as monitored by immunoprecipitation with either W6/32, a β_2m-specific mAb, or an antibody (HC10) that recognizes denatured HLA α chains (Stam *et al.*, 1990). This antibody normally binds unassembled HLA-B α chains in detergent extracts, but not assembled molecules or free HLA-A α chains. The two HLA allomorphs whose assembly was compromised by tunicamycin were, however, recognized by HC10, which indicates that the chains were aberrantly folded in the absence of N-linked oligosaccharides.[36] For at least two HLA-B allomorphs, it was the process of glycosylation itself and not the maturation of the high-mannose oligosaccharide that was required for proper folding, as an inhibitor of oligosaccharide maturation had no effect on acquisition of W6/32 reactivity.[37]

These findings indicate that the proper folding of some HLA class I allomorphs requires the addition of oligosaccharides, whereas others do not. Studies with other cellular and viral glycoproteins reveal a similar sporadic requirement for glycosylation, and it is well established that glycosylation is not absolutely necessary for the folding of at least some integral membrane proteins. It remains to be determined whether glycosylation influences the function of those allomorphs that fold normally in deglycosylated form. Of particular interest is whether peptide binding is subtly influenced by oligosaccharide structure. There are a number of studies in which target cells treated with glycosylation inhibitors or with glycosidases were found to be recognized less efficiently by T_{CD8+}, which is consistent with this possibility

[36] The more important implication of this finding is that the failure of β_2m-free, glycosylated α chains to bind HC10 results from their folding into a form in which antibody does not bind, and not from other factors. Thus, free HLA α chains are not in a completely denatured form.

[37] Oligosaccharide maturation was blocked using 1-deoxymannojirimycin, which acts by blocking Golgi mannosidase I. Prior to this step, the original $Man_9GLcNac_2Glc_3$ is usually trimmed by ER enzymes to $Man_8GlcNac_2$, so to be precise, these findings indicate that additional trimming beyond this form is not required for folding and assembly.

(Black *et al.*, 1981; Boog *et al.*, 1989; Neefjes *et al.*, 1990). Even if these findings could be shown not to result from a reduction of class I assembly or transport, the global effects of these treatments on all cellular glycoproteins still cloud interpretation. To avoid this difficulty, site-directed mutagenesis was used to remove the three glycosylation sites in L^d (Miyazaki *et al.*, 1986). Cells transfected with the gene encoding nonglycosylated L^d were recognized at similar levels by L^d-restricted, vesicular stomatitis virus-specific T_{CD8+} as cells transfected with wild-type genes, despite the decreased surface expression of deglycosylated L^d. This finding demonstrates that glycosylation is not absolutely required for class I molecules to function normally in presenting endogenously synthesized proteins. It remains to be determined, however, whether the number and types of oligosaccharides attached to class I molecules can exert subtle influences on the conformation of some allomorphs, altering the specificity of the binding site for certain peptides or the manner in which the peptide is bound to the site.[38]

ii. Palmitylation. Palmitylation is a fairly common post-translational modification of integral membrane proteins (reviewed in Grand, 1989; Sefton and Buss, 1987). Palmitate is added via a thioester bond to cysteine residues present in cytosolic domains. Palmitylation probably occurs in the intermediate compartment between the GC and ER or in the cis-GC (Bonatti *et al.*, 1989). It it thought that the conjugated palmitate inserts the membrane, tethering the modified cysteine to the inner surface of the membrane. The functional consequences of palmitylation of membrane proteins are uncertain; for a number of viral proteins it appears to have little or no effect on structure or function.

Although it has been established that class I molecules can be palmitylated, this has not been extensively characterized. Using Epstein–Barr virus transformed human B cells, HLA-B7 was shown to be palmitylated, whereas HLA-A2 derived from the same cells was not (Kaufman *et al.*, 1984). Based on protease digestion of B7, it was concluded that palmitate was added to a cysteine residue near the carboxy terminus of the transmembrane domain. Notably, A2 has a tryptophan at this position. The lack of palmitylation of A2 therefore suggests that not all cytoplasmic cysteine residues can be modified, as A2 does have a cysteine residue located closer to the carboxy terminus.

[38] The increased binding of class I-specific mAbs to cell surfaces observed following neuraminidase treatment of cells (Boog *et al.*, 1989; Liberti *et al.*, 1979) would suggest that oligosaccharides subtly influence the conformation of class I molecules, but again, this observation might be more related to the global effects of neuraminidase on the cell surface rather than a specific effect on class I molecules.

The extent to which other allomorphs are palmitylated and the degree to which palmitylation varies between cells of different lineages remain to be determined. Most importantly, of course, is the effect of palmitylation on class I folding or intracellular transport. This awaits further experiments using genetically altered class I molecules in which cysteine residues known to be palmitylated have been replaced by other residues.

iii. Phosphorylation. A third post-translational modification observed with class I molecules is phosphorylation. This is believed to occur either shortly before class I molecules have reached the plasma membrane, or after their arrival. Virtually nothing is known about the effect of phosphorylation on class I structure and function. As there is limited evidence that phosphorylation is involved in internalization of cell surface class I molecules, phosphorylation is described in Section III, which describes the properties of cell surface class I molecules (see Section III.C.2.b).

F. Control of Class I Export to the Cell Surface

1. General Considerations

Export of newly synthesized proteins from the ER can be a highly regulated event (for reviews, see Hurtley and Helenius, 1989; Rose and Doms, 1988). It is presently believed that in the absence of specific retention signals, membrane and soluble proteins are exported from the ER. There appear to be four types of retention signals. Some proteins (e.g., those in the protein translocation complex) appear to be retained in the ER by forming complexes that are retained either by being too large to be included into transport vesicles or perhaps by tethering to the cytoskeleton. Soluble proteins, such as BiP, with carboxy-terminal sequences related to Lys–Asp–Glu–Leu (or in single-letter code, KDEL) appear to be retained based on retrieval from a post-ER compartment (the intermediate compartment or possibly the early GC) by a receptor for this sequence that cycles between the retrieval compartment and the ER (Pelham, 1989, 1991a). Membrane proteins with sequences related to the adenovirus E3/19K glycoproteins are retained based on recognition of their cytosolic tails, possibly only a small number of residues close to the carboxy terminus (Jackson *et al.*, 1990; Nilsson *et al.*, 1989). This may be due to recycling of these proteins from the intermediate compartment or early GC; indeed, the receptor for KDEL-like sequences might possess such a sequence, in which case a single mechanism (yet to be defined) would account for ER retention of soluble and membrane bound proteins.

Finally, other proteins are retained by associating with proteins that reside in the ER based on the first three mechanisms. The last category appears to be used in some cases as a mechanism to prevent the export of incomplete or misfolded proteins. For several proteins, it is possible to demonstrate interaction with BiP, which is believed to bind misfolded proteins based on the exposure of hydrophobic peptides that are normally buried in native proteins.

2. *Retention of Free α Chains*

Evidence for such conformational monitoring of class I molecules comes from studies of cells lacking β_2m, where it has been found that transport of α chains to the cell surface is compromised, with retained α chains persisting in an endo H-sensitive form (Hyman and Stallings, 1976; Ploegh *et al.*, 1979; Sege *et al.*, 1981; Williams *et al.*, 1989). The extent of the transport blockade varies with class I allomorphs, as at least one class I molecule is transported to the cell surface (albeit inefficiently) (Allen *et al.*, 1986) in β_2m-deficient cells, whereas others have not been detected at the cell surface.[39] As discussed above (Section II.E.2.b), in the absence of β_2m, class I molecules fail to properly fold, as assessed by the binding of mAbs specific for the $\alpha 1\alpha 2$ domains. Conformational alterations in α chains can also be detected by their partitioning into the detergent TX-114 (Williams *et al.*, 1989). Although assembled class I molecules, like most membrane proteins, largely partition into the detergent phase, retained K^b and D^b molecules in β_2m-deficient cells partition exclusively into the aqueous phase. This finding indicates that the hydrophobic surfaces in free α chains are not available to solvent, which could reflect conformational changes within a single α chain or the association of α chains with themselves or with molecular chaperones in a manner that buries their hydrophobic domains.

More subtle conformational alterations in a class I molecule can also prevent its efficient surface expression. Substitution of arginine for tryptophan at position 167 (located in one of the α helices that forms one side of the binding site) of K^b results in a tenfold decrease in surface expression (Williams *et al.*, 1988). Mutant molecules associate with β_2m with the same efficiency and kinetics as wild-type K^b, and maintain reactivity with a number of $\alpha 1\alpha 2$-specific mAbs. Mutant mol-

[39] It has also been reported that a mutant D^d with a tryptophan-to-arginine substitution at position 133 (located in a β sheet under the α helix of the $\alpha 2$ domain) fails to associate with β_2m or bind mAbs specific for the $\alpha 1\alpha 2$ domains, but is transported to the cell surface (Rubocki *et al.*, 1991). This extends findings with β_2m-deficient cells to normal cells.

ecules were more sensitive to trypsin digestion than wild-type molecules, however, indicating that the mutation caused conformation alterations. Retained mutant molecules were sensitive to endo H and, based on fractionation of membrane vesicles from disrupted cells, appeared to be retained largely in the ER. Two hours following synthesis, the amount of mutant K^b molecules recovered in immunoprecipitates slowly declined. This loss was not prevented by reducing cellular ATP levels to block intracellular transport, which suggests that K^b molecules were degraded in the ER via the proposed ER degradation pathway (Bonifacino and Lippincott-Schwartz, 1991).[40] As with free α chains, any cellular molecules that might function in retaining misfolded K^b molecules have yet to be discovered.

The ability of free α chains to exit premedial GC compartments has also been examined in frog oocytes injected with mRNA from human cells size-selected to contain α-chain but not β_2m messages (Severinsson and Peterson, 1984). α Chains expressed in this manner remained sensitive to endo H digestion for at least 24 hours following synthesis. In contrast, α chains coexpressed with β_2m became resistant to endo H digestion between 4 and 8 hours following their synthesis. Furthermore, injection of β_2m RNA 20 hours after injection of α chain mRNA resulted in a substantial fraction of previously synthesized α chains acquiring endo H resistance, indicating that β_2m could rescue the transport of α chains sequestered in an early exocytic compartment, and that therefore, these molecules were not retained because of the formation of irreversible aggregates. These findings suggest that mammals and amphibians use a similar mechanism to retain free α chains in an early exocytic compartment. Although frogs express class I-like molecules, it might be expected that these would be highly divergent from mammalian class I molecules. Thus, retention of free α chains may be based not on recognition of class I-specific signals but on general features of misfolded proteins, such as exposed hydrophobic domains.[41]

[40] Class I molecules also appear to be degraded in a similar manner in RMA/S cells.

[41] A test of this hypothesis would be the expression of α chains with and without β_2m in insect cells, which are not believed to possess a MHC, or in yeast (which would have no reason to possess a MHC). Expression of HLA B27 in insect cells using recombinant baculoviruses has been reported (Lévy and Kvist, 1991). Association with β_2m occurred only after 48 hours of coexpression and at very low levels. Although α chains expressed in the absence of β_2m were almost exclusively retained intracellularly, their intracellular location was not characterized.

3. *Retention of β_2m–α Chain Complexes*

In addition to retaining misfolded class I molecules, cells regulate the export of functional α chain–β_2m complexes. The first direct evidence that export of assembled class I molecules from the ER was a regulated process was provided by Williams and colleagues (1985), who found that newly synthesized D^k became resistant to endo H digestion up to tenfold more slowly than K^k (5 hours versus 30 minutes). This could not be attributed to differences in β_2m association, as D^k, like K^k, rapidly associated with β_2m (the amount of radioactive β_2m in immunoprecipitates was near or at maximal values immediately after 10 minute of labeling with [^{35}S]methionine). Two and a half hours following synthesis, most endo H-sensitive D^k molecules were located in the ER, as determined by sucrose gradient fractionation of membrane vesicles from homogenized cells. Eventually, a similar (high) percentage of D^k was transported to the cell surface as K^k, which is consistent with the idea that the retained D^k molecules maintain their function and are not damaged, at least not irreversibly. Functional experiments are consistent with this idea. L929 cells lose the ability to present K^k-associated proteins from exogenous influenza virions by 8 hours following addition of protein synthesis inhibitors while maintaining the ability to present a D^k-associated protein for at least an additional 23 hours of incubation with protein synthesis inhibitors (J. Yewdell, L.Eisenlohr, and J.Bennink, unpublished observations). Similar differences in the kinetics of class I transport have been observed with HLA class I allomorphs, as detected by acquisition of sialic acids of immunoprecipitated α chains analyzed by isoelectric focusing (Neefjes and Ploegh, 1988).[42] In some cases, it appeared that slow association of α chains with β_2m accounted for some of the transport retardation.[43]

A molecule potentially involved in the control of class I export was recently identified by treating detergent extracts of [^{35}S]methionine-labeled cells with reversible chemical crosslinkers (Degen and Wil-

[42] It was noted in this study that sialylated class I molecules were transported to the cell surface from the trans-GC (the presumed site of sialylation) with different kinetics. This is the only reported observation that class I transport may also be regulated in the distal GC.

[43] The low affinity of L^d for β_2m also seems to contribute to its slow export following synthesis, as a chimeric molecule consisting of D^d α1 domain and amino-terminal portion of the α2 domain was exported rapidly (like D^d), and efficiently associated with β_2m (Beck *et al.*, 1986).

liams, 1991). SDS–PAGE analysis of immunoprecipitates from such extracts revealed that a portion of class I molecules migrated with a M_r of 145 kDa. Reelectrophoresis of the 145-kDa molecule after cross-links were disrupted revealed only α chains and β_2m. If, however, extracts were prepared from cells incubated with [^{35}S]methionine for 24 hours to label proteins synthesized at low rates, or if unlabeled extracts were radioiodinated, it became clear that the 145-kDa complex consisted of class I molecules complexed to a protein of 88 kDa (termed *p88*). p88 was identified in complexes with all class I allomorphs examined (K^b, D^b, K^d, and L^d). The association of p88 with class I was transient in nature; p88 was present at earliest times following labeling and was associated only with class I molecules containing endo H sensitive oligosaccharides. p88 binding to α chains appeared to be independent of β_2m association as it occurred with a shorter half-time than β_2m association. Importantly, the dissociation of p88 correlated precisely with the half-times for the various allomorphs to acquire endo H resistance. Two findings indicated that dissociation of p88 occurred prior to the transport of class I molecules through the regions of the GC in which resistance to endo H digestion is acquired. First, dissociation occurred with similar kinetics when vesicular transport was blocked by treating cells with an inhibitor of oxidative phosphorylation to decrease ATP levels, despite the fact that only 10 percent of class molecules became resistant to endo H digestion under these conditions. Second, dissociation occurred at 15°C at times following synthesis when class I molecules were still sensitive to endo H digestion. p88 association or dissociation with class I molecules was not affected by BFA treatment.

These findings suggest that p88 plays an important role in regulating the export of assembled class I molecules. The most exciting possibility is that p88 alone, or in conjunction with other proteins, binds class I molecules, retaining them in a premedial GC compartment until peptide association induces conformational alterations in class I molecules resulting in a diminished affinity for p88. The lack of effect of BFA on p88 association would be consistent with this possibility as it appears that antigen association occurs in the presence of BFA (C. Lapham, J. Yewdell, and J. Bennink, unpublished observations). On the basis of a recent report, it appears likely that p88 is identical to a 90-kDa protein found to associate with newly synthesized immunoglobulin, T cell receptors, in addition to class I (David *et al.*, 1991). The binding of p88 to other proteins would not preclude its playing a critical role in regulating class I export, but it would indicate either that p88 has multiple binding sites or that class I molecules share certain features

with other proteins that form the basis of p88 recognition. If p88 binding is truly limited to these proteins or their relatives, it would suggest that p88 coevolved to regulate the expression of members of the immunoglobulin superfamily. In this case, p88 could bind either the $\alpha 3$ domain or β_2m, which fold similarly to domains in the constant region of immunoglobulin (Bjorkman *et al.*, 1987a). It will be of interest to determine whether α chains or β_2m can independently associate with p88.

Further support that export of assembled β_2m–α chain complexes is regulated comes from findings that class I molecules in antigen processing deficient cells T2 and RMA/S are transported inefficiently from a premedial GC compartment. As discussed in Section II.E.2.b, it appears that class I molecules in these cells are assembled, but presumably lack peptides. If p88 is involved in regulating the export of these complexes, this would suggest that there is some species specificity involved in its interaction with class I molecules, as most mouse allomorphs appear to be exported normally in T2 cells transfected with the corresponding class I gene (Alexander *et al.*, 1989). In this scenario, it would be unlikely that p88 recognizes the $\alpha 3$ domain as proposed above, as cell surface expression of chimeric mouse–human exon shuffled molecules in T2 cells segregated with the mouse $\alpha 1\alpha 2$ domain (Alexander *et al.*, 1989). Obviously, it will be important to correlate these findings with the binding of human p88 to mouse and human class I molecules.

Related findings have been made regarding the transport of rat class I molecules. Using recombinant inbred rats with crossovers in the MHC, a locus was identified in the class II region (termed *cim* for class I modification) that altered the antigenicity of a class I allomorph RT1 A^a. This was detected by a mAb (JY3/84) or A^a-restricted alloreactive T_{CD8+} (Livingstone *et al.*, 1989, 1991). cim exists in at least two alleles, termed cim^a and cim^b.[44] The presence of cim^a confers higher levels of A^a expression, and greatly increases the binding of JY3/84. In cells from F_1 rats containing both cim alleles, cim^a is dominant. Mouse cells transfected with the A^a gene express the form associated with the cim^b allele, as determined by T_{CD8+} recognition. Biochemical studies indicate that in cells expressing cim^a alone, or in combination with cim^b, A^a molecules rapidly acquire endo H resistance following synthesis, whereas in cells expressing only cim^b, transport of A^a through the GC is much slower. This effect is allomorph specific, as other allomorphs

[44] Based on present evidence, it is possible that the cim^b allele represents a null gene that does not produce a functional gene product.

are rapidly transported in cim^b homozygous cells. Assembly of A^a with β_2m occurred indistinguishably in cim^b and cim^a expressing cells. Expression of A^a in mouse cells by DNA-mediated transfection revealed that A^a molecules rapidly acquired endo H resistance following synthesis, but were antigenically in the form associated with the cim^b allele.

These findings bear a number of striking similarities to those obtained with T2 cells. The cim^a gene appears to produce a factor required for the release of class I molecules from the grips of a rat class I molecule-specific ligand [p88?], located in a premedial GC compartment. As the MHC ABC genes have been cloned from rat cells, it should be possible to test their relationship to the cim gene, which maps in the same region of the MHC. If cim is one or both of the MHC ABC gene products, this would indicate that allelic differences in the transporters modify their ability to interact functionally or physically with class I allomorphs. The simplest explanation in these circumstances would be that the specificity of the transporters overlaps more with the peptide specificity of some allomorphs than others. On the other hand, it appears that A^a produced in cim^b homozygous cells is functional,[45] and presumably binds peptides. This suggests that the cim gene product interacts more directly with class I molecules to alter their conformation, perhaps by altering the distribution of newly synthesized A^a molecules in the pre-GC region of cells thereby favoring the binding of a unique set of peptides or, perish the thought, even by directly binding to A^a molecules as a (very) immunodominant antigenic peptide.

There is also limited evidence for control of class I assembly by a gene(s) located in the class I region of H-2. During study of transgenic mice expressing HLA-B27, it was noted that the cell surface expression of B27 varied between strains of inbred mice. Using recombinant inbred mice, it was found that expression levels segregated with the D end of H-2 (Nickerson *et al.*, 1990). Based on limited biochemical evidence, it appeared that diminished B27 surface expression results from impaired assembly of α chains with β_2m. At this stage, the primary importance of these findings is to serve notice that regulation of class I assembly may involve additional unsuspected MHC gene products lurking in the early secretory pathway.

[45] It should be noted, however, that the effect of cim alleles on the presentation of foreign determinants has not been examined yet.

4. *Induction of Class I Transport by Exogenous Peptides or Hypothermia*

In the initial description of the antigen presenting properties of RMA/S cells, the most significant finding in many aspects was that incubation of cells with high concentrations of antigenic peptides enhanced the surface expression of class I molecules that bind the peptide (Townsend *et al.*, 1989). Under these conditions, it appeared that transport of newly synthesized molecules was enhanced, as greater amounts of endo H-resistant-D^b were recovered from detergent lysates of pulse-radiolabeled cells using an α3-specific mAb [28-8-14] that was believed to be detect all forms of D^b. Moreover, peptide enhancement was partially blocked by BFA. Based on these results it was suggested that peptides were transported to a premedial GC compartment, where they induced assembly and transport of class I molecules.

If enhanced surface expression was based solely on this mechanism, however, the effect of BFA should have been complete, as by the extremely sensitive measure of antigen presentation, it completely blocks transport of peptide–class I complexes to the cell surface (Nuchtern *et al.*, 1989; Yewdell and Bennink, 1989). The likely answer to this paradox comes from an apparently unrelated experiment. While examining the effect of temperature on the degradation of class I molecules in RMA/S cells, it was serendipitously noticed that after culture of cells at temperatures between 20 and 31°C (optimally at 26°C), class I expression at the cell surface was considerably enhanced, as detected by cytofluorography (Ljunggren *et al.*, 1990; Schumacher *et al.*, 1990). Immunoprecipitation of surface radioiodinated class I molecules confirmed that far greater amounts of immunoreactive class I molecules were expressed on RMA/S cells (and to a lesser extent on RMA cells) following incubation of cells for 48 hours at 26°C. Incubation of detergent lysates for 60 minutes at various temperatures prior to immunoprecipitation revealed that class I molecules from RMA/S cells cultured at 26°C began to lose immunoreactivity at temperatures between 14 and 21°C. Similarly, when incubated at 37°C, cells cultured at 26°C rapidly lost reactivity (approximately 60-minute half-time) with class I molecule-specific mAbs (including the 28-8-14). The thermal denaturation of class I molecules in detergent lysates or on viable cells was completely blocked by the addition of antigenic peptides. Low-temperature incubation did not, however, enhance the ability of influenza virus-infected RMA/S cells to present influenza

nucleoprotein to T_{CD8+}. These findings led to a number of important conclusions:

1. At least part of the enhanced expression of class I molecules observed by culturing cells in the presence of peptides is due to stabilization of loosely associated α chain–β_2m complexes that are exported to the cell surface.
2. Functional class I molecules in RMA/S cells are irreversibly lost (either denatured, shed, or degraded) shortly after they arrive at the cell surface at 37°C.[46]
3. Hypothermia does not ameliorate the antigen processing defect in RMA/S cells, but rather favors the native conformation of class I molecules, thereby stabilizing class I molecules exported to the cell surface and possibly increasing the number of newly synthesized class I molecules that are exported by hoodwinking the control machinery.
4. As similar if less dramatic effects were observed with cells not deficient in antigen processing, these conclusions also apply to a subset of class I molecules in normal cells.[47]

An experiment in which RMA/S cells were cultured with class I molecule-specific mAbs for up to 24 hours at 37°C provides further evidence that class I molecules are constitutively transported to the cell surface in this cell line (Ortiz-Navarrette and Hämmerling, 1991). Coincubation of cells with mAbs specific for the $\alpha 1\alpha 2$ domains of either D^b or K^b resulted in enhanced expression of the relevant class I molecule. The magnitude and time course of enhancement were similar to those observed with antigenic peptides in side-by-side experiments.[48] The abilities of class I-specific mAbs to enhance class I ex-

[46] The partial effect of BFA on peptide enhancement of class I expression, then, reflects, at least in part, the requirement for ongoing class I delivery to the cell surface, as the steady-state levels of class I molecules capable of binding peptides are low relative to the delivery rate.

[47] Dermatologists take note. These findings suggest that greater amounts of unstable class I molecules will be expressed on the surface of cells in the skin, where the ambient temperature in most areas is less than 31°C. As these molecules appear to have an increased capacity to bind exogenous peptides, this implies that cells located in the skin might have enhanced capacity to present peptides. Could this be involved in the sensitivity of skin graft rejection relative to rejection of other tissues, or the seemingly magnified skin manifestations of autoimmune dyscrasias?

[48] In the peptide experiments, it was also shown that enhanced class I expression induced by a high-affinity nonameric peptide decayed more slowly than that induced by a longer, low-affinity peptide after peptides were removed. This supports the validity of

pression appeared to be related to their fine specificities, as the 28-14-8s mAb specific for the $\alpha 3$ domain of D^b, or a mAb specific for β_2m was able to enhance class I expression only marginally. As $\alpha 1\alpha 2$-specific antibodies are generally more specific for native, presumably peptide-containing molecules, this suggests that antibody enhancement of class I expression requires the antibody to freeze class I molecules in a conformation similar to that of peptide-bound molecules.

In the same study, lactoperoxidase-catalyzed radioiodination was used to monitor the fate of cell surface K^b molecules. After immunoprecipitating class I molecules from detergent extracts with a conformation-dependent mAb, remaining denatured K^b molecules were immunoprecipitated with anti-exon 8 antiserum (described in Section II.E.2.a). Only denatured K^b molecules were recovered from RMA/S cells cultured at 37°C. Culturing cells at 25°C greatly increased the amount of both native and nonnative K^b recovered. Consistent with the finding from cytofluorgraphic analysis, the amount of native K^b recovered from 25°C incubated cells decreased with a half-time of approximately 15 minutes if cells were shifted to 37°C prior to radioiodination. The amount of denatured K^b was enhanced to a similar extent as the decrease in native K^b, suggesting a product–precursor relationship. Somewhat surprisingly, a similar analysis of RMA cells cultured at 37°C revealed that far greater amounts of nonnative K^b were present on the cell surface than native K^b, and that this ratio was greatly reduced by incubating cells with a K^b binding peptide. This provides additional evidence that a sizable subset of class I molecules produced in normal cells are similarly unstable as the majority of class I molecules produced by RMA/S cells. As these molecules are stabilized by addition of peptides, it appears fairly certain they either lack peptide or bind to peptides in a low-affinity manner.[49] The expression of such nonnative class I molecules on live peripheral blood lymphocytes has been shown using a mAb that reacts only with nonnative HLA class I molecules (Madrigal *et al.*, 1991; Schnabl *et al.*, 1990). Thus, the existence of such molecules is not peculiar to tissue culture cell lines and is not strictly an artifact associated with biochemical manipulations.

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conclusions regarding the assembly of class I molecules made from studies of the behavior of metabolically radiolabeled class I molecules in detergent extracts.

[49] Note that although it is generally presumed that such low-affinity binding results from the properties of the peptide (improper length or composition), it is feasible that the "right" peptide is bound in the improper manner.

The observation that greater amounts of nonnative class I molecules were recovered from RMA/S cells incubated at 25°C than 37°C is consistent with the idea that low temperatures enhance the amount of class I delivered to the cell surface. As it is clear, however, that class I molecules are transported to the cell surface at 37°C, it is also possible that some, or indeed all, of the observed increase in the amount of native molecules after 25°C incubation results from diminished degradation or shedding. It should be possible to distinguish between these possibilities by measuring the amount of class I molecules shed into the medium, and by measuring the effects of agents that interfere with lysosomal protein degradation, where such molecules presumably would be degraded.[50]

An additional observation indicating that peptide induction of class I molecules on RMA/S cells is largely if not entirely a cell surface phenomenon is that the expression of D^b at the surface of cells incubated with antigenic peptide is not enhanced unless β_2m is present in the medium (Rock *et al.*, 1991b). As detailed in Section III.A.2, β_2m has been observed to enhance greatly the association of peptides on normal as well as mutant cells. Surprisingly, the induction of class I expression by 25°C incubation was also largely dependent on binding of β_2m present in the culture medium (Rock *et al.*, 1991b). This was shown in two ways. First, cells incubated at 25°C in medium with fetal bovine serum failed to demonstrate enhanced binding of mAbs specific for mouse β_2m or with mAbs whose binding to α chains requires the presence of mouse β_2m. Second, only minor increases in class I expression occurred when cells were incubated in serum-free medium, unless the medium was supplemented with bovine β_2m. These findings indicate that the increased expression of class I molecules at 25°C in RMA/S cells is not attributable solely to the increased stability of mouse class I molecules at this temperature. As with the other studies, however, it is uncertain to what extent the binding of bovine β_2m to class I molecules reflects enhanced transport of unstable mouse class I molecules at 25°C versus an enhanced ability to bind β_2m at

[50] A potentially critical observation made in the surface radioiodination experiments regarding the nature/fate of denatured class I molecules is that two unidentified proteins (M_r 67 and 110 kDa) were coprecipitated with K^b by the anti-exon 8 antiserum. These could represent molecular chaperones involved in the disposal or resurrection of the proteins. The major coprecipitate (67 kDa) might, however, represent K^b that is covalently bound to β_2m. Such complexes migrating with a similar M_r (62 kDa) were previously detected in similar circumstances (Bushkin *et al.*, 1986); their significance has proven elusive.

25°C, either because the life span of free nondenatured α chains increases or their affinity for β_2m is enhanced.

5. *Summary of Cellular Control Mechanisms*

It appears that two mechanisms control the export of class I molecules from the early exocytic pathway: the first operating to retain free α chains, and the second to retain β_2m–α chain complexes that have not bound peptide. The first mechanism is less specific, in that it operates on xenogeneic α chains (indeed, even in frogs), and probably recognizes features of free α chains common to unfolded proteins. The second mechanism looks to be species specific, and might be devoted to class I molecules and perhaps other proteins with immunoglobulin-like folding. The p88 protein that complexes with class I molecules seems to play a critical role in this second mechanism. The extent to which p88 acts with other proteins remains to be established, as does the role of p88 in retaining free α chains. It is also unclear whether other proteins can act in the absence of p88 to retain assembled complexes. The effect of temperature on the efficiency of the retention mechanisms remains to be established, and it will be important, if difficult, to determine the extent to which any such effects reflect induction of conformational alterations in class I molecules toward the native state versus alterations in the retention mechanism per se.

It is clear that the expression of unstable class I molecules is not limited to mutant cells, and that normal cells have a considerable amount of free α chains. This suggests that the mechanisms that regulate class I export might be relatively inefficient. This is obviously true in some cell lines, as it is extremely unlikely, for example, that D^b transported to the surface of β_2m-deficient cells has any functional antigen presentation capacity. This does not necessarily mean, however, that all of the free heavy chains present on the cell surface result from inefficient monitoring of class I conformation, as many of these molecules may derive from complexes with low-affinity peptides. Such complexes, while never reaching high steady-state levels, could persist for sufficient times to enable T_{CD8+} recognition. In this case, there would clearly be an evolutionary advantage for cells not to limit the export of class I molecules to the subset containing high-affinity peptides. Thus, there might be some imperfection purposely built into the system, either in the monitoring mechanism or in the ability of class I molecules not containing high-affinity peptides to mimic the conformation of stably assembled complexes. To resolve these issues more information is required regarding (1) the lengths of peptides provided to class I molecules in the early part of the exocytic

pathway [and the location of enzymes [if they exist] that trim peptides bound to class I molecules], (2) the effects of peptide affinity on class I conformation, and (3) the nature of class I molecule conformation monitoring mechanisms.

6. *Viral Subterfuges*

One of the major functions of T_{CD8+} is to eradicate virus-infected cells. In response, a number of viruses evolved mechanisms that block the expression of class I molecules. In some cases this is achieved by decreasing transcription or translation of class I mRNA (reviewed in Brown *et al.*, 1988). There are at least two examples, however, where viruses have evolved proteins that interfere with class I transport or assembly. The most extensively characterized example is the adenovirus E3/19K glycoprotein, which, as described in Section II.E.1.b, retains newly synthesized class I molecules in an early exocytic compartment and is capable of blocking presentation of cytosolic antigens *in vitro*.

Although it would be expected that E3/19K would function similarly *in vivo*, direct evidence for this is lacking. Despite the fact that E3/19K blocks presentation of viral determinants associated with certain mouse allomorphs (Cox *et al.*, 1990; Rawle *et al.*, 1989, 1991), E3/19K expression does not affect the generation of T_{CD8+} responses restricted to these allomorphs following immunization of mice with either adenovirus (Rawle *et al.*, 1991) or a recombinant vaccinia virus containing the E3/19K gene (J. Cox and G. Karupiah, personal communication). Although not inconceivable, it seems unlikely that the antigen presentation blocking activity of E3/19K is not its primary or sole function. Studies performed in cotton rats suggest that E3/19K serves to lessen the severity of the adenovirus infection, possibly by decreasing the magnitude of the T_{CD8+} response. Thus, the failure to observe effects on T_{CD8+} responses in mice probably reflects the artificial aspects of infecting mice with a human virus. To fully appreciate the function of E3/19K *in vivo* requires studies of humans infected with physiological doses of one or another adenovirus differing only in the capacity to produce E3/19K.[51]

The interaction of E3/19K with class I molecules has been examined using chimeric class I molecules produced from gene segments derived from K^d (which binds E3/19K) and K^k (which does not). E3/19K binds to a class I molecule consisting of the K^d $\alpha 1\alpha 2$ domains, with the

[51] As adenovirus is one of the few viruses still routinely administered to humans in infectious form, this experiment might actually be feasible.

rest of the molecule derived from K^k (Burgert and Kvist, 1987). This suggests that E3/19K interacts with the luminal domain of class I, a conclusion first inferred by the ability of class I to interact with a truncated E3/19K molecule in which the membrane spanning and cytosolic domains were deleted (Pääbo *et al.*, 1986). Analysis of additional chimeric molecules indicated that E3/19K could not bind class I molecules consisting of either the $\alpha 1$ or $\alpha 2$ domains derived from K^d (Burgert and Kvist, 1987), but could bind a molecule in which the $\alpha 1$ domain and first half of the $\alpha 2$ domain were derived from K^d (Jefferies and Burgert, 1991). This molecule differs by five residues in the $\alpha 2$ domain (residues 99, 102, 114, 116, and 121) from another chimeric molecule that does not bind E3/19K. These residues might directly contact E3/19K or might influence the overall conformation of class I molecules. As E3/19K is able to bind α chains in the presence or absence of β_2m (Lévy and Kvist, 1991), it seems unlikely that subtle conformation alterations in class I molecules induced by conservative substitutions would influence its interaction with E3/19K. Locating the altered residues in the HLA-A2 crystal structure reveals that residues 99, 114, and 116 have side chains pointing in toward the binding groove. As discussed in Section II.E.1.b, E3/19K probably does not block peptide binding (Cox *et al.*, 1991); thus, it is unlikely that E3/19K contacts residues in the antigen binding groove. This would suggest that residue 102 or 121 (or both) contact E3/19K. As residue 121 contacts β_2m in HLA-A2 (Bjorkman *et al.*, 1987a), and E3/19K does not displace β_2m from α chains, it seems most likely that residue 102 (located in the $\alpha 2$ domain) contacts E3/19K. The observation that E3/19K requires the K^d $\alpha 1$ domain in addition to the amino-terminal half of the $\alpha 2$ domain indicates that E3/19K probably also contacts residues in the $\alpha 1$ domain. Examination of the three-dimensional structure suggests that E3/19K might contact residues near the amino terminus of the $\alpha 1$ domain, which is in close proximity to residue 102, and perhaps residues in the vicinity of residue 24, which differs between K^d and K^k.

Human cytomegaloviruses may have evolved a similar strategy to thwart immune recognition. This virus encodes a protein (M_r 67 kDa) that binds β_2m (Browne *et al.*, 1990), presumably as a result of a homology to class I α chains (Beck and Barrell, 1988). It is presently uncertain, however, whether this actually interferes with antigen presentation or, indeed, even reduces class I assembly or exocytosis. It has been proposed that this protein serves as the virus receptor either by binding β_2m on infected cells or by using bound β_2m to bind to class I molecules (Grundy *et al.*, 1987). Although it has been argued that β_2m

cannot be used as a receptor because it could not be bound simultaneously by class I and the virus (Browne *et al.*, 1990), the viral protein, despite its homology to class I, might bind to β_2m in a manner that allows for a noncompetitive interaction. Only additional experiments will resolve whether the protein serves one or both of these functions.

Adenovirus and cytomegalovirus appear to have adopted similar strategies to escape T_{CD8+} recognition in interfering with class I export or assembly, respectively. These are but two examples of many in which large DNA viruses coopt and modify host genes such that the modified gene product interferes with the function of its progenitor (Kotwal and Moss, 1988). It now appears there are a number of cellular gene products whose function is essential to efficient antigen processing but would not be required for viral replication, or even for cellular viability (in the case of persistently infected cells). It would be surprising, therefore, if viruses have not evolved gene products that specifically interfere with either the production or intracellular transport of antigenic peptides.

G. Regulation of Antigen Processing

To understand T_{CD8+} function *in vivo* and how tolerance to self proteins is achieved, it is critical to know whether antigen processing differs qualitatively or quantitatively among different tissues and cell types. It has been clearly established in tissue culture cells that assembly of class I molecules can be regulated by physiologic environmental alterations. This was first conclusively shown using mouse tumor cells (CMT and BC2) that spontaneously express low levels of endogenous α chains and β_2m, due, at least in part, to low steady-state levels of mRNA (Klar and Hämmerling, 1989). Treatment of cells with interferon-γ greatly enhanced class I mRNA levels and surface expression of assembled molecules. To separate the effects of interferon-γ on class I gene transcription from those on assembly of α chains and β_2m, cells were transfected with genes encoding mouse β_2m and mouse or human class I allomorphs. The transfected genes were expressed at much higher levels than the endogenous genes, and protein expression was only slightly enhanced by interferon-γ treatment. Importantly, assembly of α chains with β_2m, as assessed by coprecipitation of the two chains or binding to conformationally sensitive mAbs, was detected only following interferon-γ treatment. These findings indicate that in these tumor cells at least one of the factors necessary for class I assembly is limiting and inducible with interferon-γ. In a subsequent report, it was shown that immunoprecipitable proteasomes (see Section II.C.2.c), were greatly enhanced in

CMT cells by interferon-γ treatment, indicating that one or more of the proteasome subunits, and by inference the antigenic peptides they might produce, could be one of the limiting factors in class I assembly (Ortiz-Navarrete *et al.*, 1991).

Assembly of D^d in mouse embryonic cells transfected with the D^d gene was also shown to be greatly enhanced by treatment with interferons-α and -β (Bikoff *et al.*, 1991a,b). This could not be attributed simply to increased β_2m expression, as cotransfection with D^d and β_2m genes did not enhance assembly in the absence of interferon, despite an increase in the amount of β_2m synthesized. It was further shown that interferon treatment enhanced expression of the HAM1 gene (one of the H-2-encoded ABC proteins discussed in Section II.D.1) in the one embryonal cell line tested. The induction of HAM1 mRNA appears to be relevant to physiological induction of class I expression in embryonic cells, as its expression (and class I surface expression) was also induced in embryonal cell lines by conditions that induce differentiation. This appears to reflect the situation *in vivo*, where most cells in the early embryo do not express α-chain or β_2m mRNA and would have no reason to express the putative transporter proteins (or antigen processing-specific components of the proteasome, for that matter), if indeed these proteins function only in providing antigens for class I assembly.

These findings establish that antigen processing can be regulated by modulating class I assembly, presumably by controlling levels of the putative transporter and proteasome proteins encoded by the MHC. As class I molecules are easily detected on the surface of most somatic cells, it has long been considered that virtually all somatic cells are able to process and present antigens to T_{CD8+}. The capacity of nonimmune cells to present antigens *in vivo*, however, is poorly characterized, and it is important to stress that the surface expression of class I molecules is no guarantee of a cell's capacity to process and present antigens. It is now abundantly clear that the relevant parameter for presentation of endogenously synthesized antigens is the rate of assembly and transport of class I molecules and not the steady-state level of surface expression. As the rate of turnover of class I molecules on nondividing cells *in vivo* is uncertain, many cell types might replenish surface molecules at a low rate, resulting in relative inefficient processing of self proteins.[52] Moreover, as demonstrated by the various antigen processing-defective cell lines, the assembly and surface expres-

[52] Dividing cells, of course, must synthesize within their time of division at least as many class I molecules existing at the average steady-state level.

sion of class I molecules do not guarantee that the molecules carry antigenic peptides. Thus, it remains to be established to what extent various cell types process and present self proteins to T_{CD8+}, and how this relates to the expression of α chains, β_2m, and the various accessory molecules (known and yet to be discovered) that contribute to antigen processing.[53] It is easy to imagine that a low basal level of antigen processing and presentation in some (or most) cell types contributes to the overall strategy of the immune system in maintaining tolerance to self antigens. It is possible that in many circumstances, even virus-infected nonimmune cells would not efficiently present viral antigens to T_{CD8+} unless they were exposed to interferon. This possibility receives some support from recent findings that cells of macrophage/monocyte lineage are required to develop T_{CD8+} responses to influenza virus (Debrick *et al.*, 1991).

From the practical standpoint, it might be most important to understand the regulation of gene products that contribute to antigen processing and presentation in tumor cells. It has been observed that cells from many different rodent and human tumors exhibit low class I expression (Cabrera *et al.*, 1991; Festenstein, 1987; P. Wang *et al.*, 1991). As described above, mouse tumor cells have been found to be deficient in interferon-γ-inducible factors required for class I assembly. A number of human tumor cell lines have been also found to have a similar phenotype (N.P. Restifo, personal communication). Although low antigen presentation might contribute to the ability of the tumor to grow, it is not clear at present whether this property is selected by tumor-specific T_{CD8+} or is simply a reflection of the antigen presenting capacity of the normal cells giving rise to the tumor.

III. Properties of Cell Surface Class I Molecules

A. Interaction of Exogenous Peptides with Class I Molecules

One of the major discoveries concerning processing and presentation of antigens for T_{CD8+} recognition was the finding that incubating cells with synthetic peptides corresponding to the regions of proteins recognized by T_{CD8+} sensitized the cells for T_{CD8+} lysis (Townsend *et al.*, 1986a). This finding was important both conceptually, in demonstrating the continuous nature of antigens recognized by T_{CD8+}, and

[53] Perhaps the most interesting and important tissue to characterize will be the thymus, where much of the positive and negative selection of T_{CD8+} occurs.

practically, in providing a simple (if often expensive) method of determining the precise sequence recognized.

While affirming the importance of these aspects, the intriguing nature of the phenomenon itself should not be taken for granted. As the antigen processing machinery seems to have evolved to present cytosolic antigens to the immune system, it is not at all obvious why it should also maintain the capacity to present peptides provided from extracellular fluids, nor that this should inevitably follow from the type of mechanism used to present intracellular antigens. There are two central questions in the presentation of exogenous peptide antigens: (1) In which compartment does association occur? (2) Does this involve displacement of endogenous peptide, β_2m, or both components.

1. Site of Exogenous Peptide Association with Class I Molecules

Exogenous peptides could potentially interact with class I molecules in four compartments: (1) the early intracellular compartment where endogenous peptides likely associate with class I molecules, (2) a later intracellular exocytic compartment, (3) the plasma membrane, (4) an endocytic compartment containing class I molecules internalized from the plasma membrane. Ostensibly supporting the first possibility is the finding that RMA/S cells incubated for prolonged periods in high concentrations of peptides demonstrated increased assembly and "transport" of newly synthesized class I molecules, as detected by immunoprecipitation of endo H-digested class I molecules recovered from detergent extracts of [^{35}S]methionine-labeled cells (Townsend *et al.*, 1989). Subsequently, it was shown that under the conditions employed, cells internalize sufficient amounts of peptides that are subsequently released by detergent extraction to stabilize endo H-sensitive class I molecules, as well as the minor population of endo H-resistant class I molecules that manage to leave the early exocytic compartment (Townsend *et al.*, 1990). Although this does not prove that all of the effects of peptides occurred after detergent extraction, there is as yet no evidence to the contrary. Further, the fact that such effects occur only with very high doses of peptides means that lower concentrations of peptides (but still oversaturating in ability to sensitize cells for T_{CD8+} lysis) do not associate with class I molecules in a manner similar to endogenous antigens.

Experiments in which cells have been treated with aldehydes to block metabolic activity clearly demonstrate that peptides can associate with class I molecules located at the plasma membrane. Cells fixed by 15-second incubation with 0.05 percent glutaraldehyde presented an ovalbumin peptide to T_{CD8+} as efficiently as unfixed cells, as mea-

sured by a standard chromium-51 release assay (Hosken *et al.*, 1989). This treatment reduced protein synthesis by more than 99 percent, and prevented presentation of ovalbumin delivered to the cytosol. It is uncertain, however, to what extent the exocytic and endocytic pathways maintained function, as this was not examined. Cells fixed with 0.5 percent paraformaldehyde for 15 minutes and then incubated with saturating amounts of an influenza virus nucleoprotein peptide were equally efficient as unfixed cells at blocking lysis of labeled influenza virus infected cells by nucleoprotein-specific T_{CD8+} (Yewdell and Bennink, 1989). The effect of this fixation treatment on cells was not characterized, but using the identical protocol, it was previously reported that a different cell line was unable to process intact protein antigens for recognition by class II-restricted T cells (Eisenlohr *et al.*, 1987), which suggests that this treatment prevents the endocytic pathway from functioning. Using higher concentrations of paraformaldehyde to treat cells (1% for 10 minutes), it was found that fixed cells actually presented ovalbumin and nucleoprotein peptides at 30- to 100-fold lower concentrations than untreated cells to the appropriate T_{CD8+} hybridoma cells,[54] when antigen presenting cells and T_{CD8+} were co-cultured for 18 hours in the presence of peptides (Rock *et al.*, 1992). Similar findings were made when live or fixed antigen presenting cells were exposed to peptides for 3 hours and then washed before being added to T_{CD8+}, demonstrating that decreased presentation by live cells was not due to their destruction or sequestration of peptides. Using radioiodinated peptides, it was further demonstrated that more peptide bound to immunoprecipitated class I molecules derived from fixed cells than live cells. Although the metabolic activity of these cells was not characterized, it is likely that the cells were capable of little, if any, endocytic or exocytic activity.

The interaction of radiolabeled peptides with live cells was investigated in two prior reports in which binding was quantitated following immunoprecipitation of class I molecules from detergent lysates. Using a K^d-binding peptide coupled to a radiolabeled photoaffinity probe, it was found that labeling of K^d was inhibited by either low temperature [4°C versus 37°C] or by treatment of cells with azide, cycloheximide, or BFA (Luescher *et al.*, 1991). Similarly, binding of radioiodinated peptides to HLA-B27 or HLA-Aw68 molecules was

[54] Using hybridoma cells, it was possible to measure target cell recognition by the release of interleukin-2 from the hybridoma cells. This is a much more reproducible and sensitive assay than unlabeled target cell inhibition. It is limited, however, relative to chromium-51 release assays, in providing no information regarding the percentage of cells capable of presenting antigen.

inhibited by treating cells with cycloheximide or BFA, and enhanced by treating cells with interferon-γ (Benjamin *et al.*, 1991). Although the mechanisms by which these various treatments interfere with peptide–class I association are not established, and may differ, all would diminish the delivery of newly synthesized class I molecules to the cell surface. This would seem to conflict with earlier reports that BFA does not inhibit the ability of cells to present peptides to T_{CD8+} (Nuchtern *et al.*, 1989; Yewdell and Bennink, 1989). In these studies, however, peptides were used only at saturating doses, and even relatively large effects of BFA on the amount of peptides required for target cell sensitization would not have been detected.

The negative effects of hypothermia and drugs on peptide binding to class I molecules have two interpretations: either they block delivery of the peptide to intracellular class I molecules, or they block the delivery of intracellular class I molecules to the peptides. As discussed above there is as yet no compelling evidence that peptides associate with intracellular class I molecules. By contrast, it has been amply documented using different approaches that a population of class I molecules transported to the surface of cells with normal antigen processing capacity is conformationally unstable and rapidly denatures. Furthermore, the enhanced binding of peptides to class I molecules from paraformaldehyde-fixed cells demonstrates that increasing the stability of class I molecules increases their peptide binding capacity, as paraformaldehyde was found to increase the stability of class I molecules, as assessed by the binding of conformation-specific antibodies to RMA/S cells and the presence of α chain–β_2m complexes observed in SDS–PAGE following reduction and boiling of immunoprecipitates derived from detergent lysates (Rock *et al.*, 1992).

Taken together, these findings are consistent with the idea that exogenously added peptides associate with class I molecules located in the plasma membrane, particularly those recent arrivals lacking high-affinity peptides that require exogenous peptides to prevent irreversible denaturation. While not eliminating the possibility that peptides associate with class I molecules in intracellular endosomal or exocytic compartments, there is as yet no direct evidence that such association is relevant to presentation of peptides to T_{CD8+} or, indeed, occurs even after incubating cells in high concentrations of peptides.

2. *Mechanism of Peptide Binding to Cell Surface-Associated Class I Molecules*

There are three ways that exogenous peptides could potentially associate with class I molecules at the cell surface: (1) The β_2m–α

chain complex remains intact as exogenous peptide displaces endogenous peptides. (2) Exogenous peptide binds weakly to noncomplexed α chains and is locked in by binding of free β_2m. (3) Exogenous peptide binds to "empty" β_2m–α chain complexes.

Using T_{CD8+} specific for ovalbumin or influenza virus nucleoprotein in association with K^b or D^b, respectively, it was found that at relatively low concentrations, peptide sensitization of target cells required β_2m, whereas at high peptide concentrations, β_2m was not required (Rock *et al.*, 1990b, 1991a). In the former case, sensitization was not affected by incubation of cells with azide or ricin; in the latter case it was completely blocked. β_2m-dependent sensitization using ovalbumin peptides was blocked by antibodies specific for the type of exogenous β_2m present in the extracellular medium, providing important functional evidence that exogenous β_2m constituted part of the complex recognized by T_{CD8+}. These findings suggest that peptides can associate by at least two mechanisms, one of which requires exogenous β_2m and is not affected by metabolic inhibitors, and the other, less efficient in terms of the concentration of peptide, that does not require β_2m but does require high cellular ATP levels and protein synthesis.

Using cells grown in serum free medium to ensure the absence of exogenous β_2m from the cell surface or endosomal compartments, a similar requirement for exogenous β_2m was observed using K^d- or D^b-restricted, influenza virus nucleoprotein specific T_{CD8+}(Vitiello *et al.*, 1990). Some β_2m-independent presentation of peptide was also observed, but it was not determined whether this presentation was more sensitive to metabolic inhibitors. When cells deficient in β_2m biosynthesis were propagated in serum free medium, they were completely unable to present peptides in the absence of exogenous β_2m, whereas cells propagated in serum free medium supplemented with β_2m behaved similarly to β_2m synthesizing cells. Pretreatment of β_2m-deficient cells with β_2m allowed the cells to present a D^b-restricted peptide added in the absence of β_2m, whereas the opposite was not true. This establishes that at least one class I allomorph synthesized in the absence of β_2m has the capacity to recover its native conformation even after reaching the plasma membrane.[55] Importantly, β_2m-deficient cells grown in β_2m-containing medium still did not detectably present endogenously synthesized antigens. This demonstrates

[55] Note that D^b is somewhat peculiar among class I allomorphs in being transported to the cell surface relatively efficiently in the absence of β_2m, possibly because its folding is less dependent on complex formation. It is important to determine to what extent this property extends to other mouse allomorphs and human class I molecules.

that free α chains do not carry antigenic peptides to the cell surface, at least not in a way that can be stabilized by the subsequent addition of β_2m.

β_2m was also found to enhance presentation of a D^d-restricted peptide, using either live cells or purified D^d attached to polyvinyl (Kozlowski *et al.*, 1991). The enhancement of antigen presentation paralleled the binding of β_2m to D^d when the concentration of β_2m was varied. Similar experiments with purified D^b, K^d, and K^b molecules revealed that as with live cells, enhancement of peptide presentation was achieved by preincubation of class I molecules with β_2m, although maximal effectiveness was achieved when β_2m and peptides were simultaneously present (Kane *et al.*, 1991).

Taken together, these findings indicate that exogenous β_2m can participate in the formation of class I complexes containing exogenous peptides and, indeed, appears to be present in the majority of such complexes produced in an energy-independent manner. It is likely that in these circumstances, peptides bind to empty β_2m–α chain complexes, as cells can be pretreated with β_2m prior to the addition of peptides. This conclusion is supported by the observation that T2 cells transfected with mouse α-chain genes, which presumably express empty mouse class I molecules, present peptides in a β_2m-independent manner, even when cultured in the absence of β_2m (Vitiello *et al.*, 1990). The energy-dependent association of peptides, as discussed in the preceding section, could represent peptide association with recently exported surface molecules or, less likely, newly synthesized intracellular class I molecules. In the former case, this could represent substitution of a weakly bound endogenous peptide by the exogenous peptide. In the absence of this substitution, these complexes would rapidly dissociate, resulting in free α chains that require exogenous β_2m to bind peptide. This scenario is supported by the observation that paraformaldehyde-fixed cells present exogenous peptides more efficiently than nonfixed cells in the absence of β_2m (Rock *et al.*, 1992), presumably because such empty complexes are stabilized by paraformaldehyde.[56] It would also explain the enhanced presentation of peptide observed when cells are incubated with an anti-HLA mAb prior to the addition of peptide, if by stabilizing class I structure, this antibody increased the number of empty complexes (Bodmer *et al.*, 1989).

Thus, it appears that exogenous peptides bind predominantly to

[56] Note that peptide dissociation could occur after fixation, contributing to the number of empty complexes, which at any one time might be expected to be few in number.

empty molecules either that transiently exist following the arrival of class I molecules at the surface that are devoid of peptide or contain low-affinity peptides or that can be formed by the binding of exogenous β_2m to free α chains. Empty molecules formed in these two circumstances might differ in peptide specificity and stability. If so, this could account for the different efficiencies of the complexes in presenting peptides. Alternatively, this difference might simply be a reflection of the balance of empty complexes produced by the two pathways.

It is noteworthy that studies performed to date documenting the requirement for exogenous β_2m used peptides longer than the naturally derived, highest-affinity form.[57] It is possible that the highest-affinity peptides will not demonstrate this dependence, as it is logical that they would more efficiently displace endogenous peptides. Also, as only these peptides have been demonstrated to bind to free α chains in detergent extracts devoid of β_2m (discussed in Section II.E.2.b), it might be possible to demonstrate enhanced presentation if they were used to pretreat cells later incubated with β_2m.

3. *Extracellular Processing of Synthetic Peptides*

In extending their studies of the β_2m requirement for peptide binding to class I molecules, Sherman and colleagues tested the ability of β_2m-deficient serum to enhance peptide sensitization (Sherman *et al.*, 1992). Although this serum failed to enhance the activity of two of the peptides tested, it did enhance presentation of two other peptides. One of these peptides consists of the natural K^d-associated determinant from nucleoprotein (147–155) plus two amino acids corresponding to nucleoprotein residues 157 and 158 (the missing residue is arginine, hence the peptide is referred to as 147–158R$^-$). Using HPLC to purify 147–158R$^-$, it was found that the residual antigenic activity obtained in the absence of serum was due to contaminating species. Incubation of HPLC-purified 147–158R$^-$ with serum resulted in the production of two new species detected by HPLC. One of the species was highly active antigenically, and proved to be the 147–155 peptide; the other species consisted of the cleaved dipeptide. The serum dipeptidase is likely to be angiotensin converting enzyme which is known to be present in serum, as angiotensin converting enzyme greatly en-

[57] At least the investigators thought they were using longer peptides. It is possible, however, that the antigenically active species was actually an octameric or nonameric contaminant present in the preparation or produced by serum or cellular proteases (described in the next section).

hances the antigenicity of 147–158R$^-$ in the absence of serum, and angiotensin converting enzyme inhibitor blocks the enhancing effect of serum. Angiotensin converting enzyme did not enhance the potency of an influenza virus nucleoprotein peptide, whose activity is also enhanced by serum, suggesting that serum contains additional proteases (or possibly other factors) that enhance peptide presentation.

These findings provide a clear demonstration that serum proteases can cleave exogenous peptides. At the very least, this serves as an important warning that the antigenicity of exogenously added peptides can be influenced by such proteases. It also underscores the possibility of peptide processing by peptidases secreted by cells or associated with the cell surface. More relevant, perhaps, to antigen presentation *in vivo*, as cells are normally bathed in 100% serum or plasma, these findings raise the possibility that exogenous proteases trim some class I bound peptides to optimal size, raising the specter that the final cleavage step in the production of some natural peptides occurs at the cell surface.

4. *Physiological Relevance of Exogenous Peptide Association*

Defining the site and mechanism of exogenous peptide association with class I molecules is clearly an interesting intellectual exercise. Its importance, however, ultimately rests with its physiological relevance. At present it is unclear whether such association ever occurs *in vivo*. It has been argued that the low concentration of β_2m in serum would disfavor such interactions, and that class I molecules evolved such a loose association of the two chains to prevent cells from presenting foreign peptides derived from other cells (Rock *et al.*, 1991d). Although this could well be true in most circumstances, it is possible that the concentration of β_2m or peptides in extracellular fluids might be sufficiently high in localized regions to result in sensitization, particularly in regions of chronic inflammation. In such areas, peptide formation might be favored by high concentrations of extracellular proteases, and complex formation might be favored by the effects of interferons, which would increase the amount of class I molecules on the cell surface and, concomitantly, increase the amount of β_2m released from cells, possibly increasing local plasma β_2m concentrations.

Whether or not such sensitization occurs in normal or pathological circumstances, the presentation of exogenous peptides is clearly relevant to the use of peptide vaccines to induce T_{CD8+} responses. Peptides have been found to induce T_{CD8+} responses in some circumstances, but it is unclear whether this reflects the operation of the exogenous or endogenous routes. It will be interesting, and possibly

important, to examine whether administering β_2m enhances the ability of peptides to elicit T_{CD8+}.[58]

B. Association of Exogenous β_2m with Class I Molecules

The requirement for exogenous β_2m in peptide binding provides a clear demonstration that exogenous β_2m can bind to class I molecules at the cell surface. The binding of exogenous β_2m to cells was first made a number of years before (Bernabeu *et al.*, 1984; Hester *et al.*, 1979; Kefford *et al.*, 1984; Kubota, 1984; Schmidt *et al.*, 1981; Ward and Sanderson, 1983), and substitution of natural β_2m with that of other species has been found to alter the reactivity of class I molecules with a number of mAbs that recognize assembled complexes (Jefferies and MacPherson, 1987; Mierau *et al.*, 1987; Nieto *et al.*, 1989). Studies with β_2m conjugated to fluorescein indicated that β_2m is capable of binding to approximately 2×10^5 class I molecules per cell, which is similar to the number of class I molecules usually detected by mAb binding (Hochman *et al.*, 1988). A similar number of sites on mouse cells was detected using radioiodinated human β_2m (Rock *et al.*, 1991d). Most of the exogenous β_2m (on the order of 90%) associated with cells after a 60-minute incubation appeared to bind to free α chains, as only a slight decrease in surface levels of mouse β_2m was detected. β_2m binding was not affected by treating the cells with azide, suggesting that binding occurred at the cell surface and not in an endosomal compartment. Binding of radioiodinated β_2m to mouse class I molecules was directly demonstrated by immunoprecipitation of β_2m using α chain-specific mAbs. Labeled β_2m was not precipitated by mAbs that either recognize mouse β_2m or require mouse β_2m to bind to class I molecules, indicating that if intermediates consisting of α chains simultaneously complexed to mouse and human β_2m exist, they are either short-lived or unstable following detergent extraction and immunoprecipitation.

The most important implication of these findings is that the quantity of free α chains present on the cell surface is similar to the amount of β_2m–α chain complexes. This not unique to cells in culture, as similar amounts of β_2m were found to bind to freshly isolated splenocytes (Rock *et al.*, 1991c). The presence of free α chains on the cell surface is supported by the binding of the LA45 mAb to the surface of some cells. This antibody binds free HLA α chains but not assembled complexes

[58] Indeed, it has been reported that β_2m binds to mouse peritoneal exudate cells following intraperitoneal injection, which indicates that β_2m association with cell surface class I molecules is not just an *in vitro* phenomenon (Jefferies and MacPherson, 1987).

(Madrigal *et al.*, 1991; Schnabl *et al.*, 1990). Unlike the binding of β_2m, however, the binding of LA45 varied considerably between cell types, reacting well with Epstein–Barr virus transformed B cells and T cells activated by phytohemagglutinin, but not resting T cells or T cells activated by other means. This discrepancy has two general explanations. First, the amount of free α chain may truly vary more among human T cell populations than among mouse tissue culture cells and splenocytes. Second, the binding of β_2m or LA45 to the cell surface may depend on more than just the amount of free α chains. For example, resting T cells might post-translationally modify class I molecules in a manner that blocks bindings of LA45. Alternatively, LA45 might bind only a subpopulation of free α chains that is unable to bind β_2m. A direct comparison of LA45 and β_2m binding to the various cell types would help sort out the various possibilities. It is important to determine whether cells do vary in their surface expression of free α chains for several reasons, not the least of which is that this could provide a useful tool determining the source(s) of free α chains. As discussed above, possibilities include free α chains transported to the surface, empty complexes, complexes with low affinity peptides, and complexes with high-affinity peptides.

C. Internalization of Class I Molecules

1. Evidence for Internalization

The internalization of cell surface class I molecules into endosomes was first described following the crosslinking of class I molecules with anti-class I antibodies (Huet *et al.*, 1980). Similarly, internalization occurs following crosslinking of many different types of cell surface molecules. As internalized molecules, including class I molecules, are often delivered to lysosomes for degradation (Dasgupta *et al.*, 1988), this likely is a mechanism for cells to remove nonfunctional molecules. The main relevance of this pathway for immunologists is that antigens can be efficiently delivered to the class II molecule processing pathway if they are crosslinked to anti-class I molecule antibodies (for review, see Lanzavecchia, 1990).

More important to the natural function of class I molecules is their spontaneous internalization. It was first established that class I molecules are spontaneously internalized by activated T cells, but not B cells or L929 cells (Tse and Pernis, 1984), as detected by immunofluorescence staining of intracellular vesicles containing class I molecules. The endosomal nature of this compartment (as opposed to the exocytic nature) was demonstrated by the presence of class I molecules labeled

at the cell surface with class I antibodies. Staining of this compartment was reduced by 70% following 3-hour incubation with cycloheximide to block protein synthesis. Under these conditions, antibody-bound cell surface class I molecules were still delivered to the compartment, but at a reduced rate, suggesting that the effect of cycloheximide reflects a general requirement for protein synthesis for efficient operation of the internalization process. As the definition of the endosomal nature of this compartment depended on the use of antibody-labeled class I molecules, it was important to demonstrate that the compartment also existed without exposing the cells to antibodies. This was accomplished using fluorescein-conjugated β_2m, which, following incubation of cells at 37°C, colocalized to an intracellular compartment stained by an anti-class I molecule mAb (Hochman *et al.*, 1991). Based on ultrastructural examination of fixed mouse T cells indirectly stained with an anti-K^k mAb, followed by gold-conjugated anti-immunoglobulin antibodies, it appears that at least some of the class I molecules are internalized via coated pits, which are thought to be exclusively used by cells for receptor-mediated endocytosis (Machy *et al.*, 1987).

Spontaneous internalization of class I molecules has also been shown using biochemical techniques. By incubating cells with class I molecule-specific antibody at 0°C and washing the cells prior to detergent extraction, it is possible to selectively immunoprecipitate cell surface class I molecules from detergent extracts. Subsequent addition of antibody to extracts depleted of surface molecules in this manner identifies the intracellular pool of class I molecules. Using this method, it was found that approximately 10% of class I molecules in a human T leukemia cell line surface radioiodinated at 0°C were present intracellularly once steady-state conditions were reached between 40 and 60 minutes after incubation at 37°C (this method was also used to assess internalization signals in HLA-A2, described in the next section) (Vega and Strominger, 1989). Class I molecule internalization was also detected using a radioiodinated probe that can be selectively added and removed from cell surface proteins. When used to label a human B lymphoblastoid cell line at 0°C, it was found that 3% of class I molecules were internalized following 30 minutes of incubation at 37°C (Reid and Watts, 1990). The amount of internalized class I molecules was increased to 20% by incubating cells with primaquine, which is known to slow the recycling of receptors such as transferrin that continuously cycle between the surface and endosomal compartments. Using primaquine-treated cells, it was determined that class I molecules were internalized with a half-time of approximately

35 minutes. Importantly, it was shown that class I molecules accumulated intracellularly in the presence of primaquine were transported to the cell surface either in the presence of primaquine or after its removal. This represents the first evidence obtained in any of the systems used that the intracellular pool of class I molecules is recycled and not simply degraded in endosomes or lysosomes.

These two biochemical studies clearly demonstrate that class I molecules can be located in a compartment inaccessible to membrane impermeant reagents. As intracellular molecules were recovered in both studies with the W6/32 mAb, it is clear that stably assembled class I molecule are internalized. Despite this, it is still possible that internalization is restricted to class I molecules damaged by labeling procedures. To more firmly establish the validity of these results for native class I molecules, it will be important in future studies to correlate the intracellular pool sizes observed in biochemical studies with those observed by immunofluorescence using cell types with differing amounts of intracellular class I molecules. If, as seems likely, the biochemical findings are an accurate means of measuring class I recycling, it will be important to determine whether class I recycling occurs in all cell types or only lymphoid cells, and to what extent this is affected by physiological alterations in antigen processing induced by virus infections and interferon treatment, for example.

2. *Internalization Signals*

a. Region of Protein

Studies of receptor endocytosis suggest that a limited region of the cytoplasmic domains of membrane proteins, consisting of four to six residues with a propensity to form tight turns, confers the ability to be rapidly internalized (Trowbridge, 1991). A somewhat homologous region is present in HLA-A2 (Vega and Strominger, 1989), and there is evidence consistent with its playing a role in internalization. The cytoplasmic domain of HLA-A2 consists of 1, 16, 11, and 5 residues encoded by exons 8, 7, 6, and 5, respectively. It was found that A2 molecules without exon 6 are internalized by human T leukemia cells, whereas those missing exon 7 are not (Vega and Strominger, 1989). The authors noted that five of the residues in exon 7 were similar to regions of receptors known, or thought to influence internalization. Somewhat different results were obtained using mouse cells expressing wild-type L^d molecules, or genetically altered L^d molecules either missing the entire 31-residue cytoplasmic tail or having the carboxy-terminal 24 residues replaced by 18 unrelated residues (Capps *et al.*, 1989). Constitutive L^d internalization was not diminished by loss or replacement

of the cytoplasmic residues. It was observed, however, that while wild-type L^d internalization was increased by treatment of cells with phorbol myristic acetate (which has been observed to affect internalization of receptors), internalization of either of the mutants was not enhanced. The discrepancy between the results with A2 and L^d might be related to the differences between the cell types studied. Perhaps more likely, however, is that the differences are related to differences in the methods used to measure internalization. Although internalized A2 was measured biochemically as described in the preceding section, L^d internalization was measured by incubating cells with radioiodinated anti-L^d antibody. Thus, much of the L^d internalized in non-PMA-treated cells may have been induced by antibody crosslinking.

b. Is Phosphorylation Involved?

Class I molecules are known to be phosphorylated post-translationally in serine residues located in the cytoplasmic domain (Pober *et al.*, 1978). A number of findings are consistent with the idea that phosphorylation targets class I molecules for internalization. First, the steady-state levels of phosphorylated molecules represent a fraction of total class I molecules (Loube *et al.*, 1983; Pober *et al.*, 1978). Second, phosphorylated class I molecules are resistant to endo H digestion and neuraminidase treatment, which indicates that mature molecules are phosphorylated. Consistent with this finding, class I molecules retained by E3/19K are not phosphorylated (Lippe *et al.*, 1991). Third, residues encoded by exon 7, but not exon 6, were found to be required for both phosphorylation and internalization (Vega and Strominger, 1989). Finally, PMA-induced internalization of L^d was accompanied by increased L^d phosphorylation in lymphoid cells (Capps *et al.*, 1989).

On the other hand, it has not been established that phosphorylation occurs after delivery of class I molecules to the cell surface, which might be expected if phosphorylation was serving as an internalization signal. Also, it was found that PMA increased L^d phosphorylation in nonlymphoid cells without increasing internalization. At best, this suggests that although phosphorylation may be necessary for internalization, other factors are required. At worst, it is consistent with internalization occurring independent of phosphorylation. Obviously, additional studies are required to establish the functional relevance of class I molecule phosphorylation.

c. Functional Significance of Class I Molecule Internalization

The present findings provide good evidence that class I molecules are internalized by T cells and suggest that this process might also occur in other cells. This could contribute to the amount of free α chains present on the cell surface, as internalized molecules would be expected to traverse compartments with a pH of 5.5 or so. At this pH, β_2m dissociation has been reported to double (Hochman *et al.*, 1991). Alternatively, the low pH might favor the dissociation of weak binding peptides from intact class I molecules. In this case, such empty molecules could represent the energy-dependent, protein synthesis-dependent pool of class I molecules that can be pulsed with peptide in the absence of endogenous β_2m (Rock *et al.*, 1990b, 1991a) (see Section III.A.2), because, as discussed in Section III.C.1, class I internalization also requires energy and, apparently, protein synthesis (Tse and Pernis, 1984).

The functional significance of class I recycling remains to be established. It would seem wasteful for this process to occur for no purpose. It is conceivable that recycling reduces the stability of peptide–class I complexes, allowing cells to avoid immune destruction if a virus infection has been eradicated, for example. It is possible, however, that all surface molecules are recycled at the relatively low rate observed for class I molecules as a by-product of some other necessary function.

That internalization appears to be specifically enhanced in T cells is intriguing. It is possible that this allows T cells to process exogenous antigens for class I association in a manner analogous to the class II processing pathway. It might be predicted that a unique set of determinants would be produced by such processing, meaning that detection would require immunization with T cells exposed to exogenous antigens. Why T cells would want to process exogenous antigens in this manner is baffling. Alternatively, class I internalization might allow T_{CD8+} to separate CD8 from its own class I molecules, as such complexes have been detected when class I molecules and CD8 are expressed by the same cell (Blue *et al.*, 1988; Bushkin *et al.*, 1988).[59]

Given that peptide binding to class I molecules appears to occur in an early exocytic compartment, it would be logical for cells to transport empty class I molecules back to their origins if such recycling required less energy than synthesizing new α chains. Although such a pathway

[59] It must be mentioned, however, that T_{CD4} also internalize class I molecules, indicating either that this explanation is wrong or, less likely, that class I internalization occurs for no good reason on CD8-negative T cells.

in cells has not yet been defined under normal conditions, incubation of cells with BFA results in the redistribution of proteins in the trans-GC to the ER (Chege and Pfeffer, 1990), so it seems possible that class I molecules could travel in such a retrograde fashion if they reached the trans-GC. It has been reported that class I molecules coupled to antibodies visit the trans-GC in cells of monocytic origin (Dasgupta *et al.*, 1988); whether such transport occurs spontaneously is not certain. Two findings provide the slightest hint that surface-to-ER traffic of class I molecules occurs in cells. First, there is some evidence that class I molecules serve as receptors to simian virus 40 (Atwood and Norkin, 1989). Second, exogenous simian virus 40 virions have been detected in the ER of cells (Kartenbeck *et al.*, 1989). Could delivery be mediated by class I molecules?

IV. Class Ib Molecules

A. General Aspects of Structure and Function

In addition to the "classical" class I genes that are known to present peptides from foreign antigens to T_{CD8+}, numerous other loci encoding similar molecules exist. These have been termed class Ib molecules (Flaherty *et al.*, 1990; Strominger, 1989; Stroynowski, 1990; Widack and Cook, 1986).[60] In mice, most of these genes are located telomeric to the D region on chromosome 17, in regions termed Qa, Tla, and Hmt. Although the expression of only a handful of class Ib gene products has been characterized, based on gene organization, some mouse strains would seem to have the capacity to express more than 30 class Ib genes. Far fewer class Ib genes have been detected in humans, but it is uncertain whether this is due to technical difficulties in detection or variability between the individuals in the number of genes.

The structure of class Ib molecules is likely to be very similar to that of class Ia molecules as they bind β_2m, share the same exon organization, and share a number of the residues conserved between class Ia molecules of different species (Stroynowski, 1990). Many class Ib molecules possess the consensus sequence identified for binding of class Ia molecules to CD8 (Potter *et al.*, 1989; Salter *et al.*, 1989, 1990), and a representative class Ib molecule has been shown to bind CD8 (Teitell *et al.*, 1991), supporting less direct evidence for CD8 involvement in T_{CD8+} recognition of class Ib molecules (Aldrich *et al.*, 1988). The

[60] In this context, the classical class I gene products are referred to as class Ia molecules.

expression of several class Ib molecules is enhanced by interferon. On the basis of these similarities, it appears that the function of most of these molecules is to transport peptides to the cell surface for immune recognition. As discussed below, several of the mouse class Ib molecules have been shown to possess the capacity to present synthetic peptides to T cells and, in one case, to present a peptide from an endogenously synthesized cellular protein. Moreover, the viability of mice lacking β_2m (Koller *et al.*, 1990; Zijlstra *et al.*, 1989) indicates that if some class Ib molecules have a nonimmunological function, it is unlikely to be essential for growth or development, unless it does not require β_2m.

There are two important differences between class Ia and Ib molecules that should provide clues to the function of class Ib molecules. First, the tissue distribution of many of the class Ib molecules appears to be more limited than that of class Ia molecules. Second, although there are allelic differences between class Ib molecules derived from different strains, these usually amount to only a few amino acid substitutions.

It is not hard to imagine scenarios involving an antigen presentation function in which a limited tissue distribution would be beneficial. For example, expression of the identical antigen might represent either a normal or pathological condition depending on the tissue. In this case, expression of the antigen presenting class I molecule in the normal situation would have one of two deleterious effects: induction of tolerance to the antigen or stimulation of an immune response to normal tissue. Or, it may simply be more economical to express the class I molecules only in those tissues that express peptides that bind the molecule.

Explaining the limited polymorphism of class Ib molecules is somewhat tricky as the evolutionary selection pressures resulting in class Ia polymorphism are hardly well understood themselves. One obvious possibility, however, is that the types of antigens presented by Ib molecules are less variable than those presented by Ia molecules, representing either self molecules with no need to vary or foreign molecules that cannot vary without loss of function.

Despite their vast numerical superiority to class Ia molecules (at least in terms of loci), much less is known about virtually every aspect of class Ib molecule assembly, transport, and structure. Although studies of class Ib molecules were initially hampered by the lack of immunological reagents specific for individual gene products, with the present genetic techniques, class Ib molecules should be no more difficult to study than their more popular cousins. Furthermore, it

should now be possible to directly identify the types of self peptides bound to class Ib molecules using the techniques devised for class Ia molecules, and to study their requirements for assembly using cells deficient in class I assembly. With these tools, assigning functions to the myriad class Ib molecules could well be akin to "shooting fish in a barrel" as has been suggested (albeit in a slightly different context) (Fischer Lindahl *et al.*, 1991).

B. Characteristics of Individual Class Ib Gene Products

1. Q Region Gene Products

Based on present knowledge, the most interesting feature of the class I gene products encoded by the Q region is that some (Q10, Qa-2, Qb), are secreted by cells. Secretion is based on modification of the carboxy terminus that normally anchors class I molecules to the plasma membrane. At least two and possibly three mechanisms are used by the Q region gene products in the process of secretion. With the recent appreciation of the intricate nature of class I assembly and transport, it is plausible that the modified carboxy termini of Q-region class Ib molecules alter their assembly and transport in some interesting way.

a. Q10

Q10 is the only class I molecule detected in the serum of mice, and is present at levels ranging from 0 (Q10 is not produced by H-2^f mice) to 70 μg/ml in the highest expressing strains (Lew *et al.*, 1986). This is probably due exclusively to secretion by liver cells, as Q10 mRNA is detected only in the liver (Cosman *et al.*, 1982; Kress *et al.*, 1983). The secretion of Q10 results from its lack of a membrane anchor. The Q10 gene has a termination codon located in what normally is the middle of the typical class I transmembrane region; further, a number of the normally hydrophobic residues are replaced with charged residues (Cosman *et al.*, 1982; Mellor *et al.*, 1984). The secretion of Q10 does not depend on liver-specific factors because it is also secreted from L929 cells transfected with the Q10 gene (Devlin *et al.*, 1985). The assembly and transport of Q10 in various cell types have not been carefully characterized, however, and it is possible that liver cells express factors that facilitate its assembly (e.g., a liver-specific peptide).

The function of Q10 is uncertain. One possibility is that Q10 delivers peptides to cells bearing a specific Q10 receptor. Such peptides might be derived from liver or serum. In the latter case, Q10 could serve as a sort of distant early warning system, signaling the immune system that

a pathogen is lurking in regions inaccessible to normal immune surveillance mechanisms. If Q10 acts to bind serum peptides, it might be expected that Q10 is secreted in a peptide-free form. This should be possible to test using antigen processing mutant cells. More directly, it might be possible to recover serum protein-derived peptides from Q10 molecules recovered from serum.[61]

b. Qa-2

Qa-2 molecules were originally defined by alloantisera. The same region of H-2 was found to induce alloreactive cytotoxic T cell responses. Qa-2 antigens are encoded by multiple genes in the Q region; in the H-2^b haplotype Qa-2 molecules include at least two gene products encoded by the Q7 and Q9 genes (Soloski *et al.*, 1988). Apparently all Qa-2 molecules share the property of being attached to the plasma membrane by a glycophosphatidyl anchor in lieu of a peptide anchor. Newly synthesized Qa-2 possesses a peptide anchor that is replaced within minutes of synthesis by the glycophospholipid anchor [glycolipid anchoring of proteins is reviewed in (Cross, 1990)]. It is thought that peptide cleavage may occur simultaneously with glycophosphatidyl addition, in which case Qa-2 would be constantly tethered to the membrane in the ER. In addition to its cell surface form, Qa-2 is secreted by activated (but not resting) T cells (Soloski *et al.*, 1986). Based on the detection of Qa-2 in the culture medium of cells that had been surface radioiodinated, it was proposed that Qa-2 is released from the cell by the cleavage of its glycophosphatidyl anchor (Robinson, 1987), which can be achieved experimentally by incubation of cells with phospholipases. Recently, however, it was shown that in addition to the glycophospholipid anchored form of Qa-2, cells produce an intracellular soluble form of the molecule that is secreted and seems to account for most, if not all, of the secreted Qa-2 detected following [^{35}S]methionine-labeling (Einhorn *et al.*, 1991). This water-soluble Qa-2 might result from a failure to attach glycophospholipid anchor to molecules in which the peptide anchor has been removed. More likely, however, the Qa-2 is produced without an anchor, as Q7/Q9 mRNA has been found to exist in two forms, one of which is lacking exon 5 encoding the membrane anchor sequence (Stroynowski, 1990).

Other proteins with glycophospholipid anchors are known to function in transmembrane signalling, suggesting this function for Qa-2.

[61] Of course many of these proteins are secreted by the liver, so this would not necessarily discriminate whether peptide binding occurred intracellularly or extracellularly.

This would be consistent with observations that its expression, at the cell surface anyway, is limited to cells of hematopoieitic origin. It has been shown that antibody binding to Qa-2 can activate lymphocytes, and that lymphocytes from transgenic mice expressing class I molecules consisting of the Qa-2 $\alpha 1\alpha 2\alpha 3$ domains and class Ia transmembrane and cytoplasmic domains are not activated by this treatment (Robinson *et al.*, 1989). Conversely, T cells from mice expressing a transgene encoding a chimeric molecule consisting of the D^b $\alpha 1\alpha 2\alpha 3$ domains connected to the Qa-2 anchor were activated by a D^b-specific mAb unable to activate T cells from normal mice. These findings suggest that Qa-2 molecules have a role in regulating T cell function. It should be noted, however, that antibody binding to class Ia molecules has also been observed to result in T cell activation (Houlden *et al.*, 1991; Wacholtz *et al.*, 1989), which somewhat clouds the relevance of antibody-induced activation.

The functioning of Qa-2 as a signaling molecule would not necessarily preclude a role for the class I molecule peptide binding site very likely to exist on Qa-2. Perhaps the natural ligand for Qa-2 binds with high enough affinity to trigger activation only when the proper peptides are present in the Qa-2 binding site. This mechanism would allow T cells to regulate their function through the use of various peptides. In considering the possible peptide specificity of Qa-2 , it is worth noting that the Q7b and Q9b gene products that are coexpressed in cells from $H\text{-}2^b$ mice differ at only a single position, residue 165. This residue is in the $\alpha 2$ domain helix that forms one of the sides of the antigen binding site. In HLA-A2, the side chain is oriented away from the site, presumably toward the T cell receptor. This suggests that Q7 and Q9 differ more in their interactions with their natural ligands than in their peptide binding specificity. Only a single report addresses (and then only obliquely) whether the glycophospholipid anchor of Qa-2 molecules affects their peptide binding properties. Using transgenic mice expressing the chimeric Q9-D^b molecules described above, it was found that alloreactive recognition of Q9 was not affected by exchanging the D^b membrane anchor and cytoplasmic tail for the normal glycophospholipid anchor (Mellor *et al.*, 1991). This indicates that Q9 molecules do not require a glycophospholipid anchor to bind the appropriate peptides if, in fact, allorecognition of Q9 is dependent on the presence of a peptide in the binding site. Indeed, if allorecognition requires certain Q9-specific peptides, this would suggest that Q9 binds peptides in the same compartment as class Ia molecules, as the glycophospholipid anchor would not be required to direct Q9 to a special peptide-containing compartment.

c. Qb-1

Qb-1 molecules are defined by alloantisera. Alloreactive T cell responses have not been described. To date the Q4 gene is the only gene known to encode Qb-1 molecules (Robinson *et al.*, 1988). The Q4 gene has a stop codon in exon 5 predicted to result in a shortened carboxy terminus whose function as a membrane anchor is possibly compromised.[62] Consistent with the genetic structure, Q4 is secreted by activated lymphocytes (Soloski *et al.*, 1986) and by 3T3 cells transfected with genomic Q4 DNA. In either cell type, immunoprecipitation from detergent extracts of [^{35}S]methionine-labeled cells demonstrates that Qb-1 is assembled with β_2m and rapidly secreted. To determine whether the soluble form of Qb-1 is derived from a membrane-bound precursor, microsomes prepared from pulse-radiolabeled cells were treated with sodium carbonate to release non-membrane-bound material, and the membrane-bound and -soluble fractions immunoprecipitated. Although all D^b was recovered from the membrane fraction, all Qb-1 was present in the soluble fraction, demonstrating that Qb-1 is synthesized in soluble form. These findings indicate that class I molecules do not require a membrane anchor to be properly assembled and transported. The observation that transport was as efficient in 3T3 cells as in T cells suggests that T cells do not produce the special accessory molecules necessary to facilitate the folding or assembly of soluble class I molecules.

Unlike other Q region genes, Q4 is transcribed in a number of tissues. In embryo fibroblasts from H-2^p mice, Qb-1 is expressed at the cell surface and is secreted at low levels, if at all (Day and Frelinger, 1991). Lymphocytes derived from the same animals detectably express only soluble Qb-1, so the surface expression observed with fibroblasts is cell type specific and not related to allelic differences in Qb-1 or other genes. The differential membrane association of Qb-1 in lymphocytes versus fibroblasts might reflect alternative splicing of mRNA, resulting in different carboxy termini. Alternatively, it might reflect differential handling of the same protein core by different cells. This is not likely to be due to addition of a glycophosphatidyl tail, as Qb-1 is not removed from fibroblasts by phospholipase treatment (P. Day and J. Frelinger, personal communication). Thus another post-translational modification might be involved, or perhaps subtle differences in the

[62] The carboxy terminus of Q4 is not compromised to the same extent as Q10, however, being four amino acids longer and more hydrophobic. On the other hand, Q4 does lack the three positively charged residues present in most membrane-anchored class I molecules that are thought to bind to the inner surface of the membrane through charge interactions.

ER translocation machinery in the cell types leads to secretion in one case and membrane anchoring in another. The latter explanation is favored by the ambiguous nature of the Q4 hydrophobic tail.

In fibroblasts, Qb-1 was found to be assembled and acquire resistance to endo H digestion with the rapid kinetics observed with class Ia molecules (Day and Frelinger, 1991). Despite this, Qb-1 was detected via immunofluorescence of fixed and permeabilized cells largely in the ER, using either a Qb-1-specific mAb or an antiserum prepared against a peptide corresponding to the carboxy terminus. In the same cells, staining with a D^p-specific mAb resulted in largely a surface pattern of staining. These findings suggest that the intracellular trafficking of Qb-1 might differ from that of other class I genes, perhaps recycling from a postmedial GC compartment to the ER. On the other hand, the possibly tenuous nature of the interaction of Qb-1 with the membrane might result in cell surface Qb-1 being unusually detergent extractable following fixation, leaving a false impression of the ratio of intracellular to surface expression.[63]

2. *TL Region Gene Products*

The Qa-1 locus is located in the TL region of H-2.[64] This locus was originally defined serologically using alloantisera, and subsequently via allogeneic cytotoxic T cell responses. In theory, such alloresponses could be directed against peptides derived from the Qa-1 locus in association with other class I molecules, or the T cell antigen receptor could recognize Qa-1 molecules in an empty state or bearing a peptides. In favor of the latter explanation, a T cell hybridoma was identified that recognizes target cells treated with the synthetic copolymer poly($Glu^{50}Tyr^{50}$) (Vidovic *et al.*, 1989). Most interestingly, the T cell antigen receptor expressed by the hybridoma consisted of $\gamma\delta$ chains. Two lines of evidence suggested that the synthetic peptide was presented by Qa-1; recognition required the target cell to express the appropriate Qa-1 allele and was blocked by anti-Qa-1 antiserum. At the time of this study, the gene encoding the Qa-1 protein was not iden-

[63] Using similar methods to fix cells prior to detergent permeabilization, even influenza hemagglutinin, which has a well-defined transmembrane domain, is preferentially extracted from the plasma membrane relative to internal organelles (J. Yewdell, unpublished observations).

[64] Ironically, the designation *Qa* was used to distinguish the newly discovered serological reactivity from those mapping to the TLA region. The MHCs of critical mouse strains used to type the first of these antigens (Qa1) were erroneously typed, and instead of being located in a new region of chromosome 17, Qa1 resides right in the middle of TLA ("the best laid schemes o' mice an' men . . .").

tified, so the ability of the isolated gene to confer target cell sensitization could not be tested.

Recently, however, the gene encoding Qa-1 was recently shown (Imani and Soloski, 1991; Wolf and Cook, 1990) to be the pH-2^d-37 gene (now termed the *T23^b gene*) identified while cloning H-2-related transcripts present in liver (Cochet *et al.*, 1989). The gene appears to be ubiquitously expressed in various tissue types. Its export to the cell surface is greatly reduced by tunicamycin treatment, suggesting that addition of carbohydrates is important for folding (Wolf and Cook, 1990). This finding is consistent with a previous report that the recognition of target cells by some Qa-1 allospecific cytotoxic T lymphocytes can be effected by treating cells with tunicamycin (Jenkins *et al.*, 1985).

In L929 cells transfected with the T23^b gene, most Qa-1 protein detected by Western blot analysis contains the simple carbohydrates characteristic of proteins located prior to their transport through the medial GC (Imani and Soloski, 1991). Similar findings were made much earlier using splenocytes (Rothenberg and Triglia, 1981). In this early report it was also observed that several hours after synthesis, most Qa1 was not associated with β_2m, in contrast to another TLA antigen examined. "Heat shocking" of transfected L929 cells by incubation for 1 hour at 42°C increased the amount of Qa-1 with mature oligosaccharides, and concomitantly increased the surface expression of Qa-1 as detected by cytofluorography (Imani and Soloski, 1991). In contrast, the surface expression of K^k was not altered. A similar increase in Qa-1 expression was achieved by incubating cells for 24 hours at 26°C. If 26°C incubated cells were shifted to 37°C, the amount of Qa-1 with mature carbohydrates detected by Western blotting diminished with a half-time less than 30 minutes, unless cells were incubated with a tryptic digest of mycobacterium HSP65 or with poly($Glu^{50}Tyr^{50}$). Incubating cells with poly($Glu^{50}Tyr^{50}$) and, to a lesser extent, HSP65 decreased the amount of lower-molecular-mass products detected in the Western blots by anti-Qa-1 antisera. These products presumably represent proteolytic fragments of Qa-1 that are produced in the absence of stabilizing peptides bound in the antigen binding site.[65]

These intriguing findings suggest that Qa-1 molecules function to present peptides to T cells, possibly selectively to those expressing

[65] These findings provide the first evidence, for any type of class I molecule, that unstable, presumably empty molecules stabilized at 26°C are actually degraded by cells following thermal inactivation, and not simply shed or rendered totally nonantigenic.

γδ T cell antigen receptors. The increased assembly and surface expression of Qa-1 on heat shocked cells suggest that Qa-1 binds either peptides derived from heat shock proteins themselves or peptides whose formation or delivery is enhanced by heat shocking cells. The known homology between HSP65 and cellular HSPs would favor the former possibility, as would observations that peptides from HSP65 can be recognized by γδ T cells induced by HSP65 (Born *et al.*, 1990; Haregewoin *et al.*, 1989). As only a digest of HSP65 was examined for its capacity to stabilize Qa-1, however, it is unclear whether this property is restricted to HSPs or would have been achieved by tryptic digests from other proteins.

There are two additional reports suggesting that the γδ T cell antigen receptor can recognize class Ib molecules. The reactivity of an alloreactive γδ T cell antigen receptor-positive, $T_{CD4-CD8-}$ line was found to map to the TL or more telomeric region of chromosome 17 (Bluestone *et al.*, 1988). More directly, a γδ hybridoma whose reactivity mapped to the TL region (Bonneville *et al.*, 1989) could be shown to recognize cells transfected with a TL gene termed 27^b but not nontransfected cells (Ito *et al.*, 1990; Van Kaer *et al.*, 1991). Based on mRNA levels, this gene appears to be expressed in numerous tissues. It seems to be directly recognized by T cells, and not as a source of peptides presented by class Ia molecules, as T cells recognize an embryonic cell line that expresses class Ib but not class Ia gene products.

Based on two reports it also appears that αβ T cell antigen receptor-bearing T cells can recognize class Ib molecules encoded by TL or more telomeric regions. The first described an alloreactive αβ T cell antigen receptor-positive, $T_{CD4-CD8-}$ line whose reactivity mapped to this region (Bluestone *et al.*, 1988). In the second report, a 12-residue peptide derived from an influenza virus protein was found to induce a peptide-specific T_{CD8+}, whose reactivity with peptide-treated target cells again mapped to this region, when tested against cells derived from various congenic mouse strains (Milligan *et al.*, 1991). It is likely that cells recognized the peptide in association with a class Ib molecules, as cells deficient in class Ia expression were recognized whereas cells deficient in β_2m expression were not. Importantly, peptide-specific T_{CD8+} did not recognize cells synthesizing the relevant influenza protein or even an 18-residue peptide including the antigenic peptide. This study raises a number of important issues. First, it is important to determine the CD8 dependence of recognition, as the importance of this interaction in T cell recognition of class Ib molecules is not well characterized. Second, it is unclear whether the

failure of cells to present endogenously synthesized antigens reflects the inability of cells to produce and/or deliver the appropriate peptide to the early exocytic compartment where association with class Ia molecules occurs or is due to the inability of class Ib molecules to bind peptides delivered to this compartment. This latter possibility would explain, at least in part, why presentation of foreign peptides with class Ib molecules has not been reported. This a difficult problem to solve, however, as there are no guarantees that cells are able to transport any antigenic form of the peptide as it was identified solely on the basis of its activity as an exogenous antigen. Although a negative finding would be insignificant, one approach to this problem would be to identify an octameric or nonameric antigenically active peptide and determine whether it could be presented when synthesized endogenously. As such peptides are now known to be transported from the cytosol, this might provide the best chance of bypassing whatever machinery might be lacking in processing a larger form of antigen.

Finally, mention should be made of an intriguing report in which recognition of Qa-1 by some of a panel of alloreactive T cell clones was influenced by a gene mapping to the H-2D region (Aldrich *et al.*, 1988). Similar findings have been made regarding a rat class Ib gene whose recognition depended on a gene in the class Ia region (Davies *et al.*, 1991b). These findings are reminiscent of those made regarding cim control of rat class Ia gene products and H-2D control of HLA-B27 expression in transfected mice (discussed in Section II.F.3). This might reflect the operation of some H-2D-encoded molecule that somehow alters or abets class I folding or peptide delivery. Alternatively, as suggested (Aldrich *et al.*, 1988), this might reflect presentation of an H-2D region gene product in association with Qa-1. Time will tell.

3. *Hmt*

The only class Ib molecule directly demonstrated to present peptides from an endogenously synthesized protein is Hmt (histocompatibility dependent on a maternally transmitted factor). The discovery of Hmt resulted from a 12-year quest to understand the non-class Ia-restricted T_{CD8+} cell recognition of a maternally inherited minor histocompatibility antigen (Mta)[66] (see Fischer Lindahl *et al.*, 1991, for a stirring account).

[66] Mta refers to the Hmt–antigen complex; the antigen presented is known as maternally transmitted factor (MTF).

a. Nature of Hmt

The class I-like nature of Hmt was inferred from the failure of two β_2m-deficient cell lines to present Mta and the blocking of Mta recognition by β_2m-specific antibodies. Screening colonies of wild mice unearthed animals with null or allelic varieties of Hmt. Crossing these animals with standard inbred strains allowed identification of the Hmt region of chromosome 17. By use of a DNA probe from a conserved region of exon 4 (encoding the α3 domain), a number of class I-like genes were identified. mRNA for only one of the genes was readily detected in Northern blots. Transfection of this gene into nonpresenting cells conferred the presentation of Mta, thus identifying the gene as Hmt (C.-R Wang *et al.*, 1991).

Sequencing the Hmt gene revealed the protein to be at least as similar to class Ia molecules as to class Ib molecules. Many residues conserved between class Ia allomorphs are present in Hmt. This similarity and the requirement for β_2m demonstrated functionally suggest that Hmt is similar in structure to class Ia proteins. The major structural difference between Hmt and class Ia molecules lies in the cytoplasmic domain, where Hmt has only 8 residues, compared with the 30 to 40 residues present in mouse class Ia molecules. Comparison between Hmt and an allele unable to present MTF reveals only three substitutions in the mature protein. Two of the substitutions are located in the $\alpha 1\alpha 2$ domains; one of these is present in the floor of the peptide binding sites and could alter peptide binding, accounting for the lack of MTF presentation.

b. Nature of Antigen Presented by Hmt

The maternal nature of the inheritance of the antigen provided a critical clue to the source of the peptide that associates with Hmt; only an infectious agent or mitochondria would be expected to be transmitted in this fashion. Two findings strongly implicated mitochondria as the agent of transmission. First, in creating somatic cell hybrids between Mta^+ and Mta^- cells, it was found that treating Mta^+ donor cells with a mitochondrial poison blocked transfer of Mta to hybrids (Smith *et al.*, 1983). Second, Mta^+ cells treated with chloramphenicol (which blocks mitochondrial but not cytosolic protein synthesis) for 17 hours no longer expressed Mta (Han *et al.*, 1989). This indicates that the half-lives of Hmt–peptide complex and the source of the antigenic peptide are approximately 6 hours or less. Screening numerous mouse strains revealed that at least four allelic forms of MTF exist (Fischer Lindahl *et al.*, 1991). Comparing amino acid sequences of mitochondrial proteins deduced from DNA sequencing revealed that in only a

single residue in one of the 13 proteins encoded by mitochondria did all four alleles demonstrate differences from each other. This was the sixth residue from the highly hydrophobic amino terminus of the ND1 protein, a subunit of NADH dehydrogenase. Armed with this knowledge, synthetic peptides were made corresponding to this region and were found to sensitize cells for lysis by Mta-specific T cells (Loveland *et al.*, 1990; Shawar *et al.*, 1990). Unlabeled peptide-treated cells completely blocked lysis of Mta^+ cells by T cell populations, indicating that the ND1 determinant was the major, if not sole, determinant recognized by the populations (Loveland *et al.*, 1990).

Comparison of the antigenic activity of peptide analogs revealed that the amino-terminal methionine of the peptide (which corresponds to the amino terminus of ND1), had to be formylated (Loveland *et al.*, 1990; Shawar *et al.*, 1990); peptides with unsubstituted or even acetylated methionine were not recognized. The critical nature of the formyl group was indicated by the antigenic activity of peptides in which formyl-methionine was replaced with formyl-valine or formyl-phenylalanine (Shawar *et al.*, 1990). The presence of amino-terminal formylated residue was not sufficient, however, to ensure binding to Hmt, as a number of formylated peptides failed to block sensitization with an antigenic peptide. Although information on the affinity of Hmt for peptides of different sizes is limited, it appears that the number of residues accommodated by the Hmt binding site might be slightly more than that accommodated by the class Ia binding site, as maximal antigenic activity was achieved with a dodecameric peptide, whereas hexameric and octameric peptides were less active. Furthermore, using the dodecameric peptide, the concentration and time dependence for target cell sensitization was similar to that observed using similar-length peptides that bind to class Ia molecules (Shawar *et al.*, 1990).

These remarkable findings provide two important insights into antigen processing. First, they demonstrate that peptides from mitochondrial proteins can find their way to the exocytic pathway for association with class I molecules. Such interactions are not limited to class Ib molecules, as a mitochondrial antigen, defined only as not being derived from ND1, has been found to be presented in association with a rat class Ia gene product (Davies *et al.*, 1991a). It remains to be determined whether such peptides are generated in the mitochondria or only after delivery of mitochondrial peptides to the cytosol (this question might be of most significance for understanding cellular regulation of mitochondrial proteins). The blocking effect of chlroamphenicol on MTA presentation indicates that peptide generation is linked

either to the process of mitochondrial protein synthesis or to levels of ND1 present in mitochondria. The observation that RMA/S cells exhibit reduced presentation of Mta (and Qa-1) suggests that the peptides that bind these class Ib molecules are handled in a similar manner as peptides that bind class Ia molecules (Hermel *et al.*, 1991).

It appears that Hmt functions to present peptides from either mitochondria or bacteria, as the cytosolic protein synthesis machinery in eukaryotes is not believed to use formyl-methionine in any circumstances. Given the obvious threat posed by intracellular bacteria, it might seem more likely that Hmt evolved to monitor cells for the presence of intracellular bacteria.[67] Only a very small fraction of bacterial peptides would, of course, derive from the amino terminus, however, so unless such peptides are preferentially secreted into the cytosol by intracellular bacteria, it is unclear why class Ia molecules would not be sufficient to present bacterial peptides. On the other hand, some of the mitochondrial proteins may not be processed under normal conditions at the same rate as ND1, which is recognized only as an allodeterminant, and probably not by self T cells. If presentation of other mitochondrial peptides was significant only after some insult to cells, this would be a neat way of disposing of irreversibly damaged cells.

V. Practical and Evolutionary Considerations

A. Clinical Relevance of Antigen Processing and Presentation

1. Tumor Immunology

It has long been hoped that T cell responses could be manipulated to prevent or treat malignancies. The normal incidence of tumors in T cell-deficient mice suggests either that T cells do not normally eradicate tumor cells or that other immune mechanisms are capable of replacing the T cell function in immune surveillance. Although immunodeficient humans have been less well characterized, individuals lacking T cell responses generally succumb to infectious diseases and not neoplasias.

Although T cells may not play a deciding role in the outcome of malignancies, a number of findings suggest that this could be achieved through appropriate intervention. Thus, it has been shown in both

[67] Or even extracellular bacteria, if the major function of Hmt-restricted T cells was nonlytic in nature.

rodents and humans that T cells can specifically respond to tumor antigens and, in certain circumstances, can prevent tumors or even eradicate established tumors. Also, human tumors frequently exhibit deficiencies in class I expression that could result from selection by tumor-specific T_{CD8+} (Cabrera *et al.*, 1991; P. Wang *et al.*, 1991).

To manipulate antitumor T cell responses effectively, it is necessary to define peptides presented in a tumor-specific manner. Based on results in nontumor systems it is clear that virtually any type of protein produced by the cell could be a class I molecule-restricted tumor antigen. This includes nuclear, cytoplasmic, membrane, and even mitochondrial proteins. As peptides from normal cellular proteins have been found complexed to class I molecules, the only requirements for tumor-specific peptides are that they bind to class I molecules and are not viewed by the immune system as a self antigen. Peptides could escape immune tolerance by possessing mutated residues or by being expressed at superphysiological levels. Mutations resulting in antigen peptides need not be related to the malignant state: each cell must possess some mutations in its genome as a result of mistakes in DNA replication. Such mutations would not necessarily have to be located in the antigenic peptide; mutations in flanking regions could enhance the liberation or transport of cryptic antigenic peptides. Mutations within peptides could act similarly, and in addition could enhance antigenicity by increasing binding to class I molecules or by abrogating tolerance. Despite the multitude of ways in which antigenic peptides could be created by mutations, such events might still occur rather infrequently in the absence of mutagenic agents. In these circumstances most tumor antigens might derive from normal proteins expressed at higher levels in tumor cells or by tumor-induced alterations in the antigen processing machinery resulting in enhanced presentation of some peptides.

In mouse plasmacytoma cells both normal and mutant peptides have been shown by Boon and colleagues to be recognized by tumor-specific T_{CD8+} (Sibille *et al.*, 1990; Szikora *et al.*, 1990; Van den Eynde *et al.*, 1991). These cells were treated with a chemical mutagen, however, and the roughly equal frequency of mutated and normal peptides might not reflect the situation with natural tumors. Indeed, the single human tumor antigen that has been defined genetically is apparently identical in the melanoma cells recognized by tumor-specific T_{CD8+} and normal cells (van der Bruggen *et al.*, 1991). Importantly, the same antigen was presented by melanoma cells from other patients expressing the class I restriction element. This offers the hope that at least in some cases, common antigens will be presented in

association with given class I molecules by certain tumor types, making antitumor vaccines possible, at least theoretically, and circumventing the need to identify a unique peptide for each and every patient.

The melanoma antigen was identified by painstakingly screening cells transfected with DNA from tumor cells.[68] Without discounting the importance or elegance of this work, in the future it will probably be much easier to identify antigenic peptides from the peptides eluted from purified class I molecules. Using this method, it is likely in the next few years that the limiting factor in identifying tumor antigens will be only the availability of tumor-specific T_{CD8+}.

Based on the high frequency of tumor cells that exhibit reduced levels of surface class I molecules, it seems highly likely that the near future should also witness the isolation of a great number of naturally occurring antigen presentation-deficient cells. As a first step, cells could be segregated into two groups based on their α chain and β_2m mRNA levels, with cells expressing normal levels delivered to immunologists interested in antigen processing and class I assembly, and the remainder delivered to those interested in gene expression. Placing the cells with normal class I and β_2m mRNA levels into complementation groups based on recovery of class I expression following cell fusion should give a good idea of the number of gene products involved in antigen processing and class I assembly. With any luck, a few more surprises will be in store.

2. *Immune, Autoimmune, and Immunodeficiency States*

a. Vaccines

Knowledge of the precise determinants recognized by antiviral T_{CD8+} can only enhance the development of vaccines that induce T_{CD8+} responses. Such vaccines might be most useful then in treating chronic infections in which T_{CD8+} responses are low or absent. Obviously, there is tremendous interest in using this approach for human immunodeficiency virus infections. Other possibilities include infections with members of the herpesvirus genera or unicellular organisms that reside within host cells. These vaccines might not have much use in acute viral infections, where the normal T_{CD8+} response is vigorous. Also, it is abundantly clear from studies with lymphocytic chori-

[68] To maximize the chances of expression of a normal cellular gene that might be aberrantly expressed in malanoma cells, cells used for transfection were derived from tumor cells by selection with the melanoma-specific T_{CD8+}.

omeningitis virus in mice, and to a lesser extent, hepatitis B virus infections in humans, that enhancing T_{CD8+} responses can have rather severe deleterious effects.

b. Autoimmunity

It is now abundantly clear that antigen processing is a complex process involving numerous steps that distinguish between highly similar protein sequences. It is tautological that a similar pathway is used in defining self structures during tolerance induction in the thymus. Given the highly specific nature of the process, it is easy to imagine that novel self peptide–class I complexes could be created by even subtle changes in any of the various steps in the process. It seems safe to wager that alterations in the levels of expression of proteins or in the manner in which they are handled by cells contribute to some autoimmune conditions.

Based on present knowledge there are three steps in the antigen processing pathway that would seem to be most amenable to pharmaceutical intervention. If the LMP proteins are dedicated to antigen processing (whatever their functions may be), competitive inhibitors might be designed that block their function. The putative transporters might be similarly inhibited if they do not perform other tasks useful to cells. Drugs inhibiting these steps would also be useful in tissue transplantation. The simplest drugs to develop would be peptides that compete for binding to class I molecules. This would also be the most specific approach, sparing the antigen presentation capacity of other allomorphs. Such antagonists have been found to compete for presentation of class II-restricted determinants *in vivo*. Although the high affinity of class I molecules for natural peptides represents a hurdle in the development of drugs that displace such natural peptides, this might not be insurmountable, particularly if the competitors are provided in a form that allows then access to intracellular compartments.

c. Novel Immunodeficiency States

Mice deficient in β_2m not only exist, but for all appearances are healthy, at least in their pathogen-free cages (Koller *et al.*, 1990: Zijlstra *et al.*, 1989). This suggests that contrary to the hopes and expectations of some, the expression of normal class I gene products is not necessary for the growth, development, or function of mammals. It might be expected that of the five billion *Homo sapiens* on earth, some would demonstrate deficiencies in class I expression or function based on mutations in β_2m or the accessory molecules that are required for

class I assembly and transport. The challenge then for clinicians is to find these people.

B. Evolution of Cellular Immunity

1. Evolution of Antigen Processing and Presentation

Intracellular parasites are ubiquitous in nature. Probably every energy-providing organism has its own set of coevolving viruses, not to mention more complicated unicellular organisms that make their homes intracellularly (no doubt providing a haven for their own set of parasites, and so on, like microscopic Russian dolls). For unicellular organisms, the responses to intracellular parasites are limited to suicide [which might be of some benefit, if nearby progeny or (perish the thought), parents were then spared] or the production of molecules that discourage entry or habitation. For multicellular organisms, however, another strategy is useful: the selective destruction of infected cells before the progeny of the parasite can be released to infect other cells in the organism (or the selective delivery of compounds that reduce replication). This strategy required the evolution of recognition and effector mechanisms.

What is the best means of detecting parasitized cells? All viruses, regardless of their structure and mechanism of maturation, must synthesize proteins in the host cytosol. Taking advantage of this viral Achilles heel requires the evolution of a system capable of transporting the proteins from the cytosol to the plasma membrane. Because the foreign proteins, or their fragments, had no means of remaining attached to the plasma membrane, the transporting protein either had to have a membrane anchor region or had to bind to a membrane-anchored protein or ligand. At its onset, the system may have been similar to its present-day form, with the ancestral class I molecule serving strictly to carry to the cell surface peptides delivered to the exocytic compartment by diffusion or via transporters whose primary function lay elsewhere. With time, the present-day transporters could have evolved to improve the efficiency and specificity of the transport process. Alternatively, the initial antigen presenting molecule may have been an ancestor of the present day putative peptide-transporter, which acted both to transport and to present the viral proteins. In this scenario, the efficiency of the process was later enhanced by the evolution of class I molecules from, perhaps, peptide binding proteins to serve their current function: the surface delivery and display of peptides supplied to the exocytic compartment by the transporter. It might

be possible to choose between these evolutionary scenarios by phylogenetic comparison of antigen processing capabilities.

There are a number of potential explanations of why T_{CD8+} evolved to recognize peptides rather than intact proteins. First, at the inception of antigen processing, in the absence of a dedicated cytosolic export mechanism, peptides would have had a greater chance of gaining access to the exocytic compartment by diffusion, particularly as some peptides would be extremely hydrophobic in character. Second, a central feature of antigen presentation is that cells have only a limited number of molecules to present a highly diverse repertoire of antigens. To achieve this degeneracy in binding it seems intuitively easier for a receptor to bind short linear sequences as opposed to the surfaces present on intact molecules. Indeed, although hundreds or, at maximum, thousands of class I allomorphs are sufficient to recognize the universe of peptide antigens, the immune system requires millions of antibody specificities to recognize the universe of conformational antigens. Third, it might be easier for the T cell antigen receptor to discriminate foreign from self antigens using a peptide-based system.

It is worth considering why β_2m is so weakly associated with class I molecules. Clearly, it would be feasible for class I molecules to function with β_2m bound more securely. Class II molecules, for example, accomplish the same peptide binding function using more tightly associated α and β chains. More directly, a single-chain class I molecule consisting of the $\alpha 1\alpha 2\alpha 3$ domain genetically linked to β_2m by a glycine spacer between the carboxy terminus of the α-chain and the amino terminus of mature β_2m has been shown to fold normally and bind peptides (Mottez *et al.*, 1991). Thus, the loose association of β_2m almost certainly results from functional rather than structural factors. It has been suggested that the weak association of β_2m evolved to minimize the binding of exogenous peptides present in sera (Rock *et al.*, 1991c). Given the dire consequences of T_{CD8+} recognition (cell death or, at the very least, exposure to lymphokines), this would prevent cases of mistaken identity and spare the destruction of cells even in close vicinity to heavily parasitized cells. As an alternative explanation, the weak binding of β_2m might contribute toward the ability of class I molecules to discriminate between peptides. As it now appears, β_2m amplifies the affinity of the subset of peptides that optimally fit the antigen binding groove. Perhaps with a more tightly tethered β_2m, a great number of peptides would fall into this category, creating many more holes in the T cell antigen receptor repertoire, as more self peptides would bind with high affinity. The characteristics of trans-

genic mice expressing either a tightly associated form of β_2m or, perhaps, excess amounts of serum β_2m (to enhance binding of exogenous peptides) might shed some light on the relative contributions of these explanations or point to some novel reason. At the minimum, this would provide direct evidence whether the concentration of extracellular peptides ever reaches values at which sensitization would occur under conditions in which β_2m was not limiting.

2. *Evolution of the T Cell Antigen Receptor*

As difficult as it is to imagine the evolution of the antigen processing and presentation mechanism, this is just half the problem faced by the immune system; the T cell recognition system had to evolve simultaneously. The primordial antigen recognizing cells might have derived from macrophage-like cells that evolved to destroy extracellular unicellular organisms. Such cells might have had lectin-like antigen receptors that recognized oligosaccharides characteristic of the pathogen cell surface. The T cell antigen receptor might have evolved from these lectins. In this scheme, the oligosaccharides present on primordial MHC molecules may have played a prominent role in their recognition by the primitive T cell antigen receptor. For example, peptide binding might have induced conformational alterations in MHC molecules that enabled its oligosaccharides to be recognized by the T cell antigen receptor.

From its very beginnings, the cellular immune system would have to surmount the problem of self versus nonself recognition. In the scenario we have described, this would first be encountered with an oligosaccharide-based recognition system. Presumably, this would not have posed a serious problem, as the differences between prokaryotic and eukaryotic cells seems to dictate that unique oligosaccharides are displayed at the cell surface. With the recognition of peptides this immediately becomes a serious problem because of the large number of self proteins. If the peptide-based system first evolved as a means of reducing a single prevalent highly lethal virus, however, then the first antigen receptors could have been selected on the basis of recognizing one or a few viral peptides. Moreover, the requirements for negative and positive selection of receptors begins with the introduction of polymorphisms into the gene pool. As long as the antigen presenting molecule and its complementary receptor are inherited as a unit there is no difficulty with self-reactive receptors, as mutations in the receptor resulting in self reactivity would be lethal.

In the regrettable absence of time travel, only indirect approaches are available for studying the evolution of the antigen processing and

presentation machinery. As more proteins with defined functions are sequenced, the origins of the modern components of the antigen processing pathway might reveal themselves by genetic similarities. Additional clues to the origins of the antigen processing system might be gleaned from the defense systems of lower organisms. Little is known about the immune systems of lower vertebrates; even less is known about invertebrate immunity. Given the ubiquitous existence of pathogens, these organisms must have developed sophisticated methods of dealing with intracellular and extracellular parasites, some of which might be closer to the primordial immune system than those used by present-day higher vertebrates. Studies of the immune systems of simpler creatures is sure to be interesting and possibly relevant toward understanding our own immune system.

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ADVANCES IN IMMUNOLOGY, VOL. 52

Human B Lymphocytes: Phenotype, Proliferation, and Differentiation

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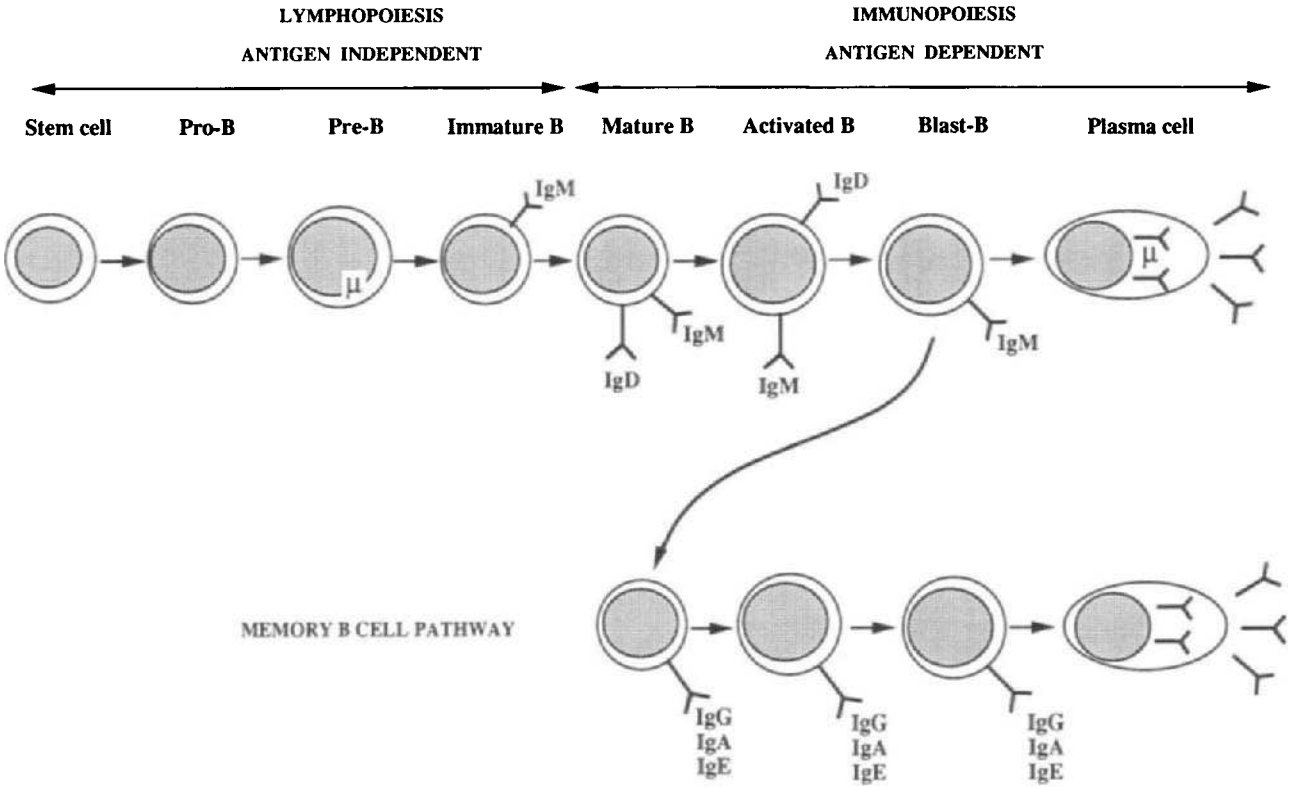
I. Introduction

The immune response results from the precisely regulated proliferation and differentiation of individual clones of lymphocytes. The main function of B lymphocytes is to produce immunoglobulins (Igs) of which nine different isotypes (IgM, IgD, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, IgA_2, and IgE) have been isolated in humans [for details on Ig gene organization, see Pascual and Capra (1991) and Walter *et al.* (1991)]. Each of these isotypes displays specific effector functions such as binding to complement components or binding to a multiplicity of Fc receptors, whose engagement results in a wide array of biological activities (Ravetch and Kinet, 1991). Immunoglobulins play a crucial role in defense against a variety of infectious microorganisms. Their absence, as a consequence of altered B cell differentiation (variable common immunodeficiency) or of a lack of B cells (severe combined immunodeficiency, X-linked agammaglobulinemia) is lethal if not compensated by repeated lifelong injections of human immunoglobulins.

The natural history of a B lymphocyte can be divided into two major stages (Fig. 1). The first stage, called B lymphopoiesis, occurs in the fetal liver and the adult bone marrow. It permits the commitment and maturation of a hematopoietic multipotent stem cell into mature B lymphocytes coexpressing IgM and IgD. It is largely independent of T cells and antigens. The first B lineage-committed cell is the pro-B cell which has its immunoglobulin genes in germline configuration. Rearrangement of the heavy-chain variable (V), diversity (D), and joining (J) gene segments then occurs, resulting in the cytoplasmic expression of μ chains, which defines pre-B cells. A schematic organization of the various genes coding for immunoglobulins is shown in Fig. 2. The pre-B cell pool expands and some pre-B cells rearrange and express light-chain genes. Cells with both functional μ and light chains then express surface IgM, thus defining the immature B cell. At this stage, it is believed that B cells expressing surface IgM (sIgM) recognizing self


LYMPHOPOIESIS
ANTIGEN INDEPENDENT
IMMUNOPOIESIS
ANTIGEN DEPENDENT
Stem cell
Pro-B
Pre-B
Immature B
Mature B
Activated B
Blast-B
Plasma cell
IgM
IgD
IgM
IgD
IgM
IgM
μ
μ
MEMORY B CELL PATHWAY
IgG
IgA
IgE
IgG
IgA
IgE
IgG
IgA
IgE

antigens are either deleted or anergyzed (Nossal, 1989). The non-self-reactive and low-affinity self-reactive cells are then induced further to express sIgD, thus defining mature B cells, also called naive or virgin B cells. Studies in rodents have shown a daily production of approximately 10^8 B cells (Osmond, 1986) and accordingly it can be estimated that the human bone marrow produces 10^{10} to 10^{11} B cells per day. A very large proportion of these newly formed B cells have a very short life span. The establishment of *in vitro* cultures has allowed much progress in the understanding of mechanisms controlling murine B lymphopoiesis (Dorshkind, 1990; Kincade *et al.*, 1989; Rolink and Melchers, 1991; Whitlock and Witte, 1982). The progenitor B cell tumors and progenitor B cell lines generated after immortalization with Epstein–Barr virus (EBV) permit us to assume that the development of human B lymphocytes follows the same rules (Uckun, 1990), although a very limited number of reports have described the growth and differentiation of human progenitor B cells *in vitro* (LeBien, 1989; Moreau *et al.*, 1992; Saeland *et al.*, 1991; Villablanca *et al.*, 1990; Wolf *et al.*, 1991).

The binding of antigen to sIg culminates in the production of highly efficient antibody-secreting plasma cells. This process has been called immunopoiesis and occurs mainly in secondary lymphoid organs such as lymph nodes, spleen, Peyer's patches, and tonsils. Following antigen invasion, resting B cells are activated which results in their enlargement and expression of new cell surface markers. With further stimulation, they enter the cell cycle (blast stage) and begin to divide intensely, so as to compensate for the relatively small number of antigen-specific B cells that is normally available. These cells will eventually differentiate into plasma blasts, which secrete antibody. Plasmablasts generated in these organs migrate to the bone marrow and mucosa where they mature into nondividing plasma cells secreting large amounts of antibody. Once the eliciting antigen has been eliminated, the B cell blasts will stop proliferating and will differenti-

FIG. 1. Simplest representation of human B lymphocyte natural history. The stem cell, precursor of all hematopoietic lineages, commits into a pro-B cell which has not yet rearranged Ig genes; following heavy chain gene rearrangement pre-B cells express cytoplasmic μ chains which yield immature B cells that express sIgM following light chain rearrangement. The immature sIgM B cells differentiate into mature B cells expressing both sIgM and sIgD. After antigen encounter and interactions with various cell types and their derived cytokines, mature B cells become activated and divide as B blasts. These blast cells can differentiate into Ig-secreting plasma cells or memory B cells. In response to a novel antigenic challenge, memory cells will mature into proliferating blasts and then plasma cells.

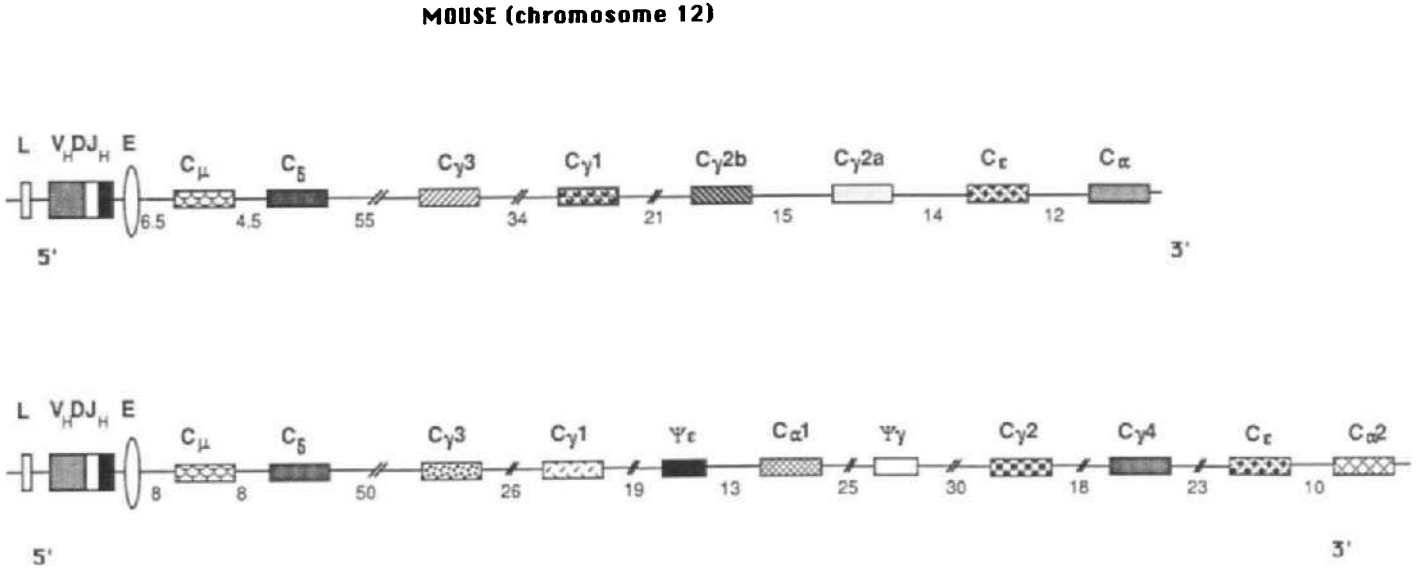


FIG. 2. Schematic representation of the loci encoding the mouse and human heavy chain loci. Figures represent intron length in kilobases.

ate into small memory B cells which express novel isotypes. These cells, the memory B cells, rapidly proliferate and differentiate in response to a repeated specific antigenic challenge. Several cell types appear to be involved in the proliferation and differentiation of B lymphocytes. They include T cells and accessory cells such as macrophages, interdigitating dendritic cells, and follicular dendritic cells. These various cells interact through cell–cell contacts involving various sets of adhesion molecules and through polypeptidic mediators called cytokines.

Numerous investigators have studied various aspects of human and murine immunopoiesis. The present review focuses on two sets of molecules involved in the proliferation and differentiation of highly purified human B lymphocytes: the molecules secreted or expressed by T cells and other accessory cells and the receptor molecules expressed on B cells. We have excluded from this review most of the information generated with whole cell supernatants or partially purified fractions to avoid inappropriate complication of this complex field. This review has been divided into distinctive sections which can be read and consulted independently so as to permit the reader to use it as a ready source of specific references.

II. Phenotype of B Lymphocytes

B cells have been distinguished by their expression of membrane immunoglobulins (Pernis *et al.*, 1970) which constitute the B cell antigen receptor. This receptor has recently been shown to form a complex of several transmembrane proteins and, thus, resembles the T cell antigen receptor (Ashwell and Klausner, 1990). The advent of monoclonal antibody technology resulted, in the early 1980s, in the establishment of monoclonal antibodies (mAbs) recognizing antigens specific to human B cells. More recently, these antigens have been molecularly characterized and, in a number of cases, their biological role has been identified. Figure 3 summarizes the expression of many of these antigens during B lymphocyte development as well as their distribution on T lymphocytes and monocytes. Flow cytometry histograms (Fig. 4) illustrate the expression of some B cell surface antigens on tonsillar B cells that have been separated according to their expression of sIgD, one of the most practical markers for the definition of naive B cells. Table I summarizes the various cell types present in human peripheral lymphoid organs and their antigenic profiles. Table II indicates the chromosomal assignment of various antigens of the B lymphocyte membrane. One of the most striking recent conclusions

FIG. 3. Expression of major B cell surface antigens on B lymphocytes at various stages of development (positivity is shown in dark). The left panels show antigen expression on T lymphocytes and monocytes. In these panels, the dark zone on the left indicates expression of the antigen on a subpopulation and while the dark zone on the right indicates expression on activated (act.) cells. The shaded area designates expression only in the cytoplasm. PC, plasmocyte.

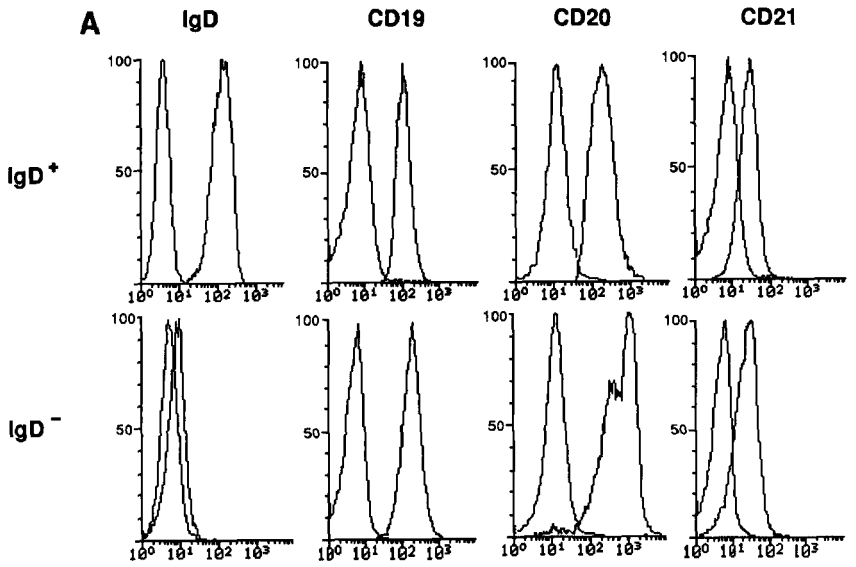


FIG. 4. Flow cytometry histograms of antigen expression on sIgD$^+$ and sIgD$^-$ B lymphocytes isolated from tonsils. First, B cells were purified from light density tonsil mononuclear cells by negative selection using rosetting with sheep red blood cells. Residual T cells and monocytes/macrophages were then removed by magnetic bead depletion following staining with anti-CD2, anti-CD3, and anti-CD14 monoclonal antibodies. sIgD$^+$ and sIgD$^-$ B cells were sorted using a magnetic cell sorting system (MACS) (Miltenyi *et al.*, 1990). Purified B cells were stained with biotinylated F(ab')$_2$ fragments of polyclonal goat anti-IgD antibody followed by fluorescein isothiocyanate (FITC)-conjugated streptavidin. Cells were then incubated with biotinylated superparamagnetic microparticles and deposited on high-gradient magnetic columns. Unlabeled sIgD$^-$ cells pass through the column; sIgD$^+$ cells are retained and then eluted. For phenotypic analysis, both populations are labeled first with the appropriate monoclonal antibody and then with phycoerythrin-conjugated anti-Fc fragment polyclonal antibody and fluorescence is analyzed with a FACScan. The antibodies were from the following companies. Immunotech: CD11a, CD18, CD19, CD21, CD23, CD35, CD37, CD39, CD44, CD54, CD72, CD77. Becton–Dickinson: CD5, CD20, CD22, CD25, CD45, CD71. Coulter: CD10, CD29, CD45RA. Dako: CD45 RO, CD45 RB. Ortho: CD38. Boehringer-Mannheim: CD22β, CD24. Amersham: IgD, IgM. Our laboratory: CD40, B7/BB1. T Cell Sciences: TS2/7. Antibodies to VLA -2, -3, -4, -5, and -6 were kindly provided by various investigators.

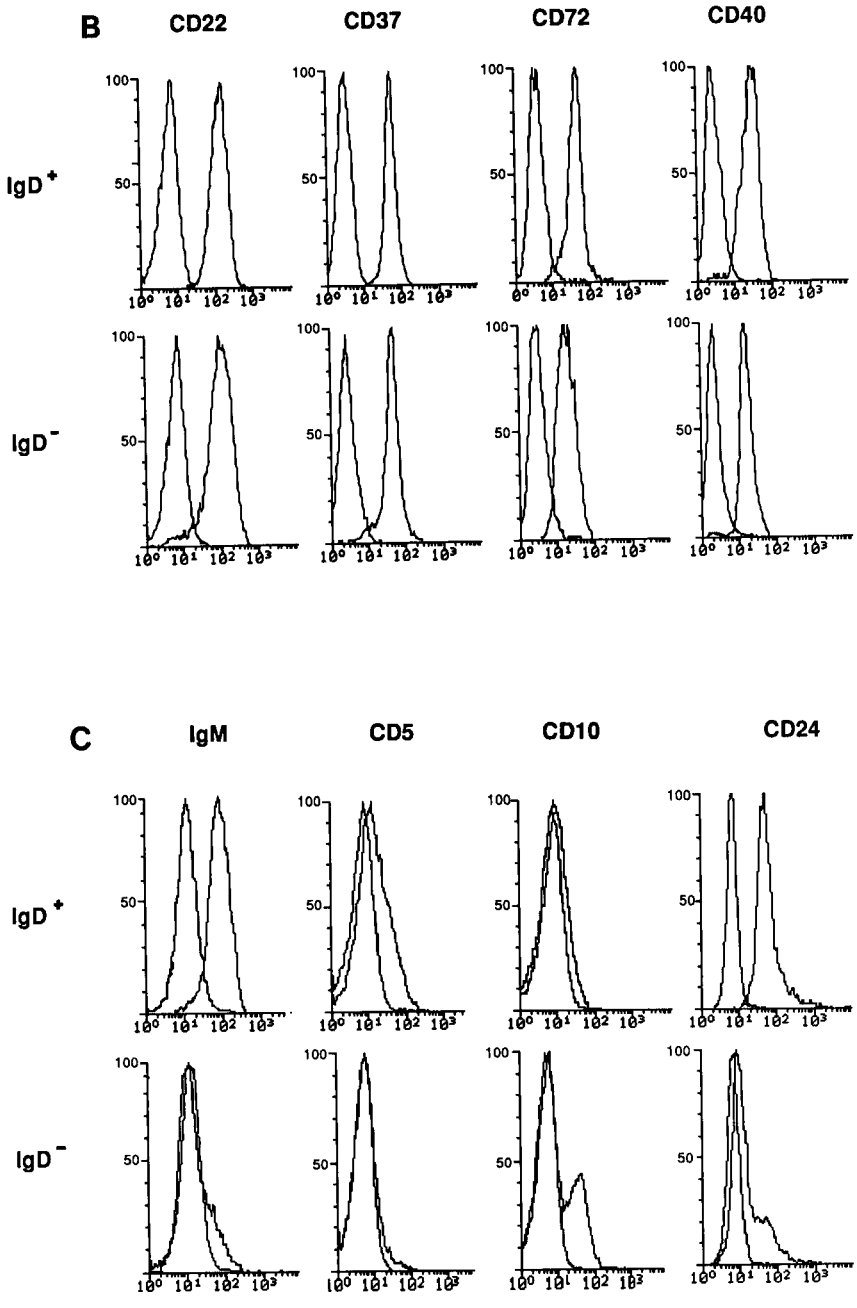
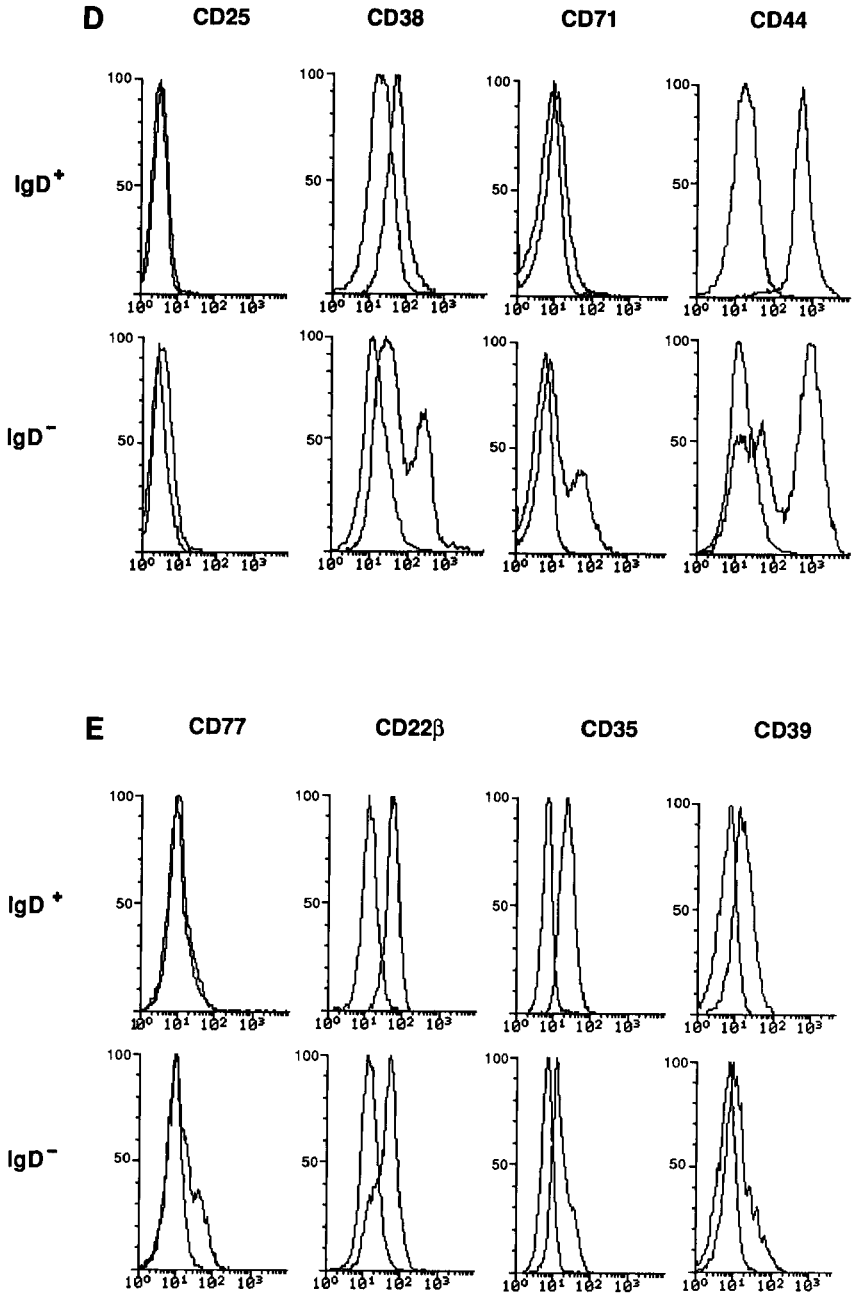


FIG. 4. Continued



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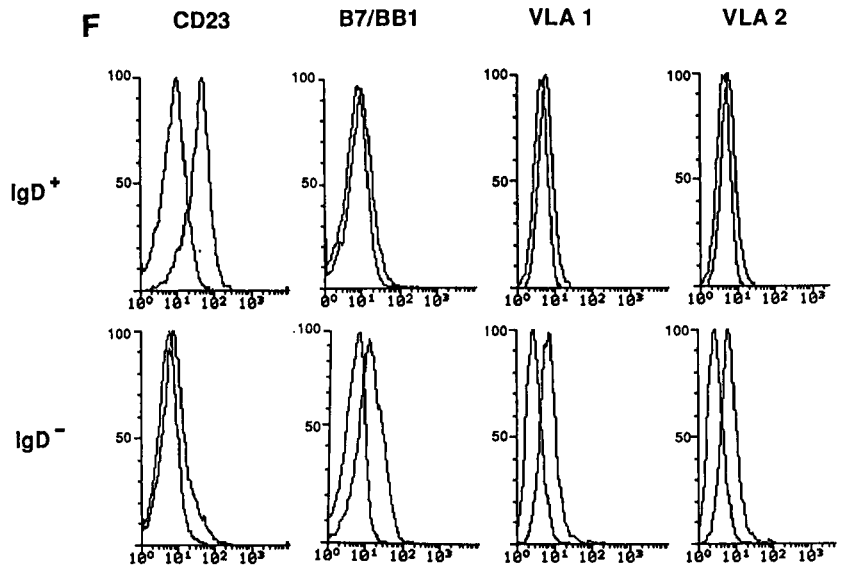

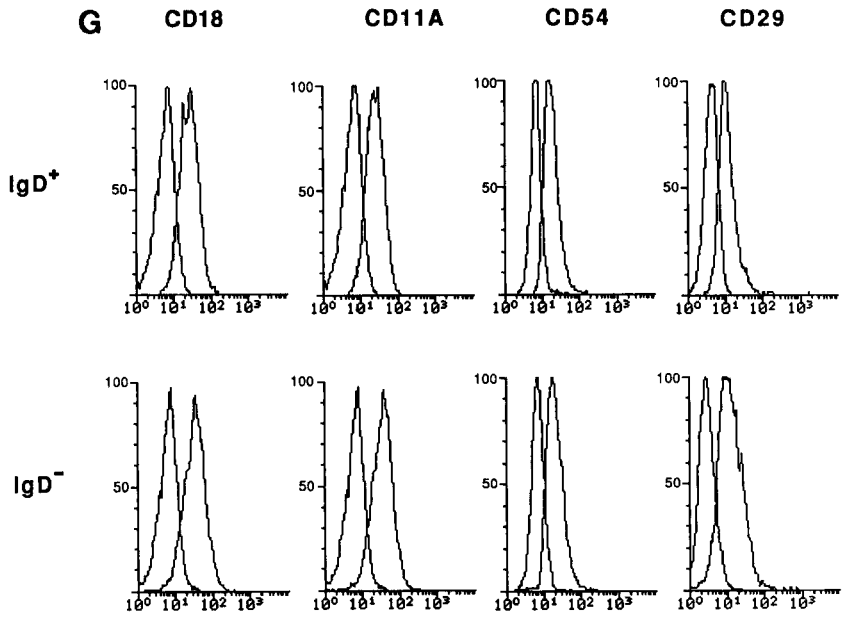

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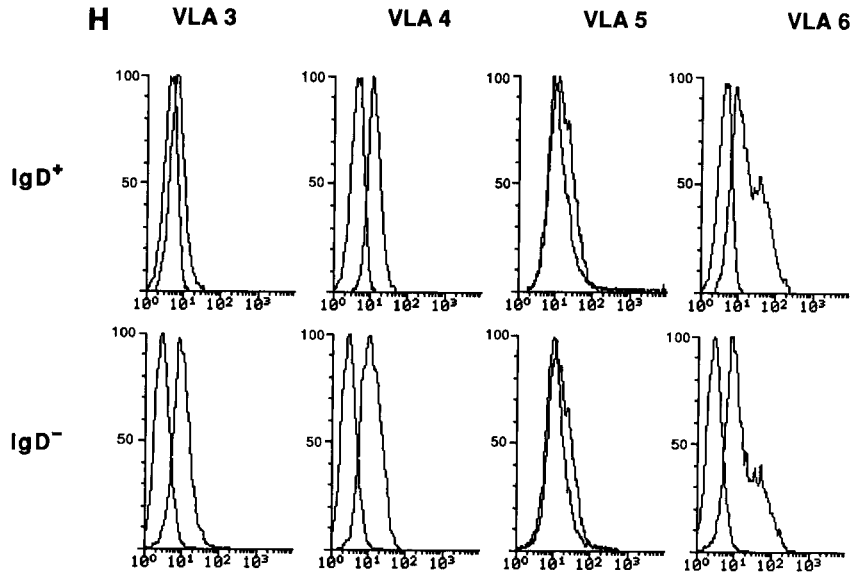


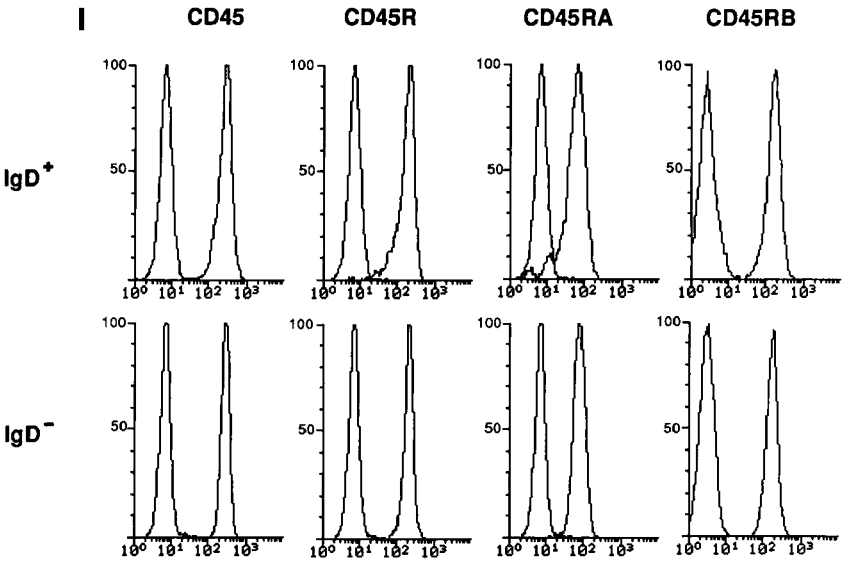


FIG. 4. Continued

TABLE I
CELL TYPES AND THEIR ANTIGENIC PROFILES WITHIN THE HUMAN LYMPHOID TISSUES

	T cell-rich areas			Follicular mantle	Germinal centers			
					Dark zone		Light zone	
	T cells	IDCs[a]	B cells (sporadic)	Resting B cells	Centroblasts	FDCs	Centrocytes	T cells (sporadic)
CD3	+	—	—	—	—	—	—	+
CD4	Some +	—	—	—	—	—	—	+
CD8	Some +	—	—	—	—	—	—	—
CD10	—	—	—	—	+	—	+	—
CD19	—	—	+	+	+	+	+	—
CD20	—	—	+	+	+	—	+	—
CD21	—	—	+	+	+	+	+	—
CD22	—	—	+	++	+	—	+	—
CD23	—	—	+	+	—	+	—	—
CD24	—	+	+	+	+/−	—	+/−	—
CD38	—	—	—	—	+	—	+	—
CD39	—	—	+	+	—	—	—	—
CD40	—	++	+	+	+	+	+	—
CD44	+	ND	+	+	+/−	—	+/−	—
CD54	+/−	+	+	+	+	+	+	+/−
CD57	—	—	—	—	—	—	—	+
CD11a	+	+	+	+	+	—	+	+
VCAM-1	—	—	—	—	—	+	—	—
CDw49d	+	—	—	+	+	—	+	—
CD76	—	—	—	+	—	—	—	—
CD77	—	—	—	—	+	—	+	—
MHC class II	—	++	+	+	+	+	+	—
Ki67	—	—	ND	—	++	—	+	—
sIgD	—	—	—	+	—	—	—	—

[a] IDCs, interdigitating dendritic cells; FDCs, follicular dendritic cells; MHC, major histocompatibility complex; ND, not determined.

regarding cell surface molecules was that one receptor may have several ligands/counterreceptors and that one ligand may bind to several receptors, a few examples being given in Table III.

A. B CELL ANTIGEN RECEPTOR

1. The Ig Complex

Triggering of sIgM with specific antibodies had been shown in the 1970s to deliver activation signals to B cells, but how the three cytoplasmic amino acids of the heavy chain could transduce an intracellular signal remained a puzzle up until recently. Several groups, using biochemical and molecular biology approaches, have now demonstrated that murine sIgM molecules are noncovalently associated with a disulfide-linked heterodimer composed of 34- and 39-kDa proteins

TABLE II
CHROMOSOMAL LOCALIZATION OF B LYMPHOCYTE SURFACE ANTIGENS

Antigen	Chromosome	References
CD1c	1q22–23	Yu and Milstein (1989)
CD5	11q13	Geurts van Kessel *et al.*, (1984)
CD9	12pter-q12	Boucheix *et al.*, (1985)
CD10	3q21–q27	Barker *et al.*, (1989); Tran-Paterson *et al.*, (1989)
CD11a	16p13.1–p11	Corbi *et al.*, (1988)
CD11c	16p13.1–p11	Corbi *et al.*, (1988)
CD18	21q22	Corbi *et al.*, (1988); Akao *et al.*, (1987a)
CD20	11q12–q13.1	Tedder *et al.*, (1989a)
CD21/CR2	1q32	Weis *et al.*, (1987); Carroll *et al.*, (1988)
CD23	19	Wendel-Hansen *et al.*, (1990)
CD25	10p15–p14	Leonard *et al.*, (1985); Ardingen and Murray (1988)
CD28	2q33–q34	Lafage-Pochitaloff *et al.*, (1990)
CD32	1q22–23	Grundy *et al.*, (1988); Sammartino *et al.*, (1988)
CD35/CR1	1q32	Weis *et al.*, (1987); Carroll *et al.*, (1988)
CD38	4	Katz *et al.*, (1983)
CD40	20	Lafage (unpublished result)
CD44	11p13	Glaser *et al.*, (1987)
CD45	1q31–32	Akao *et al.*, (1987b)
CD48	1q21–23	Staunton *et al.*, (1989)
CD54	19	Simmons *et al.*, (1988)
CD58	1p13	Sewell *et al.*, (1988)
CD72	9	von Hoegen *et al.*, (1991)
CD73	6q14–q21	Boyle *et al.*, (1988)
HLA antigens	6p21.3	Trowsdale *et al.*, (1991)
Ig heavy chain	14q32–33	Walter *et al.*, (1990, 1991)
κ	2p12	Zachau (1989)
λ	22q11.1–q11.2	Vasicek and Leder (1990)
B7-BB1	3q13.3–3q21	Freeman *et al.*, (1992)
L-selectin	1q21–24	Watson *et al.*, (1990)

referred to as IgMα and Igβ (Campbell and Cambier, 1990; Chen *et al.*, 1990; Hombach *et al.*, 1990; Parkhouse, 1990) (Fig. 5). The IgMα and Igβ components, which are respectively encoded by the genes mb-1 (Sakaguchi *et al.*, 1988; Yu and Wang, 1992) and B29 (Hermanson *et al.*, 1988), are glycosylated and phosphorylated transmembrane proteins with an extracellular Ig-like domain and a cytoplasmic tail. These α and β chains show similarity to components of the T cell receptor and are involved in the transport of the Ig to the cell surface as they permit reconstitution of an IgM receptor on fibroblasts (Venkitaraman *et al.*, 1991). The antigen receptor of normal human B cells appears to be similarly constituted; the sIgM-associated heterodimer consists of

TABLE III
PROMISCUITY IN THE INTERACTIONS BETWEEN RECEPTORS AND THEIR LIGANDS/COUNTERRECEPTORS

Receptor	Ligand/counterreceptor
TNFR p55[a]	TNF-α, TNF-β
TNFR p75	TNF-α, TNF-β
IL-1R type I	IL-1α, IL-1β, IL-1RA
IL-1R type II	IL-1α, IL-1β, IL-1RA
NGFR	NGF, NT-3, NT-4, BDNF
Il-2R α, IL-2R β	IL-2
LFA-1	ICAM-1, ICAM-2, ICAM-3
ICAM-1	LFA-1, Mac-1/CR3, CD43

[a] TNF, tumor necrosis factor; R, receptor; IL, interleukin; NGF, nerve growth factor; NT, neurotropin; BDNF, brain-derived neurotropic factor; IL-1RA, IL-1 receptor antagonist.

serine-phosphorylated glycoproteins of 37 and 47 kDa (Van Noesel *et al.*, 1990, 1991)

The sIgD molecules are also associated with a heterodimer that appears to be identical to that of sIgM, although the IgDα has a higher molecular weight than IgMα, as a result of post-translational alteration. The heterodimer also associates with murine IgG_{2b}, IgA, and IgE (Venkitaraman *et al.*, 1991).

2. *Proteins Associated with the Ig Complex*

It is possible that, like the numerous components within the T cell receptor, other undefined proteins make up the complete B cell receptor. These proteins seem to be involved mainly in the signal transduction following antigen binding. It has been demonstrated that the B cell antigen receptor is associated with tyrosine kinases (Campbell and Sefton, 1990; Gold *et al.*, 1990; White *et al.*, 1991). In particular, *blk* (Dymecki *et al.*, 1989) and *lyn* (Yamanashi *et al.*, 1990), members of the *src* family, are predominantly expressed by B cells. In addition to tyrosine kinases, the membrane-bound tyrosine phosphatase CD45 is thought to play a major role in B cell activation through the antigen receptor (for a review on phosphatases, see Fischer *et al.*, 1991b).

B. NON-IMMUNOGLOBULIN B CELL SURFACE ANTIGENS

B lymphocytes are widely distributed in the body and extremely motile and are able to interact with many other cell types, such as bone marrow stromal cells, T cells, interdigitating dendritic cells, follicular

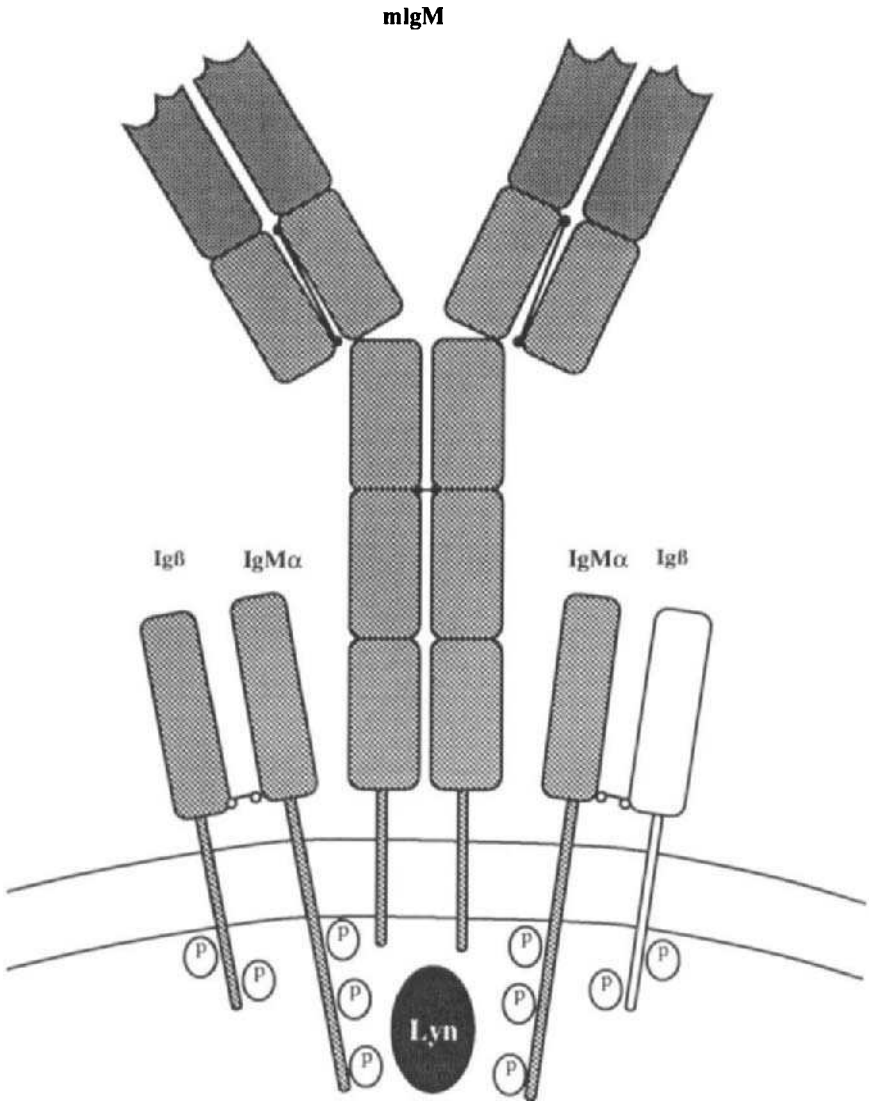


FIG. 5. Current model of the B cell antigen-receptor complex. sIgM are noncovalently associated with covalently linked heterodimers composed of Igβ (B29) and IgMα (MB-1). The complex is associated to the tyrosine kinase, *Lyn*, and the *Lyn* phosphorylation sites on the complex components are shown as P's.

dendritic cells, and endothelial cells. These interactions depend on specific sets of surface molecules. Workshops convened to define human leukocyte-associated antigens have gathered the numerous available monoclonal antibodies into clusters defining antigens, some of which are either very or only relatively specific to B cells. For detailed information about clustered antigens, readers are invited to consult the proceedings of the Leukocyte Antigen Workshops and particularly the last one edited by Knapp *et al.* (1989). Several excellent reviews on the topic of B cell surface antigens have recently appeared (Clark and Lane, 1991; Clark and Ledbetter, 1989b). We thus presently refer to the most recent and critical references on the subject and we consider these molecules particularly in the context of their presently known or suspected biological function. A schematic representation of various B cell antigens is given in Fig. 6.

1. Clustered Antigens of B Lymphocytes

a. CD5

The CD5 antigen is a 471-amino-acid (AA), 67-kDa T cell-associated glycoprotein (Jones *et al.*, 1986) expression of which in the human B cell lineage was first detected on chronic lymphocytic leukemias of B cell type (B-CLL) (Boumsell *et al.*, 1980; Wang *et al.*, 1980). It is also distributed on the vast majority of B cells in fetal lymphoid tissues (Antin *et al.*, 1986), in cord blood (Hardy *et al.*, 1987), and in the blood of patients recovering from bone marrow transplantation (Ault *et al.*, 1985). In adults, CD5 expression within the normal B cell pool is restricted to a subset represented in peripheral blood and in secondary lymphoid organs (Caligaris-Cappio *et al.*, 1982; Freedman *et al.*, 1987a; Gadol and Ault, 1986). The B cell-associated antigen CD72, which has been shown to deliver progression signals to human B cells (Kamal *et al.*, 1991), has recently been identified as the natural ligand for CD5 (Van de Velde *et al.*, 1991). Accumulating evidence now supports the notion that murine $CD5^+$ B cells belong to a separate B cell lineage with self-replenishing capacity (Hayakawa *et al.*, 1985). These studies have recently provided the basis for a new B cell nomenclature in which $CD5^+$ and $CD5^-$ B cells would be designated as B-1 and B-2 cells (Kantor, 1991); however, the question as to whether human $CD5^+$ and $CD5^-$ B cells derive from a common precursor or whether they originate from distinct progenitors remains an unresolved issue (Kipps, 1989). An additional complexity for the assignment of a specific role to human $CD5^+$ B cells comes from the observation that activation of B cells, with either phorbol esters (Miller and Gralow, 1984) or T cells and T cell supernatants (Vernino *et al.*, 1992b;

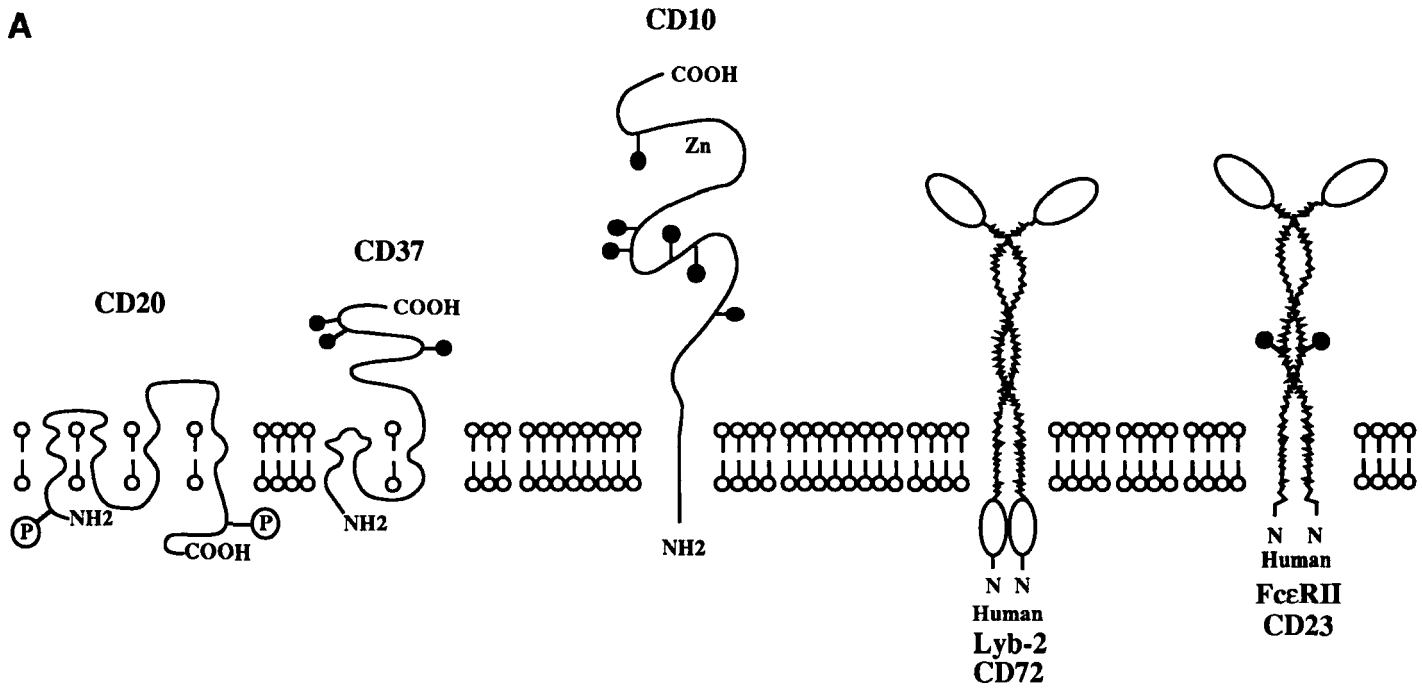


FIG. 6. Schematic representation of some of the most important antigens expressed on the surface of human B cells. (A) Molecules with type III multiple transmembrane domains as well as type II molecules with a cytoplasmic amino terminus. (B) Molecules of the Ig superfamily. (C) Other members. The open ovals represent Ig-like domains. Potential N-linked glycosylation sites and glycosphingolipid anchors are shown by ↟ and ⚲, respectively. Note that LFA-3 may also be a transmembrane protein.

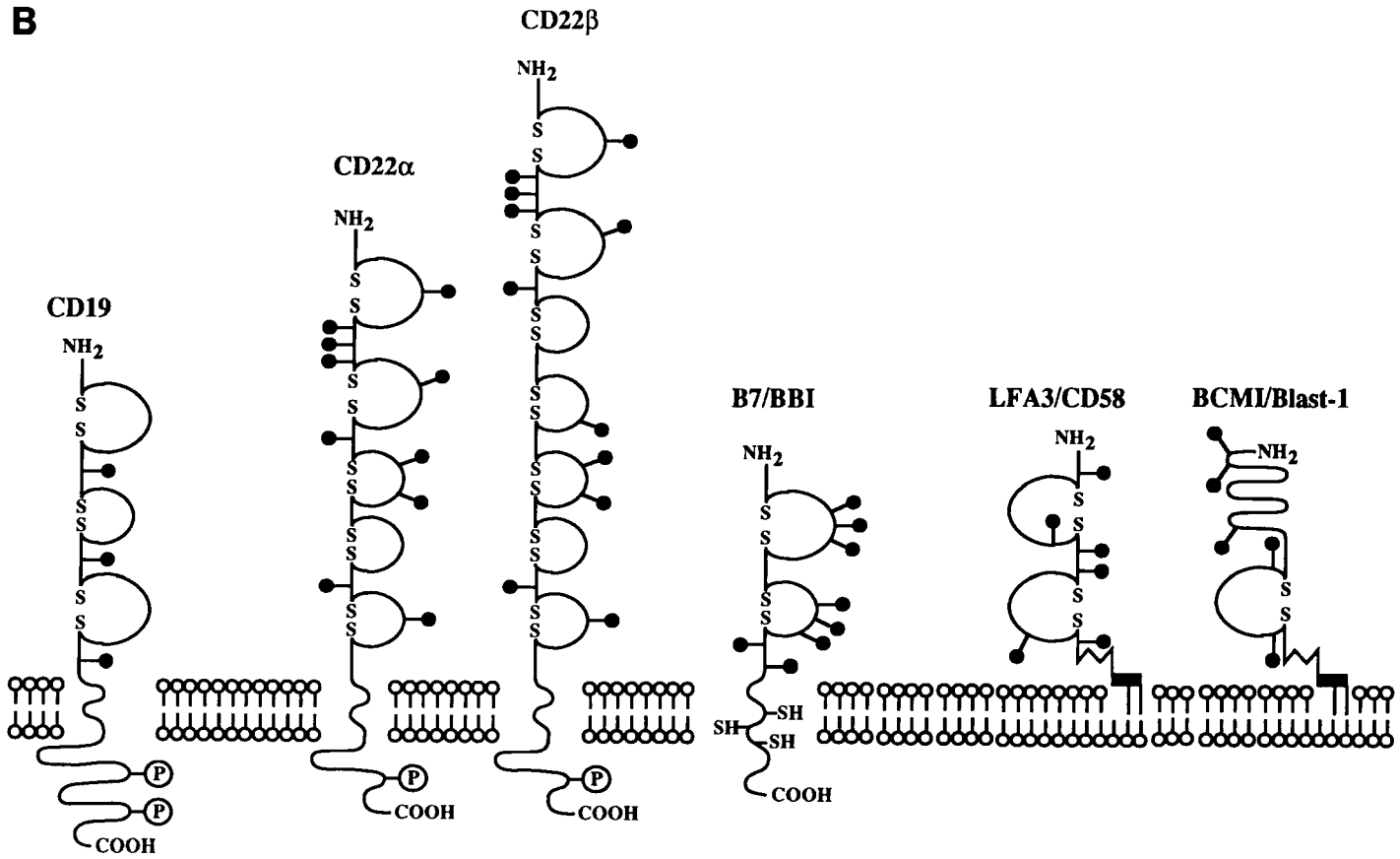


FIG. 6. Continued

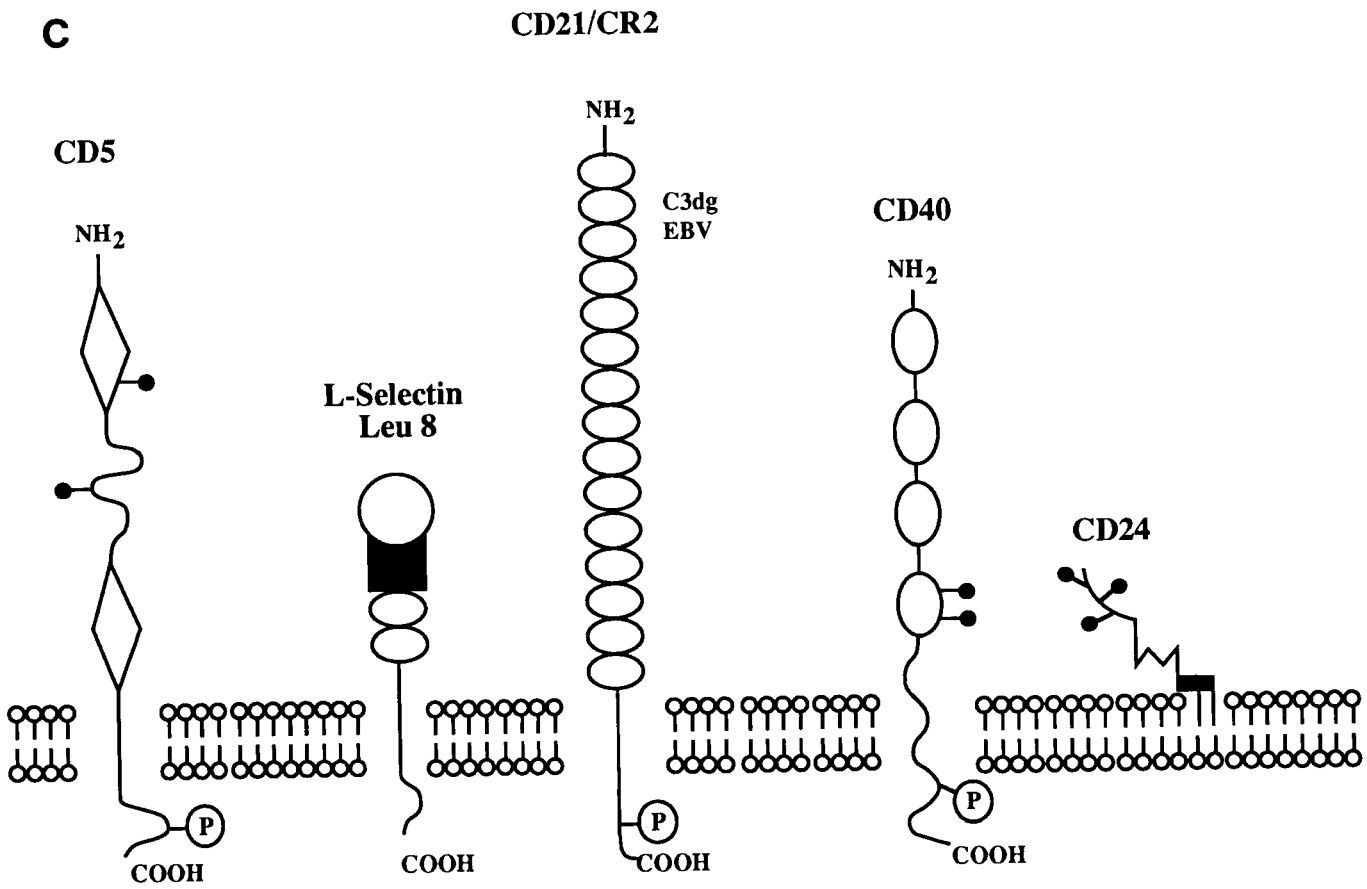


FIG. 6. Continued

Werner-Favre *et al.*, 1989), induces CD5 expression. In contrast, interleukin-4 (IL-4) downregulates CD5 expression on B cells (Defrance *et al.*, 1989). $CD5^+$ B cells produce mostly polyreactive or natural antibodies (Casali and Notkins, 1989). These antibodies, mostly of the IgM type, bind different antigens with low affinity. It has been proposed that these antibodies represent a first line of defense against invading microorganisms (Kocks and Rajewsky, 1989). These antibodies, by interacting with the large number of self constituents present in an organism, may also establish an extensive dynamic network that contributes to the general homeostasis of the organism (Avrameas, 1991). Some studies have indicated that circulating $CD5^+$ B cells may be increased in autoimmune diseases such as rheumatoid arthritis.

b. CD9

The CD9 antigen is expressed on progenitor B cells and a subpopulation of buoyant tonsil B cells (Zeleznik-Le and Metzgar, 1989). It is also present on platelets, eosinophils, basophils, and activated T lymphocytes. The protein is a 22- to 24-kDa glycoprotein of 227 AAs with four hydrophobic domains and one *N*-glycosylation site (Boucheix *et al.*, 1991). It is homologous to CD37, CD53, CD63 (Horejsi and Vlcek, 1991; Hotta *et al.*, 1988), and to an antigen of *Schistosoma mansoni.* Antibodies to CD9 induce homotypic adhesion of pre-B cell lines in a LFA-1-independent mechanism (Masellis-Smith *et al.*, 1990). Antibodies to CD9 also induce platelet aggregation in a CDw32-dependent fashion (Worthington *et al.*, 1990). The function of CD9 remains to be elucidated.

c. CD10 or CALLA

The common acute lymphoblastic leukemia-associated antigen (CD10) is a 95- to 100-kDa glycoprotein. It is expressed on lymphoid progenitor cells, germinal center B cells, renal epithelium, fibroblasts, and granulocytes. The CD10 cDNA predicts a 750-AA protein of type II (Box 1) with a short 25-AA amino-terminal cytoplasmic tail. It is a zinc metalloprotease neutral endopeptidase, earlier described as enkephalinase, that cleaves peptides at the amino terminus of the hydrophobic residues and therefore inactivates various polypeptidic mediators (LeBien and McCormack, 1989; Letarte *et al.*, 1988; Shipp *et al.*, 1988). In multiple organ systems, CD10 downregulates responses to peptide hormones. In particular, inhibition of CD10 activity was found to reduce dramatically the amount of Met-enkephalin required for neutrophil activation (Shipp *et al.*, 1991). It is not known which B cell tropic peptides are degraded by B cell CD10.

BOX 1
INTEGRAL PROTEINS OF MEMBRANES

Singer *et al.* (1987) have defined four types of structures of transmembrane integral proteins in membranes. Type I and II proteins are anchored in the membrane bilayer by a single stretch of about 20 nonionic and predominantly hydrophobic amino acids, almost certainly in an α-helical configuration. The amino terminus is extracellular in type I proteins and intracellular in type II proteins. Type III proteins have multiple hydrophobic stretches of the polypeptide chain embedded in the membrane. Type IV proteins are channel-forming subunit aggregates, with n identical or homologous subunits in the aggregates. The aqueous channel that runs through the type IV aggregate is the distinctive feature of this class of integral proteins.

d. CD19

The CD19 molecule is a glycosylated 95-kDa phosphoprotein that is expressed on all B cells throughout ontogeny and not on plasma cells. The only other cell type that may express CD19 is the follicular dendritic cell. Cytoplasmic DNAs (cDNAs) specific for CD19 have been isolated that code for a 75-kDa protein with a 273-AA extracellular domain containing five potential N-linked carbohydrate attachment sites and a cytoplasmic tail of 242 AAs. The extracellular part contains three Ig-like domains (which define the immunoglobulin superfamily) (Stamenkovic and Seed, 1988b; Tedder and Isaacs, 1989).

Recent studies have demonstrated that the CD19 antigen forms a multimolecular complex with the complement component C3 receptor CR2/CD21 and possibly three other molecules of 50, 20, and 14 kDa (Matsumoto *et al.*, 1991) (Fig. 7). The 20- and 14-kDa antigens have recently been characterized as the TAPA-1 antigen and the Leu 13 antigen, respectively (Bradbury *et al.*, 1991; Chen *et al.*, 1984; Takahashi *et al.*, 1990). It has been proposed that the CD19 molecule serves as a signal transducing element of CR2/CD21 which binds the iC3b and C3dg fragments (Fig. 8, Box 2) but whose 34-AA cytoplasmic portion appears to be too short to mediate signals (Fig. 7). The comodulation of CD19 with sIgM (Pesando *et al.*, 1989) suggests a possible association between the antigen receptor and CR2. This association may provide a mechanism by which complement-coated antigens strongly stimulate B cells through crosslinking of both surface molecules. Effect of coligation of CD19 with the sIgM is described in the Note Added in Proof (Section i).

Antibody binding to CD19 induces homotypic aggregation of B cells (Kansas and Tedder, 1991; Smith *et al.*, 1991) and a sustained increase in $[Ca^{2+}]_i$ levels. Anti-CD19 antibodies were found to inhibit anti-Ig-

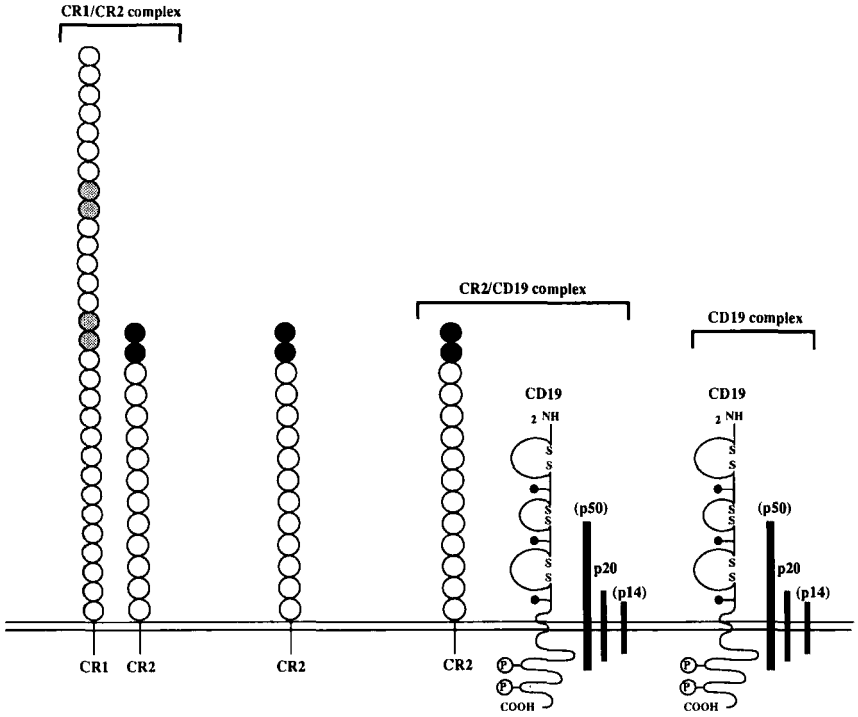


FIG. 7. Molecular complexes of CD19, CR2 (CD21), and CR1 (CD35) occurring on the surface of human B cells. The C_3b binding sites in CR1 are shown as shaded short consensus repeats (SCRs) and the iC_3b/C_3dg binding site in CR2 as black SCRs. The close contact between the C_3b binding sites in CR1 and the iC_3b/C_3dg binding site in CR2 may facilitate the transfer of C3 ligands from CR1 to CR2. The two independent CR1/CR2 and CD19/CR2 complexes may provide a mechanism for B cells to discriminate between activators of the alternative and classical complement pathways. The p50 and p14 components of the CD19 complex are shown in parentheses as their presence depends on cell sources. For details, see text and original references (Matsumoto *et al.*, 1991; Tuveson *et al.*, 1991).

induced increases in intracellular Ca^{2+}, as well as subsequent activation and proliferation of resting B cells (Barrett *et al.*, 1990; Gordon *et al.*, 1991; Pezzutto *et al.*, 1987; Rigley and Callard, 1991). Recent studies have indicated that the CD19 signal modulates proliferation in a biphasic fashion, first delaying then promoting it (Barrett *et al.*, 1990). Anti-CD19 antibodies induce activation of both phospholipase C and a protein tyrosine kinase, which seems to be different from that activated by antigen receptor triggering (Carter *et al.*, 1991a;b; Hebell *et al.*, 1991).

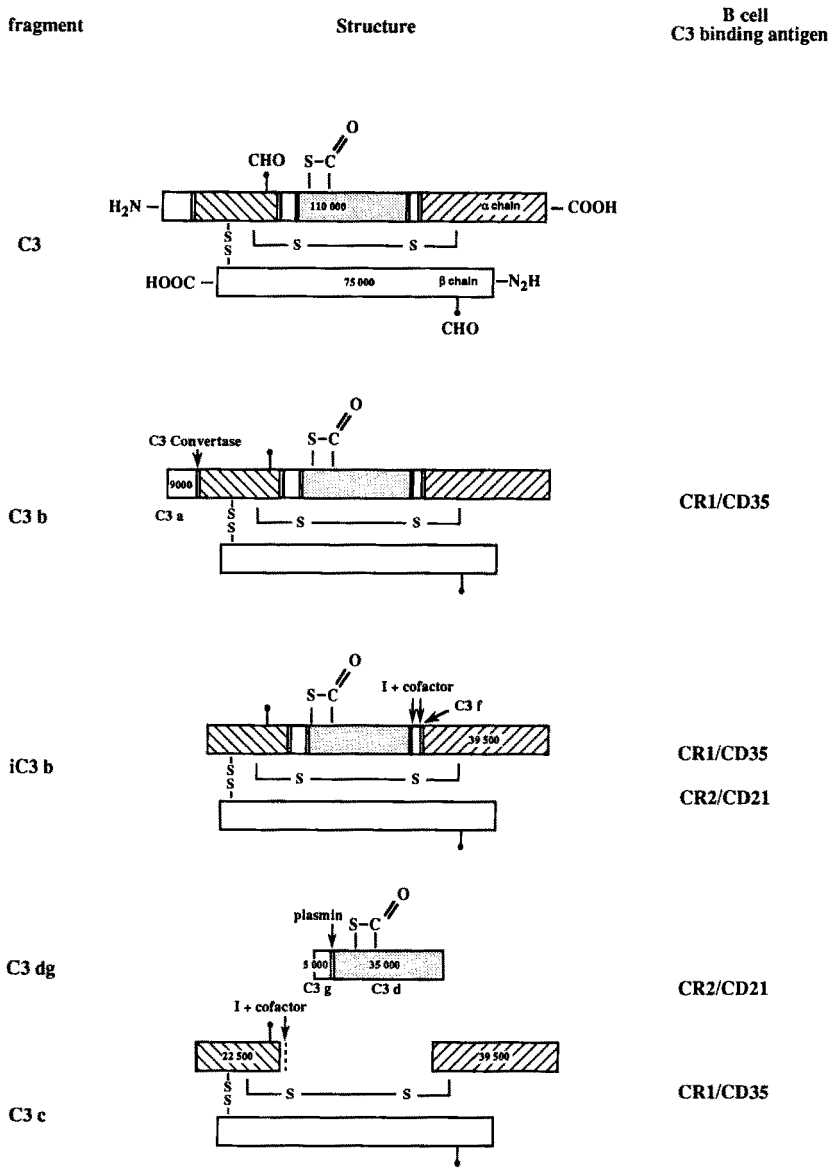


FIG. 8. C3 complement component degradation fragments (see Box 2). [Adapted, with permission, from Becherer *et al.* (1989).]

BOX 2
THIRD COMPONENT OF COMPLEMENT, C3

C3 is one of 30 complement proteins that play a pivotal role in both the classical and alternative pathways of complement activation. This 185-kD glycoprotein, the most abundant complement component in serum (1–2 g/liter), is composed of two polypeptides (a 110-kDa α chain and a 75-kDa β chain) linked by one covalent disulfide bond. Cleavage of C3 between amino acids 77 and 78 of the α chain, by C3 convertases generated by either the classical (C4b, 2a) or the alternative (C3b, Bb) pathway, leads to the production of C3b and C3a fragments. The C3b molecule can bind covalently, through an ester or amide bond, to the hydroxyl or amino groups present on cell surface molecules, soluble proteins, matrix components, or immune complexes. This covalent binding is a function of the thiolester group which involves the thiol group of Cys 988 and the γ-amide group of Gln 991, which are located in the C3d fragment. In contrast to native C3, C3b expresses multiple binding sites for other polypeptides [factors B, H, and I, C5, C4 binding protein = (C4bp), properdin, CR1/CD35, membrane cofactor protein (MCP)]. Binding of these proteins to C3b results either in amplification of C3 convertase and initiation of membrane attack complex or in inactivation by factor I cleavage to iC3b. This occurs in three steps and necessitates several cofactors. The cleavage of the α chain at two close sites liberates the 2-kDa C3f fragment and generates iC3b. Factor I further cleaves iC3b into C3c and C3dg. Finally, plasmin can further degrade C3dg into C3d and C3g. More details can be found in the book on C3 edited by Lambris (1990).

e. Complement Receptors: CR2 = CD21 and CR1 = CD35

B lymphocytes express two membrane proteins, CR1 and CR2, which interact with fragments of complement component C3. These receptors are homologous structures encoded by closely linked genes on chromosome 1q32 (Ahearn and Fearon, 1989; Cooper *et al.*, 1988). Complement receptor type 1 (CR1, CD35) is a 220-kDa protein that binds the primary activation fragment of C3, C3b (Fig. 8, Box 2). It also acts as a cofactor for the cleavage of C3b into iC3b and C3dg. CR1/CD35 is not specific to B cells as it is also expressed on erythrocytes, monocytes, follicular dendritic cells, granulocytes, and kidney glomerular podocytes. It is a glycoprotein whose extracellular domain is composed of 30 short consensus repeats (SCRs), each composed of 60–70 AAs and a short intracellular domain of 43 AAs. CR1/CD35 seems to be a rigid rodlike molecule that extends the ligand binding sites away from the membrane (Fig. 7). Immunoprecipitation studies using mild detergents preventing the dissociation of noncovalently linked polypeptide complexes have shown that most CR1/CD35 molecules form a complex with CR2/CD21 (Tuveson *et al.*, 1991). This

complex is different from the CR2/CD19 complex (Fig. 7). The CR1/CR2 complex may permit efficient capture of complement fragment C3b which is generated after activation of the alternative pathway. In keeping with this idea, the association of CR1 and CR2 should diminish the dissociation of the immunogen from the cell following proteolysis of C3b. Indeed, the conversion of C3b to iC3b generates a ligand with a 100-fold decrease in affinity for CR1 and a 10-fold increased affinity for CR2 (Kalli *et al.*, 1991).

Complement receptor type 2 (CR2/CD21) is a 145-kDa glycoprotein that binds the iC3b and C3dg fragments. The expression of this molecule is much more restricted than that of CR1/CD35. Besides B cells, it is expressed only on follicular dendritic cells and some T cells (Fischer *et al.*, 1991a). The extracellular domain is composed of 15 or 16 SCRs, thus distinguishing two isotypic forms. It also displays a short cytoplasmic tail of 34 AAs. On the surface of B cells, it can be free (or possibly associated with a 40-kDa protein of undefined function), form a complex with CR1 as discussed above, or form a complex with CD19 (Matsumoto *et al.*, 1991). Once CR2 has bound iC3b or C3dg, which results from CR1 cleavage of C3b, it may dissociate from the CR2/CR1 complex. In a second phase, it may bind to CD19 which then transduces the signal to B cells.

In addition to its complement receptor function, CD21/CR2 is also used by the EBV to attach to B cells through its gp350/220 envelope protein. The two amino-terminal SCRs are necessary and sufficient to bind both gp350/220 and C3dg (Carel *et al.*, 1990; Lowell *et al.*, 1989). Interestingly, a mAb recognizing the OKB7 epitope of CR2 blocks the binding of EBV and C3dg to B cells. Like EBV and C3dg, this mAb binds to the SCR1–SCR2 domain. Mabs binding to other SCRs do not activate B cells.

CR2/CD21 has recently been shown to bind recombinant interferon-α A (IFN-α A) inasmuch as this interferon subtype presents a motif homologous to the CR2 binding site on complement fragment C3dg (Delcayre *et al.*, 1991). As another IFN-α receptor with a totally different structure has been identified (Uzé *et al.*, 1990), this observation suggests that the IFN-α subtypes may bind to different molecules. Finally, CR2/CD21 has also been identified as the ligand for the soluble CD23 (Aubry *et al.*, 1992).

f. CD20

The CD20 molecule is a 35- to 37-kDa phosphoprotein uniquely expressed on B lymphocytes. It appears at the pro-B/pre-B transition before CD21 (Saeland *et al.*, 1991) and is lost at the plasma cell stage.

The CD20 cDNA sequence indicates that it possesses two cytoplasmic regions and four transmembrane domains (Einfeld *et al.*, 1988; Stamenkovic and Seed, 1988a; Tedder *et al.*, 1988). The multiple transmembrane domains of CD20 are reminiscent of those found in membrane transporters or ion channels. Indeed, it has been found that CD20 may be part of an ion channel or may regulate a membrane-associated calcium channel (Tedder *et al.*, 1990a). Such a function would be compatible with specific antibodies being either stimulatory (Clark *et al.*, 1985) or inhibitory (Tedder *et al.*, 1985) on B cell functions, according to whether they facilitate or inhibit opening of the channel. These stimulatory anti-CD20 antibodies share the ability of anti-IgM to induce *c-myc* gene expression and enhance the expression of major histocompatibility complex (MHC) class II antigen, CD18 (LFA-1β chain) and CD56 (LFA-3); however, in contrast to anti-IgM, anti-CD20 neither alters inositol phosphate metabolism and $[Ca^{2+}]_i$ nor enhances expression of CD54 (ICAM-1) and B1/BB7 (White *et al.*, 1991). The CD20 gene is 16 kb long and is composed of eight exons (Tedder *et al.*, 1989b). It is located on chromosome 11 near the *bcl-1* translocation site (Tedder *et al.*, 1989a). Both positive and negative *cis*-acting elements have been localized in the CD20 antigen promoter. One of these elements has been shown to bind a B cell-specific nuclear protein (Rieckmann *et al.*, 1991b).

g. CD22

CD22 is a heterodimer consisting of 130- and 140-kDa subunits. It is expressed on mature B cells, most particularly those from the marginal zone. During ontogeny, it is expressed in the cytoplasm of pre-B cells and its surface expression appears to be linked to that of CD21 (Boue and LeBien, 1988). Two cDNAs have recently been isolated that code for the two chains. They belong to the immunoglobulin superfamily (Stamenkovic and Seed, 1990; Stamenkovic *et al.*, 1991b; Wilson *et al.*, 1991). The smaller form, referred to as CD22α, has an extracellular region composed of five Ig-like domains. The larger form, CD22β, is a 847-AA glycoprotein that has a 140-AA intracytoplasmic domain and an extracellular domain composed of seven Ig-like domains. Both polypeptides have significant homology with three cell adhesion proteins: myelin-associated glycoprotein, neural cell adhesion molecule, and the vascular adhesion molecule V-CAM/I or INCAM-110 (Osborn *et al.*, 1989). CD22α mediates adhesion of B cells to erythrocytes and monocytes, whereas CD22β has recently been claimed to participate in B cell–B cell, as well as B cell–T cell interactions. Interestingly, the CD22 ligand is specifically expressed on the CD4 T cell subpopulation

and may be CD45 RO, a cell surface phosphotyrosine phosphatase. The CD22 ligand on B cells has been claimed to be another sialylated molecule, CDw75.

The pairing between T cell CD45 RO and B cell CD22α may result in the delivery of a stimulatory signal to the B cell, as addition of certain CD22 antibodies to dense resting B cells strongly enhances anti-IgM-mediated mobilization of $[Ca^{2+}]_i$ (Pezzutto *et al.*, 1988). The triggering of CD45 RO is likely to activate T cells, as a soluble form of CD22 and anti-CD45 RO antibody block anti-CD3-induced T cell activation.

h. CD23/FcεRII

CD23, which acts as a low-affinity receptor for IgE, has been the subject of many recent studies and reviews although its role is still a matter of some controversy (Conrad, 1990; Delespesse *et al.*, 1991; Gordon, 1991). It is a single-chain type II protein belonging to a family of membrane lectins including the asialoglycoprotein receptors and CD72, the counterstructure of CD5. Sequence analysis provides evidence of an extensive α-helical coiled-coil structure which forms a stalk separating the extracellular lectin heads from the membrane (Beavil *et al.*, 1992). FcεRII/CD23 appears to form dimers. It is expressed on IgM^+ IgD^+ B lymphocytes, some T cells, monocytes, Langerhans cells, platelets, eosinophils, and follicular dendritic cells. IL-4 strongly upregulates its expression on various cell types and interferon-γ (IFN-γ) inhibits IL-4-induced CD23 expression on B cells (Defrance *et al.*, 1987a; Kikutani *et al.*, 1986b). This latter effect is cell specific as IFN-γ enhances CD23 expression on Langerhans cells (Bieber *et al.*, 1989). In fact, there are two forms of CD23: the α form is specific to B cells, and the β form is expressed on many cell types including B cells (Yokota *et al.*, 1988). These two forms differ only in the first few amino acids of the cytoplasmic tail, as a result of alternate exon usage. The extracellular domain of CD23 is cleaved off through an autoproteolytic process into unstable 37- to 33-kDa fragments that subsequently yield a stable 25-kDa molecule which binds IgE (Letellier *et al.*, 1990) and also the CR2/CD21 (Aubrey *et al.*, 1992).

These soluble CD23-derived molecules have been found to display various biological effects, often in synergy with IL-1. In particular, sCD23 blocks the migration of monocytic cells (Flores-Romo *et al.*, 1990); induces the maturation of early thymocytes (Mossalayi *et al.*, 1990b), the proliferation of myeloid precursors (Mossalayi *et al.*, 1990a), and the differentiation of germinal center B cells into plasmablasts (Liu *et al.*, 1991a); and stimulates IL-4-induced IgE synthesis

(Pène *et al.*, 1988c). Soluble and membrane CD23 have also been demonstrated to display B cell growth activity (Cairns and Gordon, 1990; Gordon *et al.*, 1988a), but this observation has not been confirmed by others (Uchibayashi *et al.*, 1989). As anti-CD23 antibodies have been shown to be mitogenic for B cells (Gordon *et al.*, 1986a), it has been proposed that CD23 may be associated with the receptor for 12-kDa BCGF. This concept seems to have been abandoned (Gordon *et al.*, 1986b; Guy and Gordon, 1987). B cells expressing CD23 appear to be very efficient in IgE-dependent antigen presentation to T cells, indicating that specific IgE antibodies may amplify the antigen-specific T cell limb of the immune response (Kehry and Yamashita, 1989; Pirron *et al.*, 1990). Consistent with this, IgE and IgE-derived peptides were found to inhibit antigen presentation to T cells (Flores-Romo *et al.*, 1990).

Finally, recent studies have shown that crosslinking of CD23 on B cells with IgE immune complexes inhibits subsequent proliferation and differentiation, including IgE production (Luo *et al.*, 1991; Sherr *et al.*, 1989). This may limit the recruitment of new IgE-producing cells and, together with increased CD23-mediated antigen presenting capacity, may represent a way to facilitate IgG and IgA immune responses. This would indicate that IgE plays an amplification role in the humoral response (Mudde *et al.*, 1990). CD23 may also be involved in cellular adhesion as various cell lines form aggregates after transfection of CD23 and as $CD23^+$ cell lines can form conjugates with T cells that are blocked with anti-CD23 antibodies (Shields *et al.*, 1992).

Engagement of CD23 on activated, but not resting, normal B lymphocytes results in a marked increase in phosphoinositide hydrolysis with an early rise in the generation of inositol 1,4,5-triphosphate (Ins P3), which is followed by a rise in $[Ca^{2+}]_i$ resulting from the mobilization of an intracytoplasmic pool (Kolb *et al.*, 1991). These events involve a GTP-binding protein that is not sensitive to *Pertussis* toxin. In contrast, ligation of CD23 on monocytes does not induce early metabolic events, suggesting an association with a different set of proteins (Kolb *et al.*, 1991). The CD23/HLA-DR complex (Bonnefoy *et al.*, 1988b; Flores-Romo *et al.*, 1990) may play an important role in the transduction of these early signals as crosslinking of HLA-DR molecules also results in $[Ca^{2+}]_i$ mobilization and generation of Ins P3 (Lane *et al.*, 1990; Mooney *et al.*, 1990). Anti-CD23 antibody immunoprecipitates, together with CD23, several tyrosine phosphorylated proteins, the most prominent of which is 59 kDa. The precipitated complex includes the *src* family tyrosine kinase $p59^{lyn}$ (Sugie *et al.*, 1991).

i. CD24

CD24 is a molecule found on the surface of most human B cells , as well as on granulocytes. Progenitor B cells from the bone marrow express CD24 at high density (Duperray *et al.*, 1990). Biochemical analysis has shown that CD24 is a set of glycoproteins of 35 to 45 kDa (Pirruccello and LeBien, 1986). They are attached to the plasma membrane via a glycosyl-phosphatidylinositol (GPI) anchor (Fischer *et al.*, 1990; for reviews on GPI linked proteins, Cross, 1990; Robinson, 1991). A CD24-specific cDNA was recently isolated and found to encode a surprisingly short mature peptide of 31–35 amino acids that possesses three potential *N*-glycosylation sites and multiple *O*-glycosylation sites (Kay *et al.*, 1991). It may represent a member of a larger family that includes the murine antigen M1/69-J11d (Kay *et al.*, 1990). Anti-CD24 monoclonal antibodies suppress B cell differentiation but stimulate B cell proliferation and induce a rapid rise in $[Ca^{2+}]_i$ (Fischer *et al.*, 1990). A recent study (Stefanova *et al.*, 1991) has shown that GPI-anchored membrane glycoproteins are associated with protein tyrosine kinases, and this association may explain the signal transducing capacity of these molecules. It is not known whether the kinases interact directly with the glycolipid anchor or through interaction with other proteins. Heavy glycosylation of CD24 suggests that its putative ligand may be a lectin-like molecule.

j. CDw32/FcγRII

Receptors specific for the Fc domains of IgG molecules are present on various cell types such as B and T lymphocytes, natural killer (NK) cells, monocytes, macrophages, dendritic cells, and granulocytes. They connect the humoral arm of the immune system with these effector cells and mediate such effector functions as phagocytosis, release of inflammatory and immunoregulatory substances, antibody-dependent cellular cytotoxicity, clearance of immune complexes, activation of complement, and regulation of humoral immunity. It has been shown that monocytes/macrophages and granulocytes express the high-affinity FcγRI/CD64 and the low-affinity FcγRII/CDw32 and FcγRIII/CD16, whereas B lymphocytes bear only FcγRII/CDw32 (for reviews on FcγRs structure see Ravetch and Kinet, 1991; Unkeless *et al.*, 1988). The CDw32/FcγRII is a 40-kD glycoprotein. In human, three homologous genes—FcγRIIA, FcγRIIB, FcγRIIC—have been identified. Their extracellular portion contains two Ig-like domains. B lymphocytes essentially express the FcγRIIB form, whereas monocytes/macrophages express the three forms. Three FcγRIIB have been identified that are referred to as b_1, b_2, and b_3. They contain

identical extracellular and transmembrane domains but a distinct intracellular domain as a consequence of alternative splicing.

FcγRII/CDw32 have an important regulatory effect on antibody production of B cells, in particular, crosslinking of B cell sIg with intact anti-Ig, the Fc fragment of which binds to FcγRII/CDw32, has been shown to inhibit *in vitro* B cell activation, proliferation, and differentiation. In addition, many studies have shown an inhibitory effect of IgG immune complexes on humoral immunity *in vivo* (Sinclair and Panoskaltsis, 1987).

As noted earlier with murine B cells (Amigorena *et al.*, 1989; Snapper *et al.*, 1989), activation of human B lymphocytes results in an increased expression of FcγRII (Sarmay *et al.*, 1991). B lymphocyte activation also results in increased production of soluble fragments of FcγRII, which bind human Fc. These fragments result from the proteolytic cleavage of the membrane molecule. Studies with murine B cells have shown that supernatants of TH_1 T cell clones (see Section III.A) can enhance the expression of FcγRII on B cells (Snapper *et al.*, 1989). In contrast, supernatants of TH_2 clones inhibit, through IL-4, FcγRII expression on B lymphocytes (Laszlo and Dickler, 1988; Snapper *et al.*, 1989). This IL-4-induced downregulation of FcγRII reverses the inhibition of B cell proliferation resulting from crosslinking of FcγRII and sIg by intact anti-Ig (O'Garra *et al.*, 1987; Philips *et al.*, 1988). This effect of IL-4 would therefore be consistent with an amplification of humoral responses, a property of TH_2 cells.

k. CD37

CD37 is a 40-kDa glycoprotein expressed on all B cells. It is also weakly expressed on other leukocytes, CD34 hematopoietic progenitors, epithelium, and glial cells. It is composed of 281 AAs and possesses four transmembrane domains with both amino and carboxy termini localized intracellularly (Classon *et al.*, 1989, 1990). This structure suggests a possible role as an ion channel. It is a member of a novel family that includes CD9; CD53, a pan leukocyte antigen; and CD63, a molecule localized primarily in lysosomal membranes but that can be translocated to the cell surface on activation. This family also encompasses other unclustered molecules, such as TAPA-1,engagement of which results in inhibition of lymphoma cell proliferation (Andria *et al.*, 1991; Oren *et al.*, 1990).

l. CD38

CD38 (T10) is a 46-kDa glycoprotein expressed on cells during the early and late stages, but not the intermediate stages, of T and B

lymphocyte maturation. In particular, germinal center B cells and plasma cells (Stashenko *et al.*, 1980) express CD38. A specific cDNA has been isolated that encodes for a 300-AA polypeptide, a member of the type II integral membrane protein family. It displays four N-linked glycosylation sites and a short (19-AA) cytoplasmic tail. CD38 displays no significant homology with any other human molecule (Jackson and Bell, 1990). It may be the receptor for an as yet uncharacterized proliferation signal as crosslinking of the CD38 molecule by specific antibodies induces activation and proliferation of T cells (Funaro *et al.*, 1990).

m. CD40

The CD40 molecule is a 48-kDa glycosylated phosphoprotein, the expression of which was originally thought to be restricted to B lymphocytes, as well as certain solid tumors and carcinomas; however, CD40 has since been detected on follicular dendritic cells, Langerhans cells, dendritic cells, and activated macrophages. All these cells have potent antigen presenting capacity. CD40 was recently found on $CD34^+$ hematopoietic progenitor cells, indicating that CD40 is expressed on B cell precursors even before CD19 (Saeland *et al.*, 1992). In the human thymus, epithelial cells of both cortex and medulla, as well as interdigitating cells and B lymphocytes of the medulla, express CD40 (Galy and Spits, 1992).

The mature molecule is composed of 277 AAs with a 172-AA extracellular domain and a 62-AA intracellular tail (Stamenkovic *et al.*, 1989a). CD40 is a member of the recently identified family of the nerve growth factor (NGF) receptor (Fig. 9) (Mallett and Barclay, 1991). In addition to the low-affinity 80-kDa NGF receptor (Johnson *et al.*, 1986), this family is composed of both the 60- and 80-kDa tumor necrosis factor (TNF) receptors (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990); CD27, a molecule expressed on T cells and activated B cells (Camerini *et al.*, 1991); OX40, a rat activated T cell antigen (Mallett *et al.*, 1990); 4-1 BB, a T cell molecule (Kwon and Weissman, 1989); Fas/Apo, a lymphocyte antigen whose triggering induces apoptosis (Box 3) (Itoh *et al.*, 1991; Krammer *et al.*, 1991); SFV-T2, an open reading frame in the Shope fibroma virus, the product of which binds TNF (Upton *et al.*, 1987) ; SaIF19R, an open reading frame in the vaccinia virus (Howard *et al.*, 1991). Description of a new member of the NGF receptor family is reported in the Note Added in Proof (Section ii). Members of this family are characterized by the presence, in the extracellular domain, of three or four cysteine-rich motifs of approximately 40 AAs. In contrast to other superfamilies, in

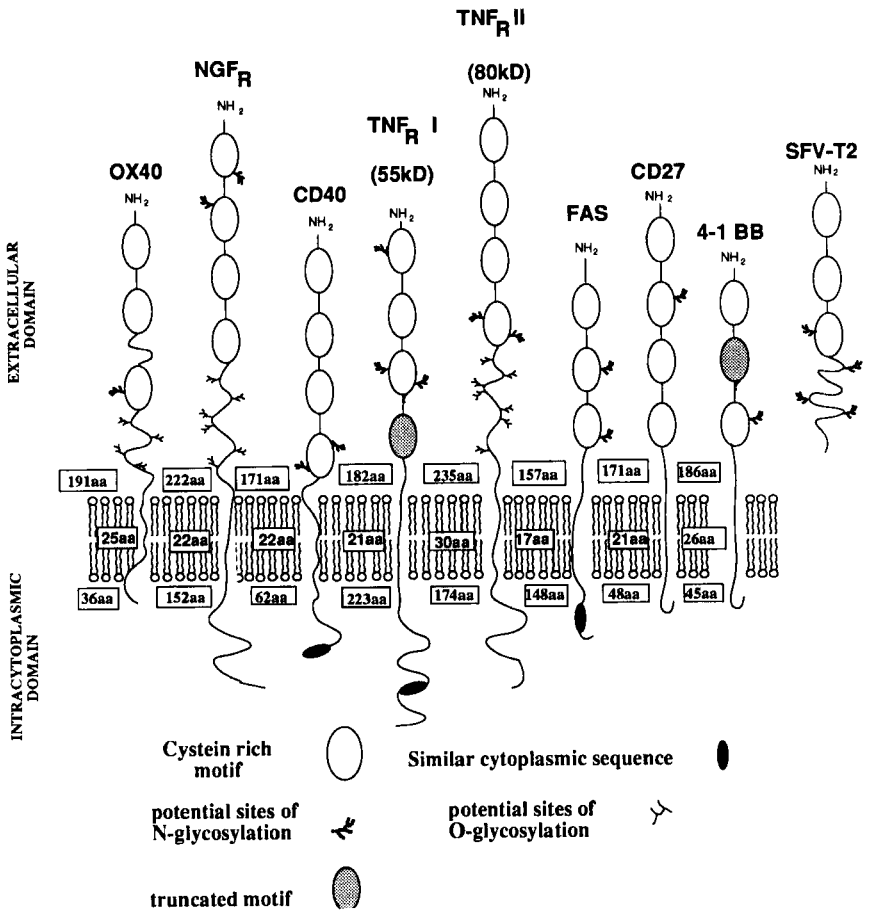


FIG. 9. Members of the nerve growth factor receptor family (see text).

which domains such as the Ig-like and epidermal growth factor-like domains are encoded by a single exon, each motif of the NGF receptor (NGF-R) superfamily is not encoded by a single exon. Interestingly, three members of this family have been shown to bind to more than one ligand: each TNF receptor (TNF-R) binds both TNF-α and TNF-β, whereas the NGF-R binds NGF (Hempstead *et al.*, 1989), the closely related brain-derived neurotropic factor (BDNF) (Rodriguez-Tebar *et al.*, 1990), neurotropin-3 (NT-3) (Squinto *et al.*, 1991), and neurotropin

BOX 3

APOPTOSIS OR PROGRAMMED CELL DEATH

Apoptosis or programmed cell death represents an active process that provides an additional means of precisely regulating cell numbers and biological activities (Cohen, 1991; Golstein *et al.*, 1991; Tomei and Cope, 1991; Williams, 1991). It is characterized by specific morphological changes that include nuclear condensation, chromatin condensation, cytoplasmic organelle compaction, degradation of DNA to oligonucleosomal fragments, and, in some cases, dependence on protein synthesis. Apoptotic cells are disposed off, before they burst, by specific recognition and phagocytosis (the tingible bodies in germinal center macrophages). There is no leakage of intracellular components, inflammation, or scar formation. The process is remarkably fast and can proceed to completion within a few hours. Entry into apoptosis is linked to the induction of a set of genes, few of which have been characterized. In particular, it involves a Ca^{2+}- and Mg^{2+}-dependent endonuclease that clips DNA at internucleosomal sites, providing the widely used biochemical marker of apoptosis (Wyllie, 1980). It also involves Ca^{2+}-dependent transglutaminases which form a detergent-resistant protein envelope under the plasma membrane by crosslinking proteins (Fesus *et al.*, 1989). The phenomenon occurs during various physiological processes, such as embryonic development and the clonal selection of T and B lymphocytes in response to environmental information. Triggering of tumor necrosis factor receptors and of the APO-1/Fas antigen results in apoptosis. In contrast, apoptosis may be prevented by triggering molecules, such as CD40. In particular, the *bcl2* protooncogene, isolated from the breakpoint of the 14–18 translocation found in most human follicular B cell lymphomas, appears to inhibit apoptosis. Germinal center B cells that do not express the *bcl2* protein and kill themselves by apoptosis express this protein in response to signals preventing their death. These stimuli are anti-Ig antibody, anti-CD40 antibody, and the combination of soluble CD23 and IL-1α (Liu *et al.*, 1989, 1991b). There are many occasions for a B cell to undergo apoptosis during its life. Pro-B cells failing to rearrange properly their heavy-chain genes and pre-B cells failing to rearrange their light-chain genes are probably deleted by apoptosis. An immature $sIgM^+$ $sIgD^-$ B cell encountering an antigen, in the absence of appropriate accessory cell/T cell help, will be clonally aborted. This represents a way to eliminate self-reactive B cells. Eighty to ninety percent of newly produced mature $sIgM^+$ $sIgD^+$ B cells have been shown to die within 24 hours, most likely because they do not encounter antigens fitting their randomly generated antigen binding sites. This may conceptually be because of the lack of invading antigens or because of a nonsense antigen binding site. Furthermore, B cells are likely to succumb to apoptosis during the hypermutation of Ig variable region genes occurring in germinal centers after antigen stimulation. The germinal center cells can be rescued by recognition of antigen following generation of surface Igs of higher affinity. Finally, it is not known whether plasma cells, which have a determined lifetime, die from apoptosis or from metabolic exhaustion.

4 (NT-4) (Hallböök *et al.*, 1991). Recently, it has been shown that the transmembrane tyrosine kinase, gp140 *trk*, the product of the proto-oncogene *trk*, contributes to the formation of the high-affinity receptor for NGF. Two other structurally related tyrosine kinase receptors, *trk* B and *trk* C, have been identified, which bind some of the neurotropic factors with high affinity (Bothwell, 1991; Lamballe *et al.*, 1991). The nature of the CD40 ligand remains to be established is described in the Note Added in Proof (Section iii).

Anti-CD40 antibodies were initially described according to their ability to enhance DNA synthesis of B cells stimulated with either anti-Ig on anti-CD20 mAb or PMA. As triggering of CD40 has now been shown to strongly activate B cells for further proliferation and differentiation, this topic is covered in detail in subsequent sections. Addition of anti-CD40 antibody to B cells results in enhanced tyrosine phosphorylation of four distinct phosphoproteins of 67, 72, 96, and 113 kDa and a rapid increase in the production of inositol 1,4,5-triphosphate, which is inhibited by protein tyrosine kinase inhibitors. CD40 crosslinking also results in activation of several serine/threonine-specific protein kinases (Uckun *et al.*, 1991). The substitution of thr^{234} for *Ala* in the CD40 cytoplasmic domain results in a mutant unable to transduce signals (Inui *et al.*, 1990). It has been proposed that CD40 may function to receive and regulate IL-6-dependent signals in B cells (Clark and Shu, 1990). Crosslinking of CD40 antigen results in a strong homotypic adhesion of B lymphocytes, which is mediated primarily through LFA-1 and ICAM-1 (Barrett *et al.*, 1991; Gordon *et al.*, 1988b; Kansas and Tedder, 1991); however, a second previously unknown adhesion pathway is also activated (Kansas and Tedder, 1991).

n. CD44

CD44 is a 90-kDa glycoprotein first identified for its role in endothelial cell recognition and lymphocyte trafficking in humans (Jalkanen *et al.*, 1986).It is expressed on virtually all blood mononuclear cells including B lymphocytes. In secondary lymphoid organs, CD44 is expressed at high density on lymphocytes within the T cell areas and the mantle zone. Germinal center B cells express CD44 at very low density. CD44 is now known to be identical to the cellular hyaluronate receptor (Aruffo *et al.*, 1990; Culty *et al.*, 1990; Miyake *et al.*, 1990), mediating the adhesion of a variety of cells to hyaluronic acid in the extracellular matrix and on cell surfaces; however, the function of CD44 as a lymphocyte homing receptor appears to involve the binding

of CD44 to mucosal vascular addressins and not to hyaluronic acid (Culty *et al.*, 1990). CD44 also acts as a receptor for type I and IV collagen (Carter and Wayner, 1988). The 90-kDa form on hematopoietic cells is a 341-AA mature protein with a 248-AA ectodomain. It is heavily glycosylated with N-linked and O-linked oligosaccharides (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989b). It displays significant homology to cartilage link protein and proteoglycan. cDNA cloning has recently demonstrated the presence of two other high-molecular-weight CD44 molecules (Dougherty *et al.*, 1991; Stamenkovic *et al.*, 1991a). CD44 polypeptides have been shown to play an important role in regulating primary and metastatic tumor development *in vivo* (Sy *et al.*, 1991) and in the development of B lymphocytes in the bone marrow (Miyake *et al.*, 1990). Finally, it remains to be determined whether engagement of CD44 on B lymphocytes may result in enhanced activation as demonstrated with T lymphocytes (Denning *et al.*, 1990; Huet *et al.*, 1989).

o. CD45

The leukocyte common antigen CD45 is a family of several membrane glycoproteins that range from 180 to 240 kDa and are found on all hematopoietic cells except erythrocytes and their progenitors. Six forms of CD45 have been isolated and the various isoforms are controlled in a cell type-specific manner (Thomas, 1989). The CD45 gene is single copy and at least 130 kb long, containing 34 exons. The differential splicing events that give rise to the various CD45 isoforms involve three exons: A, B, and C. The B lymphocytes express the highest-molecular-weight form which uses the three exons. In contrast, thymocytes splice out the three exons, giving rise to the lower-molecular-weight form, and peripheral T cells use various combinations. B lymphocytes differentiating into plasma cells lose their high-molecular-weight CD45 and express the low-molecular-weight CD45RO (Jensen *et al.*, 1989; Zola *et al.*, 1990).

The intracytoplasmic domain, common to all isoforms, is composed of 705 AAs and comprises two subdomains of internal homology of about 300 AAs. It possesses phosphotyrosine phosphatase activity (Clark and Ledbetter, 1989a; Trowbridge, 1991). It is physically associated with the B cell antigen receptor and allows the transduction of a Ca^{2+}-mobilizing signal following antigen triggering by modulating the phosphorylation state of the antigen receptor subunits (Justement *et al.*, 1991). Antibodies to CD45 can inhibit B cell proliferation and differentiation induced by anti-Ig or anti-CD19 antibodies or *Staphy-*

lococcus aureus strain Cowan (SAC) but do not inhibit anti-CD40-induced B cell proliferation (Gruber *et al.*, 1989; Ledbetter *et al.*, 1988; Mittler *et al.*, 1987; Morikawa *et al.*, 1991).

p. CD48

CD48 or Blast-1 is a 43-kDa glycoprotein expressed on activated B cells, as well as T cells and monocytes (Staunton *et al.*, 1989b; Yokoyama *et al.*, 1991). It is a member of the Ig superfamily and is linked to the cytoplasmic membrane through a GPI anchor. This molecule is similar to LFA3/CD58, the ligand to CD2, and Blast-1/CD48 acts as an adhesion molecule that binds to itself.

q. CD72

CD72 is a glycosylated homodimer of 86 kDa that resolves in a doublet of 43/39 kDa. It is the same as the murine antigen earlier known as Lyb2 (von Hoegen *et al.*, 1991), whose crosslinking induces B cell proliferation and blocks T-dependent B cell differentiation (Nakayama *et al.*, 1989; Snow *et al.*, 1986; Subbarao and Mosier, 1984). CD72 is a relatively specific B cell marker that occurs, like CD19, at the earliest stages of B cell differentiation and is lost during terminal differentiation prior to the plasma cell stage. It is also expressed on macrophages and follicular dendritic cells. It is a member of the lectin family which includes CD23 (von Hoegen *et al.*, 1990). Anti-CD72 antibody enhances DNA synthesis of B cells activated through their sIgM or with a combination of anti-CD40 and IL-4 (Kamal *et al.*, 1991). Interestingly, CD72 was recently demonstrated to bind CD5 (Van de Velde *et al.*, 1991).The pairing of these two molecules, during T cell–B cell interactions, is likely to deliver intracellular signals to both the B cells and the T cells, as antibodies to CD5 have been shown to stimulate T cell proliferation (Ledbetter *et al.*, 1985; Spertini *et al.*, 1991; Vandenberghe and Ceuppens, 1991).

r. CD73

CD73 is a 69-kDa glycoprotein expressed on most B cells, particularly those from the mantle zones. CD73 is anchored in the membrane by a GPI moiety. It is also expressed on follicular dendritic cells and a small proportion of blood T cells. CD73 acts as an ecto-5′-nucleotidase and catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to their corresponding nucleosides. This alteration probably permits the intracellular transport of nontransportable 5′-nucleotides. Interestingly, ecto-5′-nucleotidase activity is markedly reduced in B cell deficiencies. After CD73

crosslinking, B cells display activation markers and enter into proliferation. The production of IgG by pokeweed mitogen- or EBV-stimulated B cells was found to be restricted to the CD73-positive population (Thompson and Ruedi, 1988).

s. CD74

CD74, the invariant chain (Ii) associated with HLA class II antigen α–β heterodimers, is a type II integral membrane glycoprotein of 218 AAs (Claesson *et al.*, 1983). It is present at low density on the surface of B cells, and the majority of HLA class II antigens exposed at the cell surface are devoid of CD74/Ii. CD74/Ii is also expressed at low density on a proportion of monocytes, histiocytes, and some epithelial cells. The intracellular class II α–β–Ii complex is a nine-subunit transmembrane protein complex that contains three α–β dimers associated with an Ii chain trimer (Roche *et al.*, 1991). CD74/Ii, when associated with class II antigens, prevents binding of peptides to the class II peptide binding cleft (Roche and Cresswell, 1990). CD74/Ii also directs the intracellular route of class II molecules to endosomes (Long, 1989). CD74/Ii is degraded, in a post-Golgi compartment, and detached from the HLA class II molecules, which can then charge the processed antigen and express it at the cell surface. The expression of CD74/Ii on the cell surface may be the result of trafficking outside the endosome (Koch *et al.*, 1991) and its function remains undetermined.

t. CDw75

CDw75 is a cell surface glycoprotein expressed by the majority of blood B cells and a subpopulation of blood T cells. It is expressed on germinal center B cells at higher density than on mantle zone B cells. Accordingly, *in vitro* activated B cells express increased CDw75 levels and the antigen is upregulated in late G_1 before the appearance of the nuclear activation antigen Ki67 (Erikstein *et al.*, 1992). Anti-CDw75 antibodies augment DNA replication in anti-IgM-activated B cells (Erikstein *et al.*, 1989).

A cDNA associated with CDw75 expression was cloned by Stamenkovic *et al.* (1990), who suggested that CDw75 is a β-galactoside α2,6-sialyltransferase, an enzyme involved in terminal glycosylation of N-linked oligosaccharides on both glycoproteins and glycolipids; however, a more recent study (Bast *et al.*, 1992) indicated that the CDw75 is not the sialyltransferase itself, but that this enzyme regulates the expression of CDw75, CD76, and HB-6 antigen on cell surfaces. This latter antigen is specifically expressed on plasma cell precursors (Lokhorst *et al.*, 1987). Thus, α2,6-sialyltransferase is a critical

regulatory step in the formation of CDw75, CD76, and HB6 antigens and an α2,6-linked sialic acid residue is an essential component of these antigens.

u. CD76

CD76 is expressed on mature resting IgM^+ IgD^+ B cells, some T cells, and some epithelial cell types. The specific antibodies recognize a carbohydrate antigen expressed on the sialylated glycosphingolipid IV6 Neu Acn Lc4 Cer (Kniep *et al.*, 1990) and two glycoproteins of 85 and 67 kDa of presently undefined structure. The CD76 antigenic determinant can be generated by expression of the α2,6-sialyltransferase (Bast *et al.*, 1992).

v. CD77

The CD77 antigen is globotriaosylceramide (Gb3), a neutral glycosphingolipid, the sugar sequence of which is Gal α1–4 Gal β1–4 Glc (Nudelman *et al.*, 1983). It was formerly known as the antigen BLA for Burkitt lymphoma antigen. This antigen is also known as the blood group substance Pk, which accumulates in red blood cells of very rare individuals and which is a normal precursor in the biosynthetic pathway of the P blood group antigen. Apart from its relatively wide distribution in these exceptional individuals, the CD77 antigen is essentially expressed on hematopoietic cells, at high density, on a subpopulation of germinal center tonsillar B lymphocytes. In particular, CD77 is expressed on the centroblasts that constitute the dark zone. Accordingly $CD77^+$ B lymphocytes express sIgM, CD20, CD21, CD10, and CD38, but neither sIgD, CD23, or CD39. *In vitro* activation of resting B cells results in prompt induction of high levels of CD77 and thus mimics the *in vivo* antigen-dependent activation (Schwartz-Albiez *et al.*, 1990). As CD77 was initially described as being specific to Burkitt lymphoma cell lines, it has been proposed that Burkitt lymphomas would represent a tumoral development of centroblasts (Gregory *et al.*, 1987; Mangeney *et al.*, 1991).

Gb3 or CD77 is the cell surface receptor of both the *Escherichia coli* verotoxin (also known as Shiga-like toxin) (Waddell *et al.*, 1988, 1990) and the Shiga toxin produced by *Shigella dysenteriae* (Jacewicz *et al.*, 1986). Indeed, these toxins, consisting of two distinct chains, have been shown to be responsible for hemorrhagic colitis and hemolytic uremic syndrome, likely because they are cytotoxic for endothelial cells and renal cortex which express Gb3/CD77.These toxins kill a fraction of tonsillar B lymphocytes belonging to the IgG- and IgA-

committed subsets, whereas IgM-producing cells are resistant (Cohen *et al.*, 1990). The production of these toxins permits bacteria to prevent the host from developing an efficient secondary immune response against them. A recombinant verotoxin B subunit, responsible for the binding to CD77, also induces apoptosis of $CD77^+$ B cells (Mangeney *et al.*, 1992). It remains to be explained how the glycolipid can transduce the apoptotic signal inside cells. As centroblasts represent rapidly dividing B cells in which somatic mutations and isotype switching are supposed to occur and that undergo apoptosis in the absence of antigen selection (Liu *et al.*, 1989), it has been proposed that Gb3 may represent the centroblast receptor of a cytotoxic molecule triggering centroblast apoptosis. Recent studies indicate that B lymphocytes express different glycolipids at their cell surface at various maturation stages (Wiels *et al.*, 1991). In particular, germinal center B cells specifically express another neutral glycolipid, globoside (Madassery *et al.*, 1991), whereas myeloma cells express the ganglioside GM2.

w. Other Clustered Antigens

i. CD1c. The CD1c antigen is expressed on a significant proportion of adult and cord blood B lymphocytes (Durandy *et al.*, 1990; Small *et al.*, 1987). It is also expressed on some spleen and tonsil B cells.

ii. CD27. A B lymphocyte subpopulation in blood and tonsils expresses CD27 (Maurer *et al.*, 1990). These cells may represent a subpopulation of activated B cells as they express high levels of adhesion structures such as LFA-1, ICAM-1/CD54, and LFA-3/CD58. Furthermore, resting B cells can be induced to express CD27 following activation with SAC plus IL-2.

iii. CD39. CD39 is a heavily glycosylated 78-kDa glycoprotein with a 55-kDa core following removal of N-linked oligosaccharides. It is expressed on blood B cells, extrafollicular B cells, mantle zone B cells, but not germinal center B cells. Furthermore, it is also detected on activated T and B cells, macrophages, dendritic cells, and endothelial cells. Crosslinking of CD39 induces homotypic adhesion of B lymphocytes (Kansas *et al.*, 1991).

iv. CD69. CD69 is a 28/34-kDa dimeric structure representing a different glycosylation of a common 24-kDa protein backbone molecule. The CD69 antigen is expressed on T cells and B cells early after activation. Anti-CD69 antibodies costimulate with phorbol esters to induce DNA synthesis (Sanchez-Mateos *et al.*, 1989).

v. CDw78. CDw78 is a molecularly undefined antigen expressed

on B cells, as well as histiocytes and epithelial cells. Some antibodies have been proven to inhibit B cell proliferation (Kikutani *et al.*, 1986a), whereas others were found to be stimulatory (Funderud *et al.*, 1989).

2. *Nonclustered Antigens of B Lymphocytes*

a. HLA Class II Molecules

The class II antigens of the major histocompatibility complex (MHC) can be subdivided into three major isotypes (DR, DP, DQ) which are expressed at varying levels on B lymphocytes, macrophages, and dendritic cells. They are heterodimers comprising heavy (α) and light (β) glycoprotein chains that are encoded by genes located in the complex D region of the HLA system. At least six α-chain and ten β-chain genes have been identified (Trowsdale *et al.*, 1991).

The class II antigens of the MHC have a central role in the regulation of immune responses and present antigen derived peptides to T cells. In human, lack of class II antigens results in a severe combined immunodeficiency. In transgenic mice, which lack MHC class II antigen, there is a near-complete elimination of CD4 T cells. Such mice display normal numbers of B cells able to differentiate into plasma cells but very low circulating IgG levels. They are unable to respond to T-dependent antigens (Cosgrove *et al.*, 1991; Grusby *et al.*, 1991). Although the precise function of MHC class II antigens in the biological responses of B lymphocytes remains unknown, engagement of B lymphocyte class II antigens with antibodies is known to generate transmembrane signals. Immobilized anti-HLA class II antibodies were found to induce resting B cells to proliferate, particularly when cultures were supplemented with IL-4 (Lane *et al.*, 1990; Mooney *et al.*, 1989, 1990). As observed with mouse B lymphocytes (Cambier and Lehmann, 1989), the most potent stimulation is obtained with the combination of anti-IgM antibody, anti-MHC class II, and IL-4. Triggering of HLA-DR molecules by the staphylococcal toxic shock syndrome toxin TSST-1 (Box 4) or Fab fragments of some anti-HLA-DR antibodies or soluble CD4 induces an LFA-1-dependent aggregation of B lymphocytes (Kansas *et al.*, 1992; Mourad *et al.*, 1990). The observed adhesion was found to be associated with the activation of protein kinase C, but not with increased expression of LFA-1 or ICAM-1. These may reflect qualitative changes in either LFA-1 or ICAM-1, as described earlier in T cells (Dustin and Springer, 1989; Van Kooyk *et al.*, 1989). The stimulation of human B lymphocytes through MHC class II is associated with an increase of phosphatidylinositol turnover, an increase in $[Ca^{2+}]_i$, and an increase in tyrosine phosphorylation of various cellular proteins. Triggering of human B lymphocyte HLA

BOX 4
Staphylococcal Enterotoxins

Staphylococcal enterotoxins (SEs) form a family of basic secretory proteins of 22–20 kDa including SEA,SEB, SEC 1-3, SED, SEE, and the toxic shock syndrome toxin (TSST-1) (Marrack and Kappler, 1990). These toxins are responsible for various symptoms in humans including food poisoning and shock. They are among the best characterized superantigens and stimulate large numbers of T cells bearing particular Vβ gene products. T cell activation by these superantigens depends on the presence of MHC class II-bearing accessory cells but, unlike the response to nominal antigen, appears not to require processing. These toxins do not bind to the peptide groove on the class II molecule to which conventional antigens bind. They appear to engage the TCR-Vβ residues away from the complementarity-determining region. The different toxins apparently bind through their amino-terminal position (Buelow *et al.*, 1992) to distinct sites on the HLA class II molecules (Chintagumpala *et al.*, 1991). Interestingly, the toxins have detectable affinities for HLA class II antigens but have no direct affinity for the T cell receptors (Choi *et al.*, 1990; Fraser, 1989; Karp *et al.*, 1990; Mollick *et al.*, 1989; Sholl *et al.*, 1989). The shock symptoms induced by these toxins are caused by massive T cell stimulation and consequent release of cytokines, such as interleukin-2 and tumor necrosis factor (Marrack *et al.*, 1990).

class II antigens with monoclonal antibodies may not induce an increase in cAMP, in contrast to what has been reported earlier with murine B lymphocytes (Cambier *et al.*, 1987); however, the stimulation of quiescent murine B cells with IL-4 and antigen receptor ligation induces a change in the coupling of class II antigens to second messenger-generating systems. Then, triggering of class II antigens leads to tyrosine kinase-dependent activation of phospholipase C, leading to $[Ca^{2+}]$ mobilization from intracellular stores and extracellular space (Cambier *et al.*, 1991).

It is likely that the anti-HLA class II antibody-mediated activation of B cells mimics what happens during cognate T cell–B cell interactions when extensive aggregation of T cell receptor and B cell class II antigen occurs at the contacting surface (Vitetta *et al.*, 1987). Accordingly, soluble CD4, through binding to MHC class II antigens, induces protein phosphorylation and increased phosphatidylinositol turnover (Charron *et al.*, 1991). It should be emphasized that the stimulatory effects of anti-class II antibodies on human B lymphocytes have only been recently demonstrated and that most of the earlier studies in the field demonstrated such antibodies to inhibit B cell activation (Clement *et al.*, 1986; Giudizi *et al.*, 1987; Holte *et al.*, 1989; Tanaka *et al.*, 1988b). The basis for these opposite results is not clear and may

come from the antibodies and/or from differing experimental procedures, such as the use of either soluble or immobilized antibodies.

b. Adhesion Molecules

Three families of adhesion receptors have been shown to mediate adhesion of cells to other cells or to the extracellular matrix (Hemler, 1990; Kishimoto *et al.*, 1989; Springer, 1990). They include the integrin family, the immunoglobulin superfamily, and the selectin family.

i. Integrin Family. The integrin family comprises at least 15 cell surface heterodimeric glycoproteins and is divided in three subfamilies which are distinguished by their common use of a unique β chain (β1/CD29, β2/CD18, β3/CD61) noncovalently associated with several distinct α chains. Regarding the β2 subfamily, resting B lymphocytes express only LFA-1 (CD11a/CD18) and *in vitro* activation results in the expression of CD11c/CD18 (p150,95), whereas CD11b/CD18 (Mac 1) is not induced (Postigo *et al.*, 1991a). CD11c/CD18 is involved in both B cell activation and adhesion, as monoclonal antibodies against this molecule enhance DNA synthesis and block the binding of activated B cells to fibronectin. The β1 integrin subfamily includes receptors that bind to the extracellular matrix components fibronectin, laminin, and collagen. They have been designated VLA (very late activation), because two of them, VLA-1 and VLA-2, appear on lymphocytes 2–4 weeks after antigen stimulation *in vitro;* however, some of them are basally expressed on all leukocytes. In particular, VLA-4 (α4β1, CD49d/CD29) is expressed on resting blood B cells, although resident B lymphocytes of tonsils and Peyers patches virtually fail to express VLA-4. Following *in vitro* activation, a large proportion of these latter B cells express both chains of this heterodimer, as well as the α5 chain of VLA-5 (Postigo *et al.*, 1991b; Stupack *et al.*, 1991). The VLA-4 antigen of activated B cells associates with its counterstructure VCAM-1/INCAM 110 (a member of the Ig superfamily), which is expressed on follicular dendritic cells (Freedman *et al.*, 1990; Koopman *et al.*, 1991). The VLA-4 antigen (Osborn *et al.*, 1989; Rice *et al.*, 1990) expressed on progenitor B cells associates with VCAM-1 of bone marrow stromal cells (Ryan *et al.*, 1991). As VLA-4 and VLA-5 also bind to fibronectin, they may contribute to the interaction of B lymphocytes with the extracellular matrix. Like other members of the integrin family, the binding activity of VLA-4 depends on cellular activation, as resting blood B cells that express VLA-4 require activation to bind to fibronectin (Postigo *et al.*, 1991b).

ii. Immunoglobulin Superfamily. The immunoglobulin superfamily includes ICAM-1/CD54 and ICAM-2, two counterreceptors for LFA-1, as well as LFA-3/CD58, the counterreceptor for CD2. ICAM-1/CD54 is an inducible molecule that has five Ig-like domains, with the binding site for LFA-1 localized to specific residues in the first amino-terminal domain (Simmons *et al.*, 1988; Staunton *et al.*, 1988, 1990). ICAM-1 is expressed on all peripheral B cells including circulating, follicular mantle, and germinal center B cells (Dustin *et al.*, 1986). ICAM-2 is a highly glycosylated 60-kDa antigen that has only two Ig-like domains (Staunton *et al.*, 1989a). It is expressed at low levels on circulating B lymphocytes and is virtually absent from follicular mantle and germinal centers (de Fougerolles *et al.*, 1991; Nortamo *et al.*, 1991); however, ICAM-2 is strongly expressed on small clusters in germinal centers which are likely to correspond to follicular dendritic cells. Recent studies have demonstrated the presence of a third counterreceptor to LFA-1: ICAM-3 (de Fougerolles and Springer, 1992). ICAM-3 is a highly glycosylated 124-kDa protein expressed in high levels on all leukocytes including B lymphocytes. ICAM-1 has the greatest affinity for LFA-1; ICAM-2 and ICAM-3 have relatively lower affinities. The expression of ICAM-3 at high levels on resting B and T lymphocytes suggests an important role for this molecule in the generation of immune responses. ICAM-1, but not ICAM-2, binds to another molecule structurally related to LFA-1, Mac-1 (CD11b/CD18) (Diamond *et al.*, 1990); however, the binding site on ICAM-1 for Mac-1 is distinct from that for LFA-1 (Diamond *et al.*, 1991). Finally, CD43 represents a third ligand for ICAM-1 (Rosenstein *et al.*, 1991).

LFA-3/CD58, the ligand of CD2 (Selvaraj *et al.*, 1987), is a broadly distributed 40- to 65-kDa glycoprotein expressed on T and B lymphocytes, erythrocytes, granulocytes, endothelial cells, and fibroblasts. Its extracellular portion contains two Ig-like domains and is most homologous to its ligand CD2 (Seed, 1987; Wallner *et al.*, 1987). Interestingly, LFA-3/CD58 exists in two alternative forms: one is a conventional transmembrane-spanning molecule with a 12-AA intracellular domain, the other is a molecule anchored through a GPI moiety (Dustin *et al.*, 1987)

iii. Selectin Family. The selectin family is composed of three members: L-selectin (Leu-8, Mel-14, the lymph node homing receptor) (Camerini *et al.*, 1989; Tedder *et al.*, 1990b); P-selectin (CD62, GPM-140, PADGEM, a protein of activated platelets and endothelial cells); and E-selectin (ELAM-1, a protein of activated endothelial cells). Selectins have a 120-AA amino-terminal domain that is homol-

ogous to a variety of Ca^{2+}-dependent animal lectins including CD23, an approximately 40-AA epidermal growth factor motif, and a variable number of repeated units analogous to the short consensus repeats of complement regulatory proteins. Leu-8/L-selectin is expressed on many hematopoietic cells including the majority of blood B lymphocytes. Within lymph nodes and tonsils, Leu-8/L-selectin is absent from both B and T cells within germinal centers, but is present on nearly all paracortical and mantle zone B lymphocytes (Kansas *et al.*, 1985b). Accordingly, *in vitro* activation of Leu-8/L-selectin-positive B lymphocytes with anti-IgM and T cell supernatants downregulates its expression (Kansas *et al.*, 1985ba). Interestingly, the B cells giving rise to antibody-forming cells in the presence of T cells and pokeweed mitogen are essentially Leu-8/L-selectin negative, which correlates with their lack of sIgD expression (Kuritani and Cooper, 1982). It has been shown that anti-Leu-8 suppresses the Ig production of purified B cells stimulated with *Staphylococcus aureas* strain Cowan (SAC) and IL-2, which suggests that the natural ligand for Leu-8/L-selectin has important functions not only in B cell homing but also in B cell differentiation (Murakawa *et al.*, 1991).

iv. Activation of Adhesion Molecules. Transmembrane signals following binding of monoclonal antibodies to CD19, CD20, CD39, CD40, CD43, and HLA class II antigens induce rapid and strong homotypic adhesion of B cell lines (Barrett *et al.*, 1991; Kansas and Tedder, 1991). The binding of a soluble recombinant CD4/Ig heavy-chain fusion protein is able to induce homotypic adhesion comparable to that obtained with anti-HLA class II antibodies (Kansas *et al.*, 1992). Engagement of CD21, CD22, and CD23 results in lower levels of adhesion, whereas engagement of HLA class I, CD24, CD38, CD44, CD45 RA, and CD72 is inactive. Interestingly, adhesion is mediated in part through the LFA-1 pathway and in part through a presently unknown pathway (Kansas and Tedder, 1991).

c. B7/BB1

B7/BB1 is an activation antigen on B cells that is also expressed on macrophages and dendritic cells (Freedman *et al.*, 1987b; Vallé *et al.*, 1990; Yokochi *et al.*, 1981). The expression of B7/BB1 can be induced following crosslinking of sIgM or HLA class II antigens on resting B cells (Koulova *et al.*, 1991; Vallé *et al.*, 1991). It can be further upregulated with IL-2 and most notably IL-4. On monocytes, its expression can be induced by IFN-γ (Freedman *et al.*, 1991). B7 is a member of the Ig superfamily which is composed of a 216-AA extracellular do-

main, containing a single Ig-like domain, and a 16-AA intracellular domain (Freeman *et al.*, 1989).

B7 is a ligand for two homologous molecules, CD28 and CTLA-4 (Brunet *et al.*, 1988). CD28 is a 44-kDa homodimeric glycoprotein expressed on 95 percent of $CD4^+$ T cells and 50 percent of $CD8^+$ T cells (Linsley *et al.*, 1990). The triggering of T cells with specific anti-CD28 antibodies and B7 results in increased proliferation and production of cytokines (Gimmi *et al.*, 1991; Lindsten *et al.*, 1989; Linsley *et al.*, 1991). Thus the ligation of CD28 by B7 provides a signal that can amplify proliferation and lymphokine synthesis by activated T cells. If the B7-expressing cell is a monocyte or a dendritic cell, this will lead to the expansion of T cells. If the B7-expressing cell is an activated B cell, the latter will proliferate and differentiate in response to T cell cytokines, a phenomenon that may or may not be associated with the expansion of the T lymphocytes. Accordingly both anti-CD28 and anti-B7 antibodies have been found to inhibit T cell-dependent, induced B cell differentiation (Damle *et al.*, 1991). The B7 antigen also binds to CTLA-4 (Linsley *et al.*, 1991) which is a molecule homologous to CD28 (Harper *et al.*, 1991). CTLA-4–B7 interactions also appear to play an important role in T cell-dependent B cell activation, as a fusion protein of the CTLA-4 extracellular domain with an $IgC\gamma_1$ domain strongly inhibits T cell-dependent B cell differentiation. CTLA-4 has a higher affinity for B7 than CD28, although it seems to be expressed at lower density.

d. Cytokine Receptors on B Lymphocytes

The recent wave of cytokine receptor cloning identified several groups including the quickly expanding cytokine receptor family (Box 5), the NGF receptor family (discussed earlier under CD40), and the Ig superfamily (Dower *et al.*, 1990) (Fig. 10). Study of the expression of these receptors on human B lymphocytes is now in progress.

i. Interleukin-1. Two receptors that bind IL-1α, IL-1β, and IL-1RA (Dinarello and Thompson, 1991) have been molecularly identified. The 80-kDa type I receptor is a glycoprotein the extracellular portion of which displays three Ig-like domains and a 215-AA intracellular domain (Sims *et al.*, 1988). The 60-kDa type II receptor is a glycoprotein that contains an extracellular portion that also displays three Ig-like domains, but its intracellular domain is composed of only 29 AAs (McMahan *et al.*, 1991). There is evidence showing that the type II receptor is preferentially expressed on B cells (Benjamin and Dower, 1990; Bomsztyk *et al.*, 1989), whereas the type I receptor is

BOX 5
CYTOKINE RECEPTOR FAMILY

This novel receptor family is composed of type I membrane glycoproteins, characterized by two conserved motifs in the extracellular domain (Bazan, 1990; Nicola and Metcalf, 1991). The most extracellular motif, composed of approximately 60 amino acids, contains four conserved cysteines. The second motif, close to the membrane, is composed of around 50 amino acids and contains the conserved sequence Trp–Ser–X–Trp–Ser (WS–X–WS). This motif shares some homology with the type III modules of fibronectin (Patthy, 1990). This family includes the α chains of IL-3 receptor (Kitamura *et al.*, 1991), IL-5 receptor (Tavernier *et al.*, 1991) and GM-CSF receptor (Gearing *et al.*, 1989), as well as their common β chain (Gorman *et al.*, 1990), the two chains involved in the IL-6 receptor (Hibi *et al.*, 1990; Yamasaki *et al.*, 1988), the IL-2β receptor (Hatakeyama *et al.*, 1989), the IL-4 receptor (Galizzi *et al.*, 1990a; Idzerda *et al.*, 1990) the IL-7 receptor (Goodwin *et al.*, 1990), the G-CSF receptor (Larsen *et al.*, 1990), the erythropoietin receptor (D'Andrea *et al.*, 1989), the leukemia inhibitory factor receptor (Gearing *et al.*, 1991), the growth hormone receptors (Leung *et al.*, 1987) and the prolactin receptors (Boutin *et al.*, 1988). It also includes an oncogen, *v-mpl*, which is able to immortalize hematopoietic cells and which may be the receptor of a presently unidentified cytokine (Souyri *et al.*, 1990). Site-directed mutagenesis indicates a critical role of the two tryptophan residues in the proper folding of the IL-2Rβ extracellular domain and suggests that this may be true for the other family members (Miyazaki *et al.*, 1991). A recent study has suggested that the amino-terminal helix present in most cytokines contains the recognition element for the formation of high-affinity binding sites with the α subunit of their multicomponent receptors. The site of interaction on the α subunit could be the WS–X–WS motif (Shanafelt *et al.*, 1991). The intracellular domains of these receptors do not share sequence homologies altogether, although some receptors, such as those for IL-4, IL-2β, IL-3, and erythropoietin, share some similarities.

preferentially expressed on T cells and fibroblasts; however, these two receptors can also be coexpressed on various cell types. The pathways of internalization, intracellular trafficking, and overall processing of IL-1 are different following binding to type I and type II receptors (Horuk, 1991). Binding of ^{125}I-IL-1 to normal blood B lymphocytes demonstrates the presence of a major class of IL-1R (320 per cell) of intermediate affinity ($K_D = 3.8 \times 10^{-10}$ *M*) and a minor class of IL-1R (70 per cell) of very high affinity ($K_D = 4.4 \times 10^{-12}$ *M*). On B cell stimulation, the numbers of both intermediate- and high-affinity IL-1R increase to approximately 2000 and 360, respectively. Flow cytometry analysis with fluorescent IL-1α reveals that approximately 5% of the resting B cells and approximately 20% of the activated B cells express IL-1R (Tanaka *et al.*, 1988a). The proportions of the two IL-1R have however, not been established on these normal B cells.

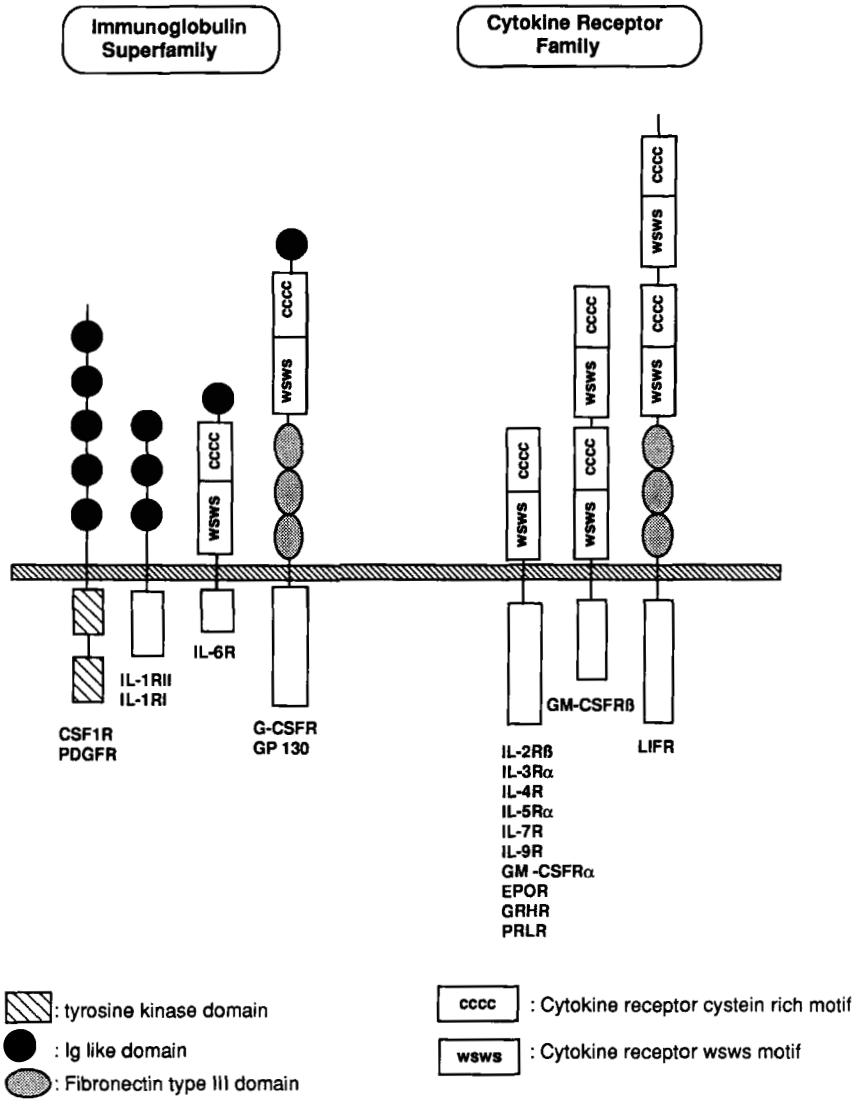


FIG. 10. Human Cytokine Receptors (see Box 5).

ii. Interleukin-2. As will be discussed in more detail later, there are three forms of cellular receptors for IL-2 based on their affinity for ^{125}I-IL-2. The high-affinity receptor results from the association of an α chain (p55, Tac, CD25) of low affinity with a β chain (p75) of intermediate affinity for IL-2. Conflicting results have been reported regarding

the expression of IL-2 receptors on B cells. It has been shown that blood and spleen B lymphocytes express low levels of p55/Tac but no p75 using specific monoclonal antibodies (Zola *et al.*, 1991). According to Tanaka *et al.* (1988a), high-density resting tonsillar B lymphocytes express neither chain, whereas large low-density B cells express the p75 β chain. Begley *et al.* (1990) detected p75 on tonsillar B cells by ^{125}I-IL-2 binding using equilibrium binding analysis and crosslinking followed by gel electrophoresis. Culturing B cells in the presence of IL-2 results in the expression of high- and low-affinity ^{125}I-IL-2 binding sites and expression of Tac/CD25. This is likely to explain the functional effects of IL-2 on B cells lacking Tac/CD25 (Bich-Thuy *et al.*, 1986; Saiki *et al.*, 1988; Tanaka *et al.*, 1987). Activation of B cells with polyclonal activators results in the expression of Tac/CD25 and the expression of high-affinity IL-2 receptors.

iii. Interleukin-4. High-density resting B cells express approximately 250 high-affinity IL-4 receptors, as determined by binding of ^{125}I-IL-4 (Zuber *et al.*, 1990). The expression of these receptors is increased following polyclonal activation to up to 1500 per cell and they may then be detected by flow cytometry using biotinylated IL-4. Crosslinking studies with ^{125}I-IL-4 show the presence of three labeled components of 70, 80, and 130 kDa as observed with B cell lines (Galizzi *et al.*, 1989). Whereas the 130-kDa component has been molecularly identified (Galizzi *et al.*, 1990a; Idzerda *et al.*, 1990), the nature of the 70- and 80-kDa components remains to be established.

iv. Interleukin-6. Binding studies with ^{125}I-IL-6 have indicated that small resting B cells express approximately 120 high-affinity IL-6 receptors, whereas large activated B cells express approximately 570 receptors per cell (Taga *et al.*, 1987). Flow cytometry analysis with a monoclonal antibody specific for the 80-kDa IL-6 receptor (Hirata *et al.*, 1989; Yamasaki *et al.*, 1988) failed to detect the 80-kDa protein on resting B cells; however, the IL-6 receptor can be detected on a small proportion of large, presumably *in vivo* activated B lymphocytes from blood and appendix (Fujihashi *et al.*, 1991; Lue *et al.*, 1991). These latter cells directly differentiate in response to exogeneous IL-6. The expression of the gp130 signal transducer (Taga *et al.*, 1989) has not yet been studied on normal B lymphocytes. More details concerning the gp130 are reported in the Note Added in Proof (Section iv).

v. Tumor Necrosis Factor α. TNF-α and -β bind with high affinity to two distinct receptors of 55 and 75 kDa (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990). As demonstrated by using specific monoclonal antibodies, resting B lymphocytes express low levels of the 75-kDa TNF-R and virtually no 55-kDa TNF-R. Follow-

ing activation, expression of the 75-kDa TNF-R is markedly increased. The 75-kDa TNF-R appears to be involved in the functional effects of TNF on B lymphocytes (Erikstein *et al.*, 1991; Heilig *et al.*, 1991).

vi. Interferon-γ. A cDNA specific for the human IFN-γ receptor has been isolated from a B cell line (Aguet *et al.*, 1988). Expression of IFN-γ receptors on normal human B lymphocytes has not been studied in detail. As IFN-γ acts on both resting and activated B cells, it is likely that they express IFN-γ receptors.

vii. Transforming Growth Factor β. ^{125}I-TGF-β_1 binding assays reveal that unactivated B cells express approximately 200 high-affinity receptors with an estimated K_D of 50 p*M* and approximately 7200 low-affinity binding sites with an estimated K_D of 1500 p*M* (Kehrl *et al.*, 1989). Crosslinking studies followed by gel electrophoresis indicate that TGF-β_1 binds to two species of 65 and 90 kDa, which are likely to correspond to the type I and type II receptors described later. B lymphocytes may not express the 200-kDa type III receptor. Interestingly, TGF-β_2 may interact with high-affinity receptors distinct from the TGF-β_1 receptors.

viii. Other Cytokines. A monoclonal antibody specific for a 90-kDa antigen, BA5, has been shown to inhibit the B cell proliferation induced by high-molecular-weight B cell growth factor (BCGF) (Ambrus *et al.*, 1988). It binds minimally to resting B cells and more significantly to activated B cells. The natural low-molecular-weight BCGF has been found to bind to activated B lymphocytes with both high and low affinity (Mehta *et al.*, 1986)

e. Other Antigens of B Lymphocytes

i. Bgp 95. The mAb G28-8 recognizes a 95-kDa glycoprotein expressed on mature B cells (Valentine *et al.*, 1988). G28-8 delivers signals to B cells comparable to those delivered by anti-IgM antibody. It induces $[Ca^{2+}]_i$ mobilization and proliferation of B cells when associated with IL-4 or anti-CD40 mAbs (Clark *et al.*, 1989).

ii. Immunoglobulin M-Binding Protein (Fcμ Receptor). Activated B lymphocytes express a 60-kDa glycoprotein that binds IgM, Fcμ receptor (FcμR). It is anchored on the cell surface through a GPI linkage (Ohno *et al.*, 1990; Sanders *et al.*, 1987). FcμR is not detectable on T cells, monocytes, or granulocytes. The function of FcμR remains to be determined.

iii. CK226 Antigen. The CK226 antigen is a 75-kDa molecule expressed on T and B lymphocytes. Antibody crosslinking of CK226 induces lymphocyte proliferation, which can be further increased by addition of IL-2 (Poggi *et al.*, 1990).

3. *Antigens of Plasmacytes*

a. PC-1: A Plasma Cell Threonine-Specific Protein Kinase

The murine plasma cell membrane glycoprotein PC-1 was discovered around 1970. PC-1 is a disulfide-linked homodimer containing two 120-kDa monomers. It is found in a small number of highly discrete locations mostly associated with epithelia. Strong expression is observed on the distal convoluted tubules of kidney, the chondrocytes, and the epididymis (Harahap and Goding, 1988). The recent cloning of both murine and human specific cDNAs indicates that PC-1 is a class II transmembrane protein comprising an 826-AA extracellular domain and a very short 24-AA intracytoplasmic domain (Buckley *et al.*, 1990; Van Driel and Goding, 1987). The extracellular domain displays an ATP binding site. The PC-1 molecule acts as a threonine-specific ectoprotein kinase. Thus PC-1 may phosphorylate certain secretory proteins or plasma membrane proteins when functionally expressed in the lumen of the endoplasmic reticulum or Golgi complex. Interestingly, PC-1 also possesses nucleotide phosphodiesterase activity (Oda *et al.*, 1991; Rebbe *et al.*, 1991). The role of PC-1 in the development of plasma cells remains to be determined.

b. Other Antigens of Plasmacytes

The T cell antigen CD28 is expressed at high levels on bone marrow plasma cells and myeloma cell lines (Kozbor *et al.*, 1987). Whether the triggering of CD28 with its specific ligands B7/BB1 and CTLA-4 occurs on plasma cells and plays a role in their proliferation remains to be established. The PCA-1 antigen, expressed at high density on plasma cells, is not detected on T and B lymphocytes, but is present at low density on granulocytes and monocytes (Anderson *et al.*, 1983).

III. Cytokines Involved in B Cell Growth and Differentiation

As it will be illustrated in subsequent sections, many cytokines affect B lymphocytes and their detailed descriptions can be found in the books recently edited by Sporn and Roberts (1990) and Thomson (1991). Some of the molecular features of these cytokines are displayed in Table IV. We describe more specifically five cytokines (interleukin-2, interleukin-4, interleukin-6, interleukin-10, and transforming growth factor β), as they appear to display quite important functions in B cells. As these cytokines are principally, although not exclusively, produced by T cells, we first briefly summarize the recent concept of TH_1 and TH_2 $CD4^+$ helper T cells which is of importance in the physiology of B lymphocytes.

TABLE IV
PROPERTIES OF HUMAN CYTOKINES

	AA[a] precursor	AA mature	M_r (kDa)	Sites of glycosylation	Residue cysteine	Gene chromosome	Exon
IL-1α	271	159	17.5	0	0	2q12–21	7
IL-1β	269	153	17.3	0	0	2q13–21	7
IL-1RA	177	152	18.2	1-*N*	2	?	?
IL-2	153	133	15–20	1-0	3	4q26–27	5
IL-3	152	133	14–30	2-*N*	—	5q23–32	5
IL-4	153	129	15–19	2-*N*	6	5q23–31	4
IL-5	134	115	45	2-*N*	2	5q23–31	4
IL-6	212	184	26	2-*N*	4	7p21	5
IL-7	177	152	20–28	3-*N*	6	8q12–13	6
IL-8	99	77	8	0	4	4q3–21	?
IL-9	144	126	20–30	4-*N*	10	5q23–31	5
IL-10	178	160	—	1-*N*	4	1	?
IL-11	199	179	—	0	0	?	?
SCF[b]	273	189	?	6-*N* (1-0)	4	?	6
IFN-γ	166	143	40–70	2-*N*	0	12q24	4
IFN-α	189/188	166/165	16–27	0	0	9p22	0
TNF-α[c]	233	157	35	0	2	6p21	4
TNF-β	205	171	60–70	1-*N*	0	6p21	4
TGF-β_1	391	2 × 112	25	0	9	19p13	7
GM-CSF	152	127	23	2-*N*	4	5q23–31	4

[a] AA, amino acid; IL, interleukin; SCF, stem cell factor; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-1RA, IL-1 receptor antagonist.

[b] SCF transmembrane protein = 273 AAs; signal peptide = 25 AAs; extracellular domain = 189 AAs; transmembrane domain = 23 AAs; intracellular domain = 36 AAs.

[c] TNF-α: the 76-AA signal peptide displays a transmembrane hydrophobic region.

A. NOTION OF TH_1 AND TH_2 $CD4^+$ HELPER T CELLS

Studies with murine T cell clones have established that IL-4 is produced mostly in conjunction with IL-5, IL-6, and IL-10 by TH_2 clones, whereas IL-2 and IFN-γ are produced by TH_1 clones. T cell clones producing IL-4, IL-2, and IFN-γ have also been identified that represent precursors (TH_0) of the TH_1 and TH_2 clones (Möller, 1991; Mosmann and Coffman, 1989a,b). These precursor cells are themselves derived from virgin T cells (TH_p), primary activation of which leads to IL-2 secretion. Recent data suggest that the same dichotomy may apply to human T cells (Del Prete *et al.*, 1991; Haanen *et al.*, 1991; Maggi *et al.*, 1991; Parronchi *et al.*, 1991; Romagnani, 1991; Wierenga *et al.*, 1990,1991; Yssel *et al.*, 1991). In particular, CD4 T cells infiltrating the conjunctiva of patients with vernal conjunctivitis or allergen-

specific T cell clones obtained from atopic patients are essentially of the TH_2 type. Furthermore, subjects with atopic asthma present an increased frequency of TH_2 cells in their bronchoalveolar lavage fluid (Robinson *et al.*, 1992). In contrast, CD4 T cells infiltrating the thyroid gland of patients with autoimmune thyroid disease and bacterial antigen-specific T cell clones are mostly of the TH_1 type.

An important feature of TH_1 and TH_2 cells is the ability of one subset to regulate the activities of the other. It occurs at the levels of the effector cells triggered by these subsets, as indicated by the inhibitory effect of IFN-γ on IL-4-induced B cell activation or those effects of IL-4 on IL-2-induced T and B lymphocyte proliferation. It also occurs directly at the level of these subsets as the products of one subset can antagonize the activation of the other: IFN-γ inhibits proliferation of TH_2 cells, whereas IL-10 inhibits cytokine production by TH_1 cells (Fig. 11). The mechanisms controlling the development of TH_1 and

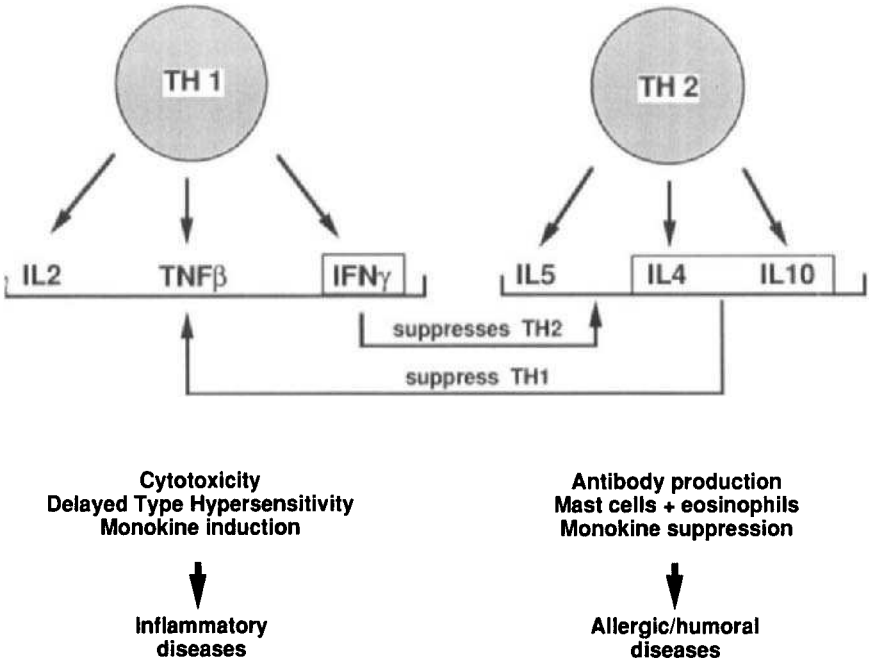


FIG. 11. Functional roles and cross-regulation of TH_1 and TH_2 helper T cells. Protective immunity results from a balanced activation of both subsets. Hyperactivation of either subset leads to immune-mediated disease. IL, interleukin; TNF, tumor necrosis factor.

TH_2 cells are poorly understood and are likely to depend on the antigen structure, the antigen presenting cells, and the steroid and cytokine environment.

In particular, recent studies have indicated that IL-4 may facilitate the development of TH_2 cells, whereas IFN-γ may allow the generation of TH_1 cells (Coffman *et al.*, 1991; Mosmann *et al.*, 1991; Swain *et al.*, 1991). IL-4 and IFN-γ would be produced by basophil/mast cells and NK cells, respectively.

TH_1 and TH_2 cells differ in their functions as might be expected from their capacity to produce different cytokines. TH_2 cells facilitate humoral responses, whereas TH_1 cells mediate delayed hypersensitivity responses and inflammatory reactions. Another major difference is their ability to differentially regulate the production of certain immunoglobulin isotypes. TH_2 cells are particularly effective in promoting IgE responses because of the critical role of IL-4 in this isotype switching (Finkelman *et al.*, 1990).

These two cell subsets normally function in a balanced fashion. Exposure to various pathogens may result in the dominant role of one subset. Predominance of TH_1 responses results in chronic inflammation, whereas predominance of TH_2 responses results in allergy and hypergammaglobulinemic states (Peltz, 1991) (Fig. 11). Helminthic parasites facilitate TH_2 responses and induce high levels of IgE (IL-4-dependent) and increased numbers of eosinophils (IL-5-dependent). Infection of animals with bacteria such as *Brucella abortus* or viruses triggers mostly TH_1 responses.

B. Interleukin-2

IL-2, a cytokine essentially produced by T lymphocytes, was characterized in 1976 for its ability to induce sustained *in vitro* growth of T lymphocytes. According to Ehlers and Smith (1991) and W. T. Lee *et al.* (1990), IL-2 is produced by both naive and memory T cells, whereas IL-4 and IFN-γ are produced mostly by memory T cells. IL-2 binds to a receptor (IL-2R) composed of at least three chains (Waldmann, 1991).

The IL-2R α chain (Tac, CD25, p55) and β chain (p75) bind IL-2 with a low affinity ($K_D = 10^{-8}\ M$) and intermediate affinity ($K_D = 10^{-9}\ M$), respectively. When they are associated, they form a high affinity receptor ($K_D = 10^{-11}\ M$). The β chain is a member of the cytokine receptor family (Hatakeyama *et al.*, 1989; Miyazaki *et al.*, 1991). A third chain, a 56-kDa protein expressed by hematopoietic cells, confers the β chain with the ability to bind IL-2 (Saito *et al.*, 1991). Several other molecules such as ICAM-1/CD54, MHC class I antigens, and glycoproteins of undefined structure appear to associate with the IL-2R. The IL-2R β

chain forms a stable complex with the lymphocyte-specific protein tyrosine kinase $p56^{lck}$, which is a member of the *src* family (Fung *et al.*, 1991; Hatakeyama *et al.*, 1991). On binding to its receptor, IL-2 induces tyrosine phosphorylation of various proteins following activation of a tyrosine phosphorylated phosphatidylinositol-3-kinase (Horak *et al.*, 1991; Merida *et al.*, 1991; Mills *et al.*, 1990) and the generation of inositol phosphate glycan (Eardley and Koshland, 1991).

IL-2 acts on T cells and NK cells to stimulate their proliferation, their differentiation into cytotoxic cells, and their production of cytokines such as IFN-γ. As will be detailed in subsequent sections, IL-2 can induce the proliferation and differentiation of B lymphocytes. IL-2 also acts on monocyte/macrophages by stimulating their cytotoxicity (Malkovsky *et al.*, 1987; Ralph *et al.*, 1988) and their cytokine production (Herrmann *et al.*, 1989). IL-2 is also a potent chemoattractant for eosinophils (Rand *et al.*, 1991).

In vivo studies have indicated that administration of IL-2 results principally in the expansion of $CD8^+$ T cells and NK cells. Accordingly, hybrid transgenic mice expressing both human IL-2 and IL-2R α chain have expanded NK cell populations (Biron *et al.*, 1990; Ishida *et al.*, 1989). Various *in vivo* studies have assigned IL-2 with a key role in thymus development and in the T cell-dependent control of pathological conditions such as autoimmunity (Gutierrez-Ramos *et al.*, 1990; Kroemer *et al.*, 1991), viral infections (Karupiah *et al.*, 1990), and tumor development (Fearon *et al.*, 1990) without any significant role in the humoral responses; however, mice in which the IL-2 genome has been interrupted by homologous recombination show normal T cell development but dramatically altered immunoglobulin levels (Schorle *et al.*, 1991). Yet, a child with severe combined immunodeficiency disease involving decreased circulating T cells, but not B cells, was found to have an apparently specific defect in the production of IL-2 (Weinberg and Parkman, 1990).

C. Interleukin-4

Interleukin 4, originally described for its ability to induce lipopolysaccharide-activated mouse spleen B cells to proliferate further and to secrete IgG_1, is now recognized as one of the most pleiotropic cytokines (Banchereau, 1991; Paul, 1991).

1. Sources and Structure of Interleukin-4 and Its Receptor

As discussed earlier, both murine and human IL-4 are produced by activated T lymphocytes as well as basophil/mast cells activated by crosslinkage of both their FcϵRI and their FcγRII (Le Gros *et al.*, 1990;

Piccinni *et al.*, 1991). IL-4 binds with a high affinity ($K_D = 10^{-10}$ *M*) to a relatively small number of receptors (100–1000) per cell and such receptors have been detected on virtually all cell types tested to date (Cabrillat *et al.*, 1987; Park *et al.*, 1987). Crosslinking studies have shown that IL-4 binds to three species of 140, 80, and 70 kDa (Galizzi *et al.*, 1989). The purified 140-kDa component has been shown to bind IL-4 with high affinity (Galizzi *et al.*, 1990a,b; Idzerda *et al.*, 1990). The 207-AA extracellular domain, which binds IL-4 with high affinity (Garrone *et al.*, 1991), contains the two motifs that are characteristic of the recently identified cytokine receptor superfamily. Soluble IL-4 receptors have been detected in mouse biological fluids, where they may act as transport proteins (Fanslow *et al.*, 1991a; Fernandez-Botran and Vitetta, 1990,1991). Soluble IL-4 receptors have not yet been identified in human biological fluids.

In human B cells, IL-4 turns on the production of the Ca^{2+} mobilizing messenger inositol 1,4,5-triphosphate and cAMP (Finney *et al.*, 1990). In contrast, studies with murine B lymphocytes have indicated a different mechanism of IL-4 action, which promotes anti-Ig-mediated protein kinase C translocation and reverses phorbol ester-mediated protein kinase C downregulation (Harnett *et al.*, 1991). Other studies with a human erythroleukemic cell line have concluded that IL-4 proliferative signal transduction involves the activation of a tyrosine-specific phosphatase and the dephosphorylation of an 80-kDa protein (Mire-Sluis and Thorpe, 1991).

2. *Pleiotropic Effects of Interleukin-4*

In vitro and *in vivo* studies have demonstrated that IL-4 stimulates the proliferation of $CD4^-$ $CD8^-$ thymocytes while inhibiting that of $CD4^+$ $CD8^+$ thymocytes. It promotes the proliferation of activated T lymphocytes (Spits *et al.*, 1987), but inhibits IL-2-dependent activation of both T and NK cells by inhibition of IL-2R induction (Gaya *et al.*, 1991; Martinez *et al.*, 1990). IL-4 enhances the generation of antigen-specific T cells (Horohov *et al.*, 1988; Widmer *et al.*, 1987) and IL-4 antagonists inhibit *in vivo* alloresponsiveness (Fanslow *et al.*, 1991b). IL-4 strongly blocks the synthesis of IFN-γ (Peleman *et al.*, 1989), its own natural antagonist, but augments the production of a lipase that may participate in cell cytotoxicity (Grusby *et al.*, 1990).

IL-4 acts at numerous stages of B cell natural history. It displays inhibitory effects on the proliferation of progenitor B cells likely as a consequence of induced differentiation (Hofman *et al.*, 1988; Pandrau *et al.*, 1992). It increases the size of resting B cells and increases the expression of antigens such as MHC class II antigens, adhesion mole-

cules (Björck *et al.*, 1992), CD40 (Vallé *et al.*, 1989), sIgM (Rigley *et al.*, 1991; Shields *et al.*, 1989), and most notably CD23/FcεRII (Defrance *et al.*, 1987a; Kikutani *et al.*, 1986b). It has been proposed that the induction of CD23 and sIgM may reflect the triggering of two distinct IL-4 receptors, as these phenomena can be modulated independently by several agents including anti-CD19 antibodies (Rigley *et al.*, 1991). Our most recent studies with neutralizing monoclonal antibodies specific for the extracellular domain of the 130-kDa IL-4 receptor demonstrate an inhibition of the IL-4-dependent induction of CD23 and sIgM on normal B lymphocytes and thus rule out the involvement of two different IL-4 receptors in these events (P. Garrone, J. P. Galizzi, and J. Banchereau, unpublished results). The effects of IL-4 on resting B cells reflect an activation allowing an improved presentation of processed antigen to T cells. As will be discussed in detail in forthcoming sections, IL-4 displays important agonistic and antagonistic effects on the growth and differentiation of mature B cells.

IL-4 alters monocyte phenotype by increasing CD23 (Vercelli *et al.*, 1988) and MHC class II expression (Te Velde *et al.*, 1988). It also blocks their production of proinflammatory cytokines (Essner *et al.*, 1989; Standiford *et al.*, 1990). Such properties of IL-4 are consistent with its facilitation of humoral responses and suppression of delayed-type hypersensitivity reactions. IL-4 affects the different granulocytes and appears to play a particularly important role in the proliferation and differentiation of precursors of basophils/mast cells and eosinophils into the mature cell type (Favre *et al.*, 1990).

IL-4 acts on fibroblasts, epithelial, and endothelial cells (Masinovsky *et al.*, 1990; Thornhill *et al.*, 1990). In particular, IL-4 increases the adhesiveness of endothelial cells for T cells and inhibits their increased procoagulant activity induced by LPS and proinflammatory cytokines (Kapiotis *et al.*, 1991).

In vivo studies with IL-4 have generated various results. Transgenic mice with IL-4 expressed under the control of the $p56^{lck}$ promoter show altered development of T cells in the thymus and a selective defect of peripheral CD4 cells, but no growth B cell abnormalities (Lewis *et al.*, 1991). In contrast, an IL-4 transgene under the influence of the H-chain enhancer results in animals with an allergic-like inflammatory disease and increased serum levels of IgG_1 and IgE (Burstein *et al.*, 1991; Tepper *et al.*, 1990). In line with this latter study, IL-4-deficient mice display a strong reduction in IgG_1 and IgE serum levels, whereas IgG_2 and IgG_3 levels are increased (Kühn *et al.*, 1991). Surprisingly, T and B cell development was normal in these mice. Finally, tumors engineered to produce IL-4 were rapidly rejected

through both T cell-dependent mechanisms (Golumbek *et al.*, 1991) and T cell-independent, eosinophil-dependent mechanisms (Tepper *et al.*, 1989).

D. Interleukin-10

IL-10 was identified first as a product of TH_2 cell clones able to block IFN-γ production by TH_1 cells (Fiorentino *et al.*, 1989), and then as a product of B cell lymphoma cell lines able to augment cytokine-induced proliferation of thymocytes and mast cells (Suda *et al.*, 1990).

1. Sources and Structure of Interleukin-10

Human IL-10 (Vieira *et al.*, 1991) is 73% homologous to murine IL-10 (Moore *et al.*, 1990) at the amino acid level. It is produced by T cells, B cells (O'Garra *et al.*, 1990), and mostly monocytes (de Waal Malefyt *et al.*, 1991b) in response to activators. Human IL-10 exhibits marked homology to an open reading frame in the EBV genome, BCRF1 (Hsu *et al.*, 1990). The similarity is most pronounced in the mature protein coding sequences where human IL-10 and BCRF1 are approximately 84% identical, and it is likely that the ancestor of BCRF1 may have been a captured host IL-10 gene (Moore *et al.*, 1991; Zlotnik and Moore, 1991).

2. Pleiotropic Effects of Interleukin-10

IL-10 is a cofactor stimulating proliferation of mouse thymocytes and T cells in combination with IL-2 and IL-4 (MacNeil *et al.*, 1990) and acts as a cytotoxic T cell differentiation factor (Chen and Zlotnik, 1991). It synergizes with IL-3 and IL-4 to stimulate proliferation of mast cell precursors (Thompson-Snipes *et al.*, 1991). Furthermore, together with IL-3 and IL-1 [or granulocyte colony-stimulating factor (G-CSF) or steel factor], IL-10 enhances the formation of colonies by isolated murine multipotent progenitor cells (D. Rennick, personal communication). IL-10 inhibits the production of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF) by monocytes and downregulates their MHC class II expression (de Waal Malefyt *et al.*, 1991a,b; Fiorentino *et al.*, 1991). This latter effect results in the subsequent inhibition of antigen-induced T cell proliferation and production of cytokines including IFN-γ, IL-2, IL-5, and IL-6. IL-10 also inhibits IL-2-induced IFN-γ production by NK cells, but, unlike IL-4, it does not inhibit IL-2-induced NK cell proliferation. IL-10, like IL-4, augments expression of MHC class II antigens on small resting mouse B cells and sustains their viability (Go *et al.*, 1990). Human IL-10 does not, however, alter MHC class II expression on purified tonsillar human B cell, probably

because of the high levels of constitutive expression of MHC class II on these cells. Nevertheless, as will be described in detail later, human and viral IL-10 display potent B cell growth and differentiation activity.

E. Interleukin-6

Human IL-6 is a mature glycoprotein of 23–32 kDa according to glycosylation (Hirano *et al.*, 1990; Van Snick, 1990). It is secreted by numerous cell types including T and B cells, monocytes/macrophages, fibroblasts, keratinocytes, and endothelial cells in response to antigenic challenge, endotoxin, or cytokines. IL-6 binds specifically to low-affinity ($K_D \approx 10^{-9}$ *M*) and high-affinity ($K_D \approx 10^{-11}$ *M*) receptors that are expressed on many cell types. In fact, IL-6 binds to an 80-kDa glycoprotein of 449 AAs (Yamasaki *et al.*, 1988) that further associates with a mature 896-AA 130-kDa glycoprotein (Taga *et al.*, 1989). This latter protein acts as a signal transducer. Both components belong to the cytokine receptor superfamily and the 80-kDa also includes an Ig-like domain.

IL-6 stimulates the growth of activated thymocytes and peripheral T cells and enhances the IL-2 dependent differentiation of cytotoxic T lymphocytes (Houssiau *et al.*, 1988a; Lotz *et al.*, 1988; Okada *et al.*, 1988). As will be described later, IL-6 stimulates proliferation and Ig secretion of differentiated B lymphocytes. IL-6 enhances cytokine-dependent proliferation of hematopoietic progenitor cells (Gardner *et al.*, 1990; Ikebuchi *et al.*, 1987), induces the maturation of megakaryocytes, and acts as a potent thrombopoietic factor (Gardner *et al.*, 1990).

IL-6 appears to play a critical role in the acute phase of inflammation. In particular, IL-6 acts directly on hepatocytes and induces the production of acute phase proteins such as fibrinogen and C reactive protein, while inhibiting the synthesis of albumin (Geiger *et al.*, 1988).

F. Transforming Growth Factor β

TGF-β_1 is one member of a superfamily composed of at least 20 molecules classified in four different families including the TGF-β family, the inhibin family, the bone morphogenetic protein family, and the müllerian inhibiting substance family. The different members of these families are 25–90 percent homologous. Three different forms, TGFβ_{1-3}, that have comparable properties have been described in mammals (Massagué, 1990).

1. Sources and Structure of Transforming Growth Factor β and Its Receptors

TGF-β_1 is a 25-kDa homodimeric molecule composed of disulfide-linked 112-AA polypeptides. Each chain represents the carboxy-terminus of a 390-AA precursor which is glycosylated and secreted. Following enzymatic cleavage, the amino terminus remains associated with the 25-kDa dimer and yields an inactive latent form of TGF-β. The latent TGF-β contains a third component, the TGF-β_1 binding protein, which is a 1374-AA mature glycoprotein containing 16 epidermal growth factor (EGF)-like sequences, the function of which is not presently known (Kanzaki *et al.*, 1990). The TGF-β dimer can also associate with α_2-macroglobulin, an abundant serum protein composed of four 185-kDa polypeptides which may be involved in the clearance of TGF-β.

TGF-β is produced by virtually all cell types and particularly by activated T cells (Kehrl *et al.*, 1986b), B cells (Kehrl *et al.*, 1986a), and monocytes/macrophages (Assoian *et al.*, 1987). Although mononuclear cells secrete latent TGF-β, there is a mechanism that converts the latent TGFβ complex into an active form and allows TGF-β to exert its biological effects (Lucas *et al.*, 1990). TGF-β binds with a high affinity (K_D = 5–50 p*M*) to two glycoproteins of 53 kDa (type I receptor) and 70–85 kDa (type II receptor) which are expressed at low density by most cell types. The type II receptor is analogous to the receptor for activin, a member of the TGF-β superfamily, the intracellular domain sequence of which predicts serine kinase activity [Mathews and Vale, 1991, see also Note Added in Proof (Section v)]. In addition, TGF-β binds with an intermediate affinity (K_D = 30–300 p*M*) to the more abundant 280- to 330-kDa β-glycan (TGF-β type III receptor), which is a proteoglycan (Kjellén and Lindahl, 1991) composed of a 120-kDa core polypeptide, a 200-kDa glycosaminoglycan moiety, and a 10-kDa N-linked glycan. The specific cDNA codes for a 853-AA core protein with a short 41- to 43-AA intracytoplasmic domain highly homologous to that of endoglin, a major membrane protein of vascular endothelium, which may act as an adhesion molecule (Lopez-Casillas *et al.*, 1991; X. F. Wang *et al.*, 1991). The large extracellular domain of β-glycan presents clustered sites for the potential attachment of glycosaminoglycan chains. The extracellular domain can be released as a soluble proteoglycan through a cleavage site near the transmembrane region. This site is identical to the highly regulated cleavage site of the membrane-anchored precursor of TGF-α. Interestingly, the heparan sulfate chain of β-glycan can also bind basic fibroblast growth factor

(FGF); however, TGF-β binds to the deglycosylated molecule, whereas FGF binds to the oligosaccharide portion. This allows for the independent regulation of TFG-β and FGF binding functions (Klagsbrun and Baird, 1991; Ruoslahti and Yamaguchi, 1991). Transfection of type III receptors in cells lacking them results in increased binding of TGF-β to type II receptors, indicating that the fairly abundant type III receptor may capture TGF-β from the pericellular environment and present it to the less abundant higher-affinity type I and type II receptors. Other lower-affinity receptors have been detected, some of which are glycolipid-anchored binding proteins specific for different TGF-β isoforms.

2. *Pleiotropic Effects of Transforming Growth Factor β*

TGF-β can inhibit, in a reversible fashion, the growth of numerous cell types of epithelial, endothelial, fibroblastic, neuronal, or hematopoietic origin. It acts by inhibiting the phosphorylation of the product of the retinoblastoma sensitivity gene, which normally acts as an intracellular suppressor of growth (Moses *et al.*, 1990). TGF-β increases cellular adhesion through stimulation of the production of extracellular matrix components, inhibition of their degradation, and increased expression of adhesion molecules such as integrins. TGF-β is also a chemotactic agent for monocytes, fibroblasts, and neutrophils (Brandes *et al.*, 1991; Fava *et al.*, 1991), as well as T lymphocytes (Adams *et al.*, 1991), and attracts these cells to inflammation sites. In keeping with this, recent studies have indicated that TGF-β also acts as an enhancer of granulopoiesis both *in vitro* (Keller *et al.*, 1991) and *in vivo* (Carlino *et al.*, 1990). Taken together, these various properties would provide TGF-β with an important role in tissue repair and remodeling. As will be discussed in a later section, TGF-β has been shown to display a strong inhibitory role in lymphocyte proliferation which could be a mechanism for controlling cellular expansion following antigen-induced proliferation; however, recent studies have shown that TGF-β can enhance the proliferation of T cells activated with immobilized anti-CD3 (Lee and Rich, 1991) and can induce rapid acquisition of a mature or memory CD45 RB^{lo} $CD44^{hi}$ phenotype. TGF-β also enhances MHC class II antigen expression on murine B cells while blocking the expression of various other antigens (Cross and Cambier, 1990). This may facilitate T cell–B cell interactions, and in this context, TGF-β has also been shown to act as a switch factor for IgA, as will be discussed later.

G. Low-Molecular-Weight B Cell Growth Factor (BCGF-12 kDa)

Low-molecular weight BCGF or BCGF-12 kDa has been identified for its ability to induce the proliferation of human B cells stimulated with anti-IgM antibody (Maizel *et al.*, 1982, 1983; Mehta *et al.*, 1985). A specific cDNA has been isolated (Sharma *et al.*, 1987) that is characterized by expression of an *alu* sequence. BCGF-12 kDa mRNA can be detected in malignant B cells, but not in normal B cells, suggesting that it might play a role in the pathogenesis of B cell neoplasia. Recombinant BCGF-12 kDa was found to induce the proliferation of a variety of established B cell lines, and a neutralizing antibody to BCGF-12 kDa was shown to block the spontaneous growth of a lymphoma cell line suggesting an autocrine mechanism of action. The BCGF-12 kDa gene maps to the chromosome 1q23–25 region, which is prone to rearrangement and translocation in diverse human tumors. It is possible that constitutive overproduction of BCGF-12 kDa may provide growth advantages to transformed B cells (Kumar *et al.*, 1990).

Many studies with this molecule have been performed with a commercial preparation which unfortunately contains many other cytokines including TNF-α (Kehrl *et al.*, 1987a). This precludes the possibility of making any conclusion relating to which cytokine would in fact be involved in the observed biological activity.

IV. Polyclonal Activation of Human B Cells

Early studies on B lymphocyte activation and proliferation were performed primarily with polyclonal activators, which can be subdivided into two groups according to whether they bind to surface immunoglobulins or to other antigens of the B cell surface. In addition, chemical agents have been used that bypass cell surface receptors such as phorbol esters which stimulate more or less specifically protein kinase C and various calcium ionophores (A23147, ionomycin) that induce Ca^+ influx within the cell. Early studies on activation of human B cells with polyclonal activators have been well reviewed (Fauci and Ballieux, 1982; Jelinek and Lipsky, 1987a).

A. Antigen Receptor

1. Anti-immunoglobulins

a. Stimulatory Effects

Anti-IgM antibodies have been used extensively to study B lymphocyte activation with the understanding that these antibodies mimic

the interaction of the antigen with the B cell antigen receptor. Initial studies with polyclonal rabbit antibodies have shown that intact immunoglobulins do not induce DNA synthesis, whereas derived $F(ab')_2$ fragments are active. The lack of effect of the whole antibody is due to the binding of its Fc fragment with the B cell FcgRII/CDw32. This delivers inhibitory signals through an uncoupling of phospholipase Cγ (PLCγ) from the antigen receptor (Rigley *et al.*, 1989); however, some murine monoclonal antibodies in soluble form or adsorbed onto the culture vessel matrix have been found to induce DNA synthesis in resting B cells (Maruyama *et al.*, 1985; Rudich *et al.*, 1985; Suzuki and Cooper, 1985). Kinetic studies have shown that DNA synthesis induced by anti-IgM antibodies peaks at Days 3 to 4 and is virtually abolished by day 6. Optimal proliferation is obtained for antibody concentration around 5 μg/ml but, recently, monoclonal anti-IgM antibody coupled to dextran has been shown to induce maximal DNA synthesis at approximately 100 pg/ml (10^{-12} *M*) (Rehe *et al.*, 1990). Such a presentation of anti-IgM antibody very closely mimics that induced by the so-called thymus-independent antigens which are usually high-molecular weight polymers with simple repeating structures. The consensus in the early 1980s was that mIg engagement with either antigen or anti-Ig antibody would induce entry of B cells into proliferation. Now it appears that the occupancy of mIg by thymus-dependent antigens will not result in entry into proliferation. It simply activates B cells to respond to the growth and differentiation effects of T cell membrane molecules and soluble cytokines. In fact, some authors have even argued that engagement of surface Igs would not even induce B cells to directly respond to cytokines and that the triggering of other B cell antigens through interaction with T cells would allow cytokine responsiveness (Abbas, 1988; Noelle and Snow, 1990).

b. Inhibitory Effects of Anti-immunoglobulin Antibodies

Just as well as immature thymocytes reacting with self components are anergyzed or deleted through a process of programmed cell death (Blackman *et al.*, 1990), it is accepted that immature IgM^+ IgD^- B cells are also anergyzed/deleted when they encounter (self) antigens (Goodnow *et al.*, 1990; Nossal, 1989; Rolink and Melchers, 1991). Such conclusions are essentially derived from studies performed with murine B cells. Moreover, studies with mature $sIgM^+$ $sIgD^+$ B cells have also indicated that crosslinking of sIgM, but not sIgD, can generate negative signals. In particular, the cell division, but not the DNA synthesis, of human $sIgM^+$ $sIgD^+$ lymphoma B cell lines is strongly inhibited by anti-IgM but not anti-IgD antibodies, following a block in

the G_2 phase of the cell cycle (Beckwith *et al.*, 1991; Kim *et al.*, 1991; Mongini *et al.*, 1989). The anti-IgM induced inhibition of line B104 may represent a novel undefined mode of programmed cell death (Ishigami *et al.*, 1992) different from that involved in anti-IgM-mediated apoptosis in the mouse lymphoma cell line WEHI-231 (Hasbold and Klaus, 1990; Scott *et al.*, 1985). Accordingly, the block of B104 cells occurs in G_2/M, whereas that of WEHI-231 cells occurs in G_1/S (Page and Defranco, 1990). Surprisingly, the anti-IgM-induced inhibition appears to be independent of phosphatidylinositol turnover and protein kinase C activation and involves tyrosine phosphorylation (Beckwith *et al.*, 1991). Anti-IgM antibodies also appear to inhibit the entry into mitosis of purified blood B lymphocytes activated with anti-IgD and IL-4 (Kim *et al.*, 1992). Anti-IgM, but not anti-IgD, also inhibited entry into mitosis of SAC activated B cells. The anti-IgM-induced inhibition of murine neonatal B cell proliferation can be overcome by addition of T cells (Chang *et al.*, 1991) and that of human B cells can be overcome by addition of IFN-α/β (Kim *et al.*, 1991, 1992). This indicates that antigen alone, although initiating B cell DNA synthesis, may finally inhibit B cell expansion if signals given by T cells and accessory cells are not provided. This represents an efficient way to avoid clonal expansion of autoreactive B cells. Furthermore, B cells triggered with anti-IgM antibodies alone fail to differentiate into Ig-secreting cells and anti-IgM antibodies have been found to inhibit pokeweed mitogen (PWM)-induced T-dependent plasma cell differentiation (Kuritani and Cooper, 1982; Maruyama *et al.*, 1985).

2. *Staphylococcus aureus Strain Cowan*

Formalinized SAC particles are able to induce resting B cells to enter DNA synthesis and appear to be more potent than anti-Ig antibodies. SAC particles stimulate a large proportion of resting B cells through the interaction of protein A with IgM and also IgG (Romagnani *et al.*, 1981). It is also possible that the staphylococcal toxins participate in the important stimulatory effect of SAC (see Box 4). These toxins bind to HLA class II antigens of B cells and then to antigen receptors of T cells. This bridging results in an important proliferation of both T and B cells (Mourad *et al.*, 1989). Furthermore, together with anti-IgM, TSST1 is directly able to induce resting B cells to enter into DNA synthesis (Fuleihan *et al.*, 1991). These different toxins bind to distinct binding sites on HLA-DR (Chintagumpala *et al.*, 1991).

Similar to anti-Ig, SAC is unable to induce purified B cells to secrete Igs. Addition of T cells and activated T cell supernatants allows Ig

secretion, which will be discussed in detail later. Staphylococcal enterotoxins also permit the differentiation of B cells in a T-dependent fashion. SAC induces rapid tyrosine phosphorylation of several proteins of 45, 68, 75, 97, and 145 kDa, which reaches a maximum after 10 minutes. Mitogenic soluble anti-IgM antibodies also induce the same pattern of tyrosine phosphorylation, whereas the nonmitogenic antibodies fail to induce phosphorylation of p68 (Roifman *et al.*, 1991). SAC is also a potent inducer of respiratory burst in B lymphocytes, resulting in the generation of O_2 and H_2O_2 (Leca *et al.*, 1991).

3. *Branhamella catarrhalis and Others*

Branhamella catarrhalis has been shown to be a T-independent activator of DNA synthesis in human B cells (Banck and Forsgren, 1978). It does so following interactions with surface IgD and class I HLA antigens, as antibodies against these structures inhibit *B. catarrhalis* -induced B cell activation (Calvert and Calogeras, 1986; Forsgren *et al.*, 1988). *Branhamella catarrhalis* appears particularly interesting as a polyclonal B cell activator, because following such activation, B cells secrete IgA in response to human IL-5 (E. Benson, personal communication) and express germline α_1 and α_2 transcripts in response to TGF-β (Islam *et al.*, 1991; Nilsson *et al.*, 1991).

Formaldehyde-fixed *Salmonella paratyphi* B (Chen *et al.*, 1981) and membrane preparations of *Klebsiella pneumoniae* (Gross and Rucks, 1983) are both able to induce purified human B lymphocytes to differentiate into Ig-secreting cells without proliferation. IL-2, however, is able to induce the proliferation of *Klebsiella*-stimulated B cells (Stüber *et al.*, 1989). B lymphocytes can also be induced to synthesize DNA in response to the *Nocardia* water-soluble mitogen (Bona *et al.*, 1979) and the mannose-specific adhesin on *Escherichia coli* type I fimbriae (Ponniah *et al.*, 1989).

B. Anti-CD40 Antibodies

Anti-CD40 antibodies have been isolated for their ability to costimulate with either anti-IgM antibodies or phorbol esters (Clark and Ledbetter, 1986; Ledbetter *et al.*, 1987; Vallé *et al.*, 1989). Soluble anti-CD40 antibodies can induce very weak DNA replication in resting B cells (Gordon *et al.*, 1988b); however, coculture of resting B cells with monoclonal antibodies to CD40 and a mouse fibroblastic cell line (L cell) that had been transfected with the human Fc receptor (FcγRII/CDw32) results in strong and long-lasting B cell DNA replication (Banchereau *et al.*, 1991). The observed proliferation is dependent on the expression of CDw32 by L cells, as untransfected L cells or L cells

transfected with an antigen such as HLA class I fail to induce B cell proliferation. Maximal DNA replication is obtained with concentrations of anti-CD40 as low as 30 ng/ml(10^{-10} *M*). Under these conditions, B cell number increases by three-to-fourfold over 2 weeks. As anti-CD40 Fab fragments and CDw32 L cells or a combination of intact anti-CD40 and untransfected L cells are unable to induce B cell proliferation, it is concluded that crosslinking of the CD40 antigen is essential for B cell growth. The soluble anti-CD40 does not directly bind to the transfected CDw32 but does so following binding to B cell CD40 antigen, most likely because its Fc portion undergoes spatial rearrangement allowing further high-affinity interaction with CDw32. This novel culture system is a powerful B cell activator in that at least 40–50% of B cells enter into the G_1 phase of cycle and 25–30% into the S phase after 48 hours of culture. It permits the proliferation of various B cell subpopulations including the mantle zone $sIgD^+$ $sIgM^+$ B cells, the $sIgD^-$ $sIgM^{+/-}$ B cells, and the $CD5^+$ and $CD5^-$ B cells (Defrance *et al.*, 1992c). Furthermore, it also induces quite significant DNA synthesis in leukemic B cells, such as non-Hodgkin B cell lymphomas and chronic lymphocytic leukemia B cells (Fluckiger *et al.*, 1992). Such culture conditions induce only minor DNA synthesis of progenitor B cells and acute lymphoblastic pre-B leukemias; however, $CD34^+$ hematopoietic progenitor cells which express CD40 (Saeland *et al.*, 1991) do not proliferate under these conditions. Inasmuch as soluble anti-CD40 antibody enhances anti-IgM-induced B cell DNA synthesis, anti-IgM antibody and SAC particles enhance B cell proliferation induced by anti-CD40 antibody and CDw32 L cells (Fig. 12).

B cells cultured with anti-CD40 antibody, in the presence or absence of CDw32 L cells, produce marginal amounts of immunoglobulins (Rousset *et al.*, 1991); however, the coculture of B cells with SAC particles, anti-CD40 antibody, and CDw32 L cells results in the production of very large amounts of IgM, IgG, and IgA without IgE (Defrance *et al.*, 1992b). Mantle zone $sIgD^+$ $sIgM^+$ B cells secrete only IgM, whereas $sIgD^-$ $sIgM^{+/-}$ B cells secrete all the IgG and IgA but smaller amounts of IgM (Table V). In fact, anti-IgM antibody can be used instead of SAC although the levels of secreted Igs are lower. This indicates that the concomitant triggering of sIg and CD40 results in a T cell independent differentiation of human B lymphocytes.

As will be discussed in subsequent sections, cytokines can efficiently alter the proliferation and differentiation of B cells triggered with anti-CD40 antibody and CDw32 L cells. This activation procedure has been called the CD40 system (Banchereau and Rousset, 1991).

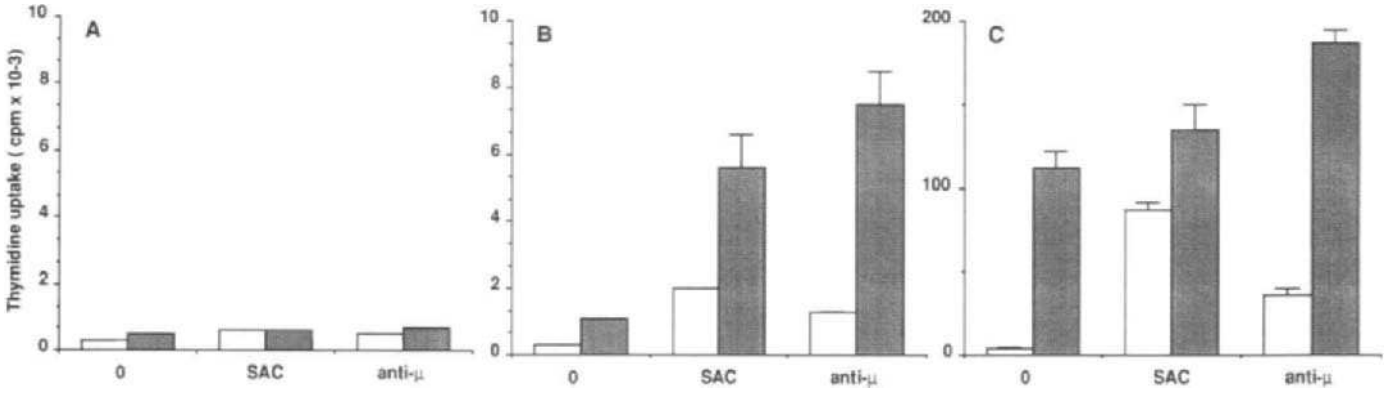


FIG. 12. Costimulatory effect of anti-IgM and anti-CD40 on B cell proliferation. Purified tonsillar B cells (2×10^4) were cultured with or without 2.5×10^3 irradiated CDw32 L cells, with or without 0.5 μg/ml monoclonal antibody 89, with or without 5 μg/ml anti-IgM antibody (used as beads), with (■) or without (□) 100 U/ml interleukin-4. [^{3}H]Thymidine was added for the last 16 hours of the culture at day 6. (A) Polyclonal activators. (B) Polyclonal activators and anti-CD40. (C) Polyclonal activators, CDw32 L cells, and anti-CD40. Note the differences in scales. SAC, *Staphylococcus aureus* strain Cowan.

TABLE V
COSTIMULATION OF PURIFIED HUMAN B LYMPHOCYTES WITH *Staphylococcus aureus* STRAIN COWAN (SAC) AND ANTI-CD40 RESULTS IN T-INDEPENDENT B CELL DIFFERENTIATION[a]

		Immunoglobulin concentration (μg/ml)		
		IgM	IgG	IgA
IgD^+	—	<0.1	<0.1	<0.1
	Anti-CD40	0.2	<0.1	<0.1
	SAC	<0.1	<0.1	<0.1
	SAC + anti-CD40	30	<0.1	<0.1
IgD^-	—	0.1	0.1	<0.1
	Anti-CD40	0.3	0.3	0.1
	SAC	0.1	0.1	<0.1
	SAC + anti-CD40	5	7	0.1

[a] $sIgD^+$ or $sIgD^-$ Tonsil B cells (5×10^4) were cocultured for 10 days with 5×10^3 irradiated CDw32 L cells in complete medium without or with SAC, or anti-CD40 mAb 89, or both. Ig levels represent the mean values of quadruplicate determinations. The standard deviation represents less than 10% of the mean value.

C. ACTIVATED T CELLS CAN INDUCE RESTING B CELLS TO PROLIFERATE AND DIFFERENTIATE

Several systems of murine and human B cell activation have recently been developed that rely on the use of activated T cells (Parker, 1990).

In pioneering studies, Zubler (1985) and Zubler *et al.* (1987) demonstrated that the mouse thymoma EL-4 can activate resting human B cells to proliferate. Addition of T cell supernatants allows activated B cells to differentiate into Ig-secreting cells. Different isotypes have been detected in the supernatants of a single B cell which indicates the switching of Ig isotypes (Tucci *et al.*, 1991; Zhang *et al.*, 1990). Under these conditions, 90% of the B cells can be induced to expand into clones reaching a few hundred cells, thus allowing an evaluation of the frequency of antigen specific circulating B cells (Wen *et al.*, 1987).

Lipsky (1990) and Noelle and Snow (1990) have shown that T cells or T cell clones activated with immobilized anti-CD3 antibodies (Hirohata *et al.*, 1988) are able to induce resting B cells to proliferate and differentiate in the complete absence of accessory cells or lectins that might favor cellular interactions. The activation of B cells requires direct T cell–B cell interaction, as physical separation by semiper-

meable membranes does not allow B cell activation. Cytokines, particularly IL-2, appear to play an important role and anti-Tac completely blocks both proliferation and differentiation of human B cells (Tohma and Lipsky, 1991). $CD8^+$ T cells are also able to induce B cells to proliferate and differentiate, although to a lesser extent than that reached with $CD4^+$ T cells. Such variations are also observed with murine T cells. Resting mouse B cells can be activated with both TH_1 and TH_2 clones, although the latter are usually more efficient (Abbas *et al.*, 1990; DeKruyff *et al.*, 1989). An important characteristics of these model systems is the lack of requirement for MHC identity between T cells and B cells. Virtually, every B lymphocyte can be induced to secrete immunoglobulins under these conditions. The secretion of specific antibodies including autoantibodies, such as rheumatoid factor and anti-DNA antibody, has been obtained. Neonatal (cord blood), naive ($sIgD^+$), postswitch ($sIgD^-$), CD5+, and $CD5^-$ B cell populations can all be stimulated under these conditions (Splawski and Lipsky, 1991). Single B cells cultured with anti-CD3-activated T cells can be induced to secrete several isotypes, therefore demonstrating induced isotype switching (Amoroso and Lipsky, 1990). This culture system results in the generation of nondividing high-rate Ig-secreting plasma cells (Vernino *et al.*, 1992a).

The interaction between LFA-1 (CD11a/CD18) on the B cell and of ICAM-1 (CD54) on the activated T cell plays a central role in the activation of the B lymphocyte (Tohma *et al.*, 1991). Other undefined receptor–ligand interactions are also likely to occur in this system as anti-LFA-1 and anti-ICAM-1 are only partly inhibitory. In particular, during the initial activation phases, the B cell RNA synthesis induced by paraformaldehyde-fixed activated T cells cannot be inhibited by anti-LFA-1 and anti-ICAM-1 antibody (Tohma and Lipsky, 1991). These interactive pairs of molecules may be the recently identified CD28-CTLA4/B7 or CD5/CD72. Interestingly, the peptide-dependent activation of antigen-specific T cells, as well as B cells, in a cognate interaction, also engages the T cell LFA-1 and the B cell CD54/ICAM-1 (Lane *et al.*, 1991). Importantly, the integrity of activated T cells is not required as their isolated membranes are able to activate murine B cells and induce them to proliferate (Brian, 1988; Hodgkin *et al.*, 1990; Sekita *et al.*, 1988). Ig secretion, however, requires addition of exogenous cytokines (Hodgkin *et al.*, 1991; Noelle *et al.*, 1991). Recent studies with activated human and murine T cell clone membranes have also indicated their stimulatory activity for human B cells (Gascan *et al.*, 1992).

Interestingly, human immunodeficiency virus (HIV)-infected hu-

man T cell clones can also directly activate autologous and allogeneic resting B cells to secrete very large amounts of immunoglobulins (Macchia *et al.*, 1991). This observation may explain the functional abnormalities of B cells observed in acquired immunodeficiency syndrome (AIDS) patients: hypergammaglobulinemia, high numbers of circulating activated B cells, increased titers of antibodies against HIV-unrelated pathogens. These patients also display autoantibodies, some of which are directed against patient lymphocytes (Amadori and Chieco-Bianchi, 1990).

D. Epstein–Barr Virus

The Epstein–Barr virus (EBV) is a member of the herpes family of viruses that infects resting human B lymphocytes *in vitro* and transforms them into blasts which can proliferate indefinitely in culture (Tosato and Blaese, 1985).

After binding to its receptor, the EBV is internalized and expression of EBV nuclear antigens can be detected within 8 to 10 hours. It takes, however, 3–4 days for EBV-activated B cells to begin to synthesize DNA and secrete Igs. Limiting dilution studies find transformation rates between 0.1 and 10 percent, according to laboratories. EBV preferentially infects cells expressing the Bac 1 antigen (Crain *et al.*, 1989), which is enriched for surface IgM and IgD expression. The B cells that can be transformed by EBV are isotype committed (Miyawaki *et al.*, 1991). EBV-transformed B cells producing IgM originate in their majority from $sIgM^+$ $sIgD^+$B cells and from some $sIgM^+$ $sIgD^-$B cells. EBV binds to cells through CR2/CD21, but there is no correlation between CD21 density and transformability (Dosch *et al.*, 1990). Instead, post-receptor binding events such as the extracellular $[Ca^{2+}]i$ flux (Dugas *et al.*, 1988, 1989), Na^+/H^+ exchange, and tyrosine phosphorylation of 55- to 60-kDa and 145-kDa proteins have been identified as critical determinants of transformability (Dosch *et al.*, 1990). Whereas the 145-kDa component likely represents the virus receptor CR2/CD21, the 55- to 60-kDa protein is the tyrosine kinase $p56^{lck}$. In fact, EBV induces a rapid and transient upregulation of $p56^{lck}$, a kinase that is expressed at very low levels in normal B lymphocytes (Cheung and Dosch, 1991). $p56^{lck}$ antisense oligonucleotides block EBV-induced B cell growth and Ig production, suggesting a critical role for $p56^{lck}$ in the EBV-directed B cell transformation into immortalized lines; however, the sustained expression of $p56^{lck}$ in transformed lines does not appear to be necessary for their propagation.

Once it gained entry, the internalized EBV genome, which is linear in the virion, undergoes a process of circularization that appears to be

necessary for the effective transformation of B cells (Hurley and Thorley-Lawson, 1988). This event is associated with upregulation of CD23 expression by the transformed cells. Interestingly, the circularization process generates a fused gene with an open reading frame that codes for the so-called "terminal protein" (Laux *et al.*, 1988).The EBV gene products initiating and maintaining B cell growth *in vitro* include six EBV-encoded nuclear antigens (EBNAs)—1,2,3A, 3B, 3C and LP (leader protein)—and three EBV-encoded integral latent membrane proteins (LMPs)—1, 2A, and 2B—which are all expressed in EBV-transformed cell lines (Kieff and Liebowitz, 1990). EBNA-2 appears to play an essential role in B cell transformation, as recombinant viruses with EBNA-2 deletions (Cohen *et al.*, 1989) or truncations (Cohen *et al.*, 1991; Hammerschmidt and Sugden, 1989) fail to transform B cells. In addition, the two naturally occurring EBV types, EBV-1 and EBV-2, which differ widely in their ability to transform B lymphocytes (Rickinson *et al.*, 1987), differ essentially at the EBNA-2 level. EBNA-2 acts through transactivation of various host and virus genes, such as CD23 (Calender *et al.*, 1987; F. Wang *et al.*, 1987, 1991), CD21 (Wang *et al.*, 1990a), and LMP-1 (Abbot *et al.*, 1990; Wang *et al.*, 1990b). Studies with Burkitt's lymphoma cell lines have indicated that LMP_1 protein may play a key role in the establishment of B cell lines, as it prevents B cell apoptosis through increased expression of the cellular proto-oncogene *bcl*-2 (Gregory *et al.*, 1991; Henderson *et al.*, 1991). The LMP-2 proteins partially colocalize with LMP-1 in the plasma membrane and associate with a B- lymphocyte tyrosine kinase (Longnecker *et al.*, 1991). Recombinant viruses encoding for a mutant EBNA LP lacking the carboxy-terminal 45 AAs were markedly impaired in their ability to transform B lymphocytes (Mannick *et al.*, 1991). As B cell lines generated with the EBNA LP mutant recombinant viruses express a large percentage of cells with bright cytoplasmic Ig staining, it has been hypothesized that EBNA-LP plays an indirect role in suppressing terminal B cell differentiation in EBV-infected cells.

EBV can transform cells other than mature B cells. In particular, progenitor B lymphocytes can generate cell lines with immunoglobulin genes that are in a germline configuration or that have undergone DJ or abortive VDJ rearrangements (Hui *et al.*, 1989; Kubagawa *et al.*, 1988). Some clones are able to undergo terminal B cell differentiation coupled with J-chain expression, whereas some clones express light-chain genes before heavy-chain gene rearrangement (Kubagawa *et al.*, 1989).

EBV is also able to bind to a small subpopulation of immature human thymocytes (Watry *et al.*, 1991) and the EBV genome can be detected

in infected thymocytes. EBV in combination with IL-2 can induce thymocyte proliferation, a finding consistent with the expression of CR2/CD21 on T lymphocytes (Fingeroth *et al.*, 1987; Fischer *et al.*, 1991a; Sauvageau *et al.*, 1990; Tsoukas and Lambris, 1988). Indeed, the ability of EBV DNA to immortalize T cells (Stevenson *et al.*, 1986) may explain the association of EBV with T cell lymphomas (Jones *et al.*, 1988; Leyvraz *et al.*, 1985).

V. Cytokines and B Lymphocyte Growth

In 1982, an assay for determining B cell growth factor activity was described. It was based on the synergy between anti-Ig antibodies and T cell-derived soluble factors for induction of DNA synthesis in purified mouse spleen cells (Howard *et al.*, 1982). This observation led to the later isolation of IL-4, a cytokine characterized by its pleiotropic effects. A comparable assay was described, at the same time, using human B cells and anti-IgM antibody coupled to beads (Maizel *et al.*, 1982, 1983). This observation led to the later isolation of the so-called low-molecular-weight BCGF or BCGF-12 kDa for which a cDNA was cloned (Sharma *et al.*, 1987), and the biological functions of which remain to be established for the most part. Other contemporary studies have used B cells costimulated with anti-IgM $F(ab')_2$ fragments (Yoshizaki *et al.*, 1983) rather than immobilized anti-IgM antibody or B cells preactivated with SAC (Muraguchi and Fauci, 1982). The effects of T lymphocyte supernatants on the DNA replication of these activated B cells have been reviewed by Kishimoto (1985).

For the review of the experimental data regarding the effects of recombinant cytokines on B cell proliferation and differentiation, we have primarily three different ways of activating B cells: following engagement of their antigen receptor, following crosslinking of their CD40 antigen, following contact with activated T cells. As discussed in the last section, these models are likely to represent *in vivo* situations.

A. Antigen Receptor Dependent Activation

1. Polyclonal Activators

a. Interleukin-2

Following the description of Tac/CD25 expression on *in vitro* activated B cells (Malek *et al.*, 1983; Tsudo *et al.*, 1984), several groups demonstrated that IL-2 induces DNA replication of B cells stimulated with either anti-Ig or SAC (Boyd *et al.*, 1985; Defrance *et al.*, 1988a; Jelinek *et al.*, 1986; Jung *et al.*, 1984; Mingari *et al.*, 1984; Mittler *et al.*,

1985; Muraguchi *et al.*, 1985; Nakagawa *et al.*, 1985, 1986, 1988; Suzuki and Cooper, 1985). IL-2 is able to induce DNA synthesis in B cells obtained from different anatomic sites, such as peripheral blood, tonsils, lymph nodes, and spleen (Jelinek *et al.*, 1986). Post-switch $sIgD^-$ B cells show a stronger DNA synthesis in response to IL-2 than naive IgD^+ IgM^+ B cells. IL-2 represents only one of the B cell growth factors of T cell supernatants as the proliferative response of B cells to IL-2 is lower than that of activated T cell supernatants. Activated B cells proliferating in response to IL-2 express CD23/FcεRII and B7/BB1, although the induced levels are lower than those obtained in response to IL-4 (Defrance *et al.*, 1987a; Hivroz *et al.*, 1989; Vallé *et al.*, 1991).

High concentrations of IL-2 are able to induce resting B cells to proliferate most likely as a consequence of binding to the intermediate affinity IL-2 receptor (Begley *et al.*, 1990; Bich-Thuy and Fauci, 1985, 1986; Mond *et al.*, 1985; Saiki *et al.*, 1988; Tanaka *et al.*, 1988a). Recent studies (Holder *et al.*, 1991) have indicated that a subpopulation of germinal center B cells can proliferate in response to low concentrations of IL-2. In fact, IL-2 also represents a preferential growth factor for B blasts generated after stimulation with T cells (Forman and Puré, 1991; Tohma and Lipsky, 1991).

b. Interleukin-4

Recombinant IL-4 strongly enhances DNA replication of B cells costimulated with insolubilized anti-IgM antibody (Defrance *et al.*, 1987b) but appears to be less active when soluble F(ab')2 fragments are used (Almerigogna *et al.*, 1989). Whereas murine IL-4 mostly acts as a competence factor (Oliver *et al.*, 1985; Rabin *et al.*, 1985, 1986), human IL-4 acts rather as a progression factor as it induces proliferation of B cells preactivated with anti-Ig antibodies (Clark *et al.*, 1989). IL-4 induces entry into mitosis of B cells whose DNA synthesis has been turned on by anti-Ig (Kim *et al.*, 1992). Furthermore, unlike mouse IL-4, it does not prepare resting B cells for a more vigorous subsequent response to mitogenic anti-Ig antibody (Gordon *et al.*, 1988b; Shields *et al.*, 1989) and, in fact, even delivers inhibitory signals. Unlike IL-2, IL-4 does not costimulate with SAC (Jelinek and Lipsky, 1988; Maher *et al.*, 1991), although it does enhance the DNA replication of SAC-preactivated B cells (Defrance *et al.*, 1987b). The DNA replication induced by IL-4 on anti-IgM- and SAC-stimulated B cells is short lasting (up to 5 days) and cannot be restimulated by readdition of IL-4, whereas that induced by IL-2 can last up to 7–8 days. It should be stressed that neither IL-2 nor IL-4 can induce the

expansion of viable B cells activated through their antigen receptor.

Paradoxically, IL-4 was found to antagonize the IL-2-induced DNA replication of B cells costimulated through their antigen receptor (Defrance *et al.*, 1988a; Jelinek and Lipsky, 1988; Maher *et al.*, 1991; Vazquez *et al.*, 1989). The inhibitory effect is particularly striking on freshly isolated leukemic B cells, such as chronic lymphocytic leukemia B cells (Karray *et al.*, 1988) and non-Hodgkin B cell lymphomas (Defrance *et al.*, 1992a) where IL-2, in contrast to IL-4, often induces a potent DNA replication. Various studies, attempting to clarify the mechanisms of the observed antagonism have yielded different conclusions. Karray *et al.* (1990) demonstrated that IL-4 strongly inhibits the expression of high-affinity IL-2 binding sites on B-CLL cells, which explains the observed inhibition of DNA synthesis. Surprisingly, this study indicated that IL-4 does not inhibit the expression of either the p55 α chain or the p75 β chain of the high-affinity IL-2R. Therefore, other mechanisms must account for the inhibitory effects of IL-4 on the high affinity IL-2R and IL-4 might either influence the state of the IL-2R α/β complex association or act on another subunit of the IL-2R. Studies with SAC-activated normal human B cells have also shown that IL-4 is able to block the IL-2-induced upregulation of both high- and low-affinity IL-2R (H. K. Lee *et al.*, 1990). Incubation of blood B lymphocytes with IL-4 alone results in increased expression of IL-2R α chain and a strong decrease in IL-2R β chain expression (Tomizawa *et al.*, 1991). Studies with murine B lymphoma cell lines confirmed the antagonism of IL-4 toward IL-2-induced B cell growth but the addition of IL-4 to the cultures did not affect the binding of IL-2 to cells in one report (Tigges *et al.*, 1989) and inhibited it in other studies (Fernandez-Botran *et al.*, 1989; Yoshimoto *et al.*, 1990). Studies with human T cells have also shown that IL-4 can inhibit the early stages of IL-2-dependent proliferation through inhibition of IL-2R expression (Martinez *et al.*, 1990) as a result of decreased expression of the IL-2R β chain (Ishikawa *et al.*, 1991; Lindqvist *et al.*, 1991). Interestingly, agents increasing intracellular cAMP levels such as cholera toxin, forskolin, and prostaglandin E_2 have been found to enhance IL-4-dependent B cell DNA synthesis but to inhibit that induced by IL-2 (Garrone and Banchereau, 1992; Vazquez *et al.*, 1991). It has been proposed that the IL-4 inhibitory signals to IL-2-dependent B cell DNA synthesis involve cAMP-dependent protein kinase activation, as IL-4 increases cAMP levels (Finney *et al.*, 1990; Taieb *et al.*, 1991) and as an inhibitor of this enzyme reverses IL-4 inhibitory effects (Vazquez *et al.*, 1991)

The inhibitory effects of IL-4 on IL-2 driven B cell proliferation

could be considered as a regulatory mechanism whereby a TH_2 cell product inhibits the TH_1 type-mediated humoral response at the B cell level. Accordingly, *in vivo* studies in the mouse have shown that TH_1 responses (e.g., response to viruses) are accompanied by increased serum levels of IgG_{2a}, whereas TH_2 responses (e.g., response to parasites) are characterized by increased IgE and IgG_1 (Finkelman *et al.*, 1990).

c. Other Stimulatory Cytokines

IL-10, of both human and viral origin, is able to enhance DNA synthesis in B cells that have been preactivated with either anti-IgM or SAC particles (Rousset *et al.*, 1992). IL-10 is, however, less active than either IL-2 or IL-4 (Table VI). It also enhances the DNA synthesis of B cells costimulated with anti-IgM antibody but is inactive on resting B cells.

TNF-α and -β are also able to enhance DNA synthesis in B cells costimulated with either anti-IgM or SAC (Jelinek and Lipsky, 1987c; Kehrl *et al.*, 1987a,b). TNF also acts as a cofactor for various B cell tropic cytokines including IL-2, IFN-γ, and IL-4 (Zola and Nikoloutsopoulos, 1989). IFN-γ, as well as IFN-α/β, costimulates with anti-IgM antibody (Defrance *et al.*, 1986; François *et al.*, 1988; Morikawa *et al.*, 1987; Romagnani *et al.*, 1986a, b; Xia and Choi, 1988). IFNs cannot

TABLE VI
INTERLEUKIN-10 INDUCES DNA SYNTHESIS IN B CELLS ACTIVATED VIA SURFACE Ig[a]

	Activation system	
Cytokine	SAC	Anti-μ
	[^{3}H]Thymidine uptake (cpm $\times 10^{-3}$)	
Medium	3 ± 0.3	2 ± 0.3
IL-10	12 ± 1.0	10 ± 0.8
IL-2	48 ± 1.2	28 ± 2.0
IL-4	20 ± 1.0	31 ± 1.9

[a] B cells were preactivated for 2 days with *Staphylococcus aureus* strain Cowan (SAC) particles or anti-IgM beads. Viable blasts were cultured with10 ng/ml interleukin-10 (IL-10). IL-2 and IL-4 were respectively used at 20 and 50 U/ml. Cells were pulsed for 16 hours with tritiated thymidine and harvested at day 3. Results are means ± SD of triplicate determinations.

induce DNA synthesis in B cell blasts, but preincubation of B cells with IFN-γ enhances their subsequent DNA synthesis induced by antigen–receptor crosslinking (Boyd *et al.*, 1987). This indicates that IFNs act at the early stages of B cell activation. IFN-γ can act as a cofactor for other B cell tropic cytokines, such as IL-4, IL-2, and BCGF-12 kDa (Jelinek *et al.*, 1986; Karray *et al.*, 1987). A recent report has indicated that low concentrations of IL-6 (1–100 pg/ml) are able to induce the proliferation of B cells purified from tonsils and spleens, whereas high concentrations (1–10 ng/ml) were ineffective (Levy *et al.*, 1990). Finally, IL-3 was recently found to enhance the DNA synthesis of B cells costimulated with anti-IgM antibody and SAC (Defrance *et al.*, 1992c; Xia *et al.*, 1992).

d. Transforming Growth Factor β

TGF-β inhibits the IL-2-dependent DNA synthesis of SAC preactivated B cells (Kehrl *et al.*, 1986a) and of B cells costimulated with anti-IgM and BCGF-12 kDa (Flescher *et al.*, 1990; Smeland *et al.*, 1987). Although TGF-β has to be present during the early culture phase, it has little or no effect on several early intermediate parameters of cell activation, such as $[Ca^{2+}]_i$, *c-myc* mRNA increase, cellular enlargement, RNA increase, and increased expression of the 4F2 activation antigen. In contrast, TGF-β blocks expression of transferrin receptor which normally appears in the late G_1 phase of the cell cycle, suggesting that TGF-β arrests cells in the middle of the late G_1 phase (Smeland *et al.*, 1987).

2. *Antigen-Dependent Activation*

The limited numbers of antigen-specific B cells that can be isolated from human lymphoid organs have hampered the development of detailed studies on antigen-dependent activation of B cells. Nevertheless, when blood B lymphocytes are cultured in the presence of trinitrophenylated polyacrylamide beads (TNP-PAA), both IL-2 and IL-4 can induce the expansion of antigen-specific B cells (Llorente *et al.*, 1990). This observation is in agreement with the IL-2 (Pike *et al.*, 1984)- and IL-4 (Alderson *et al.*, 1987)-induced proliferation of single murine antigen-specific spleen B cells cultured in the presence of their specific antigen. The *Candida albicans*-derived mannan antigen, which behaves as a TI-2 antigen, can directly activate dense tonsillar B lymphocytes of mannan-sensitized subjects and induce RNA synthesis (Mangeney *et al.*, 1989). IL-2, but not IL-4, was found to costimulate with this antigen to induce B cells from sensitized subjects to synthesize DNA.

B. CD40-Dependent Activation

Combinations of soluble anti-CD40 antibodies and either anti-IgM or phorbol esters have been shown to act in concert to induce DNA synthesis; IL-4 preferentially boosts the observed proliferation whereas IL-2 is much less efficient (Gordon *et al.*, 1987, 1988b; Vallé *et al.*, 1989). Interestingly, a combination of soluble anti-CD40 and IL-4 weakly enhances the DNA synthesis of purified resting B lymphocytes; however, none of these conditions results in long-term B cell proliferation.

In contrast, addition of IL-4 to B cells cultured in the CD40 system (composed of irradiated fibroblastic L cells that have been transfected with the human FcγRII/CDw32 antigen and anti-CD40 antibody) results in their sustained proliferation (Banchereau *et al.*, 1991). The cells grow in tight clumps and within 5 weeks the total B cell population can expand up to 1000-fold. This results in the generation of factor-dependent long-term normal B cell lines that are negative for EBV infection (Fig. 13). Cell lines can be generated from B cells

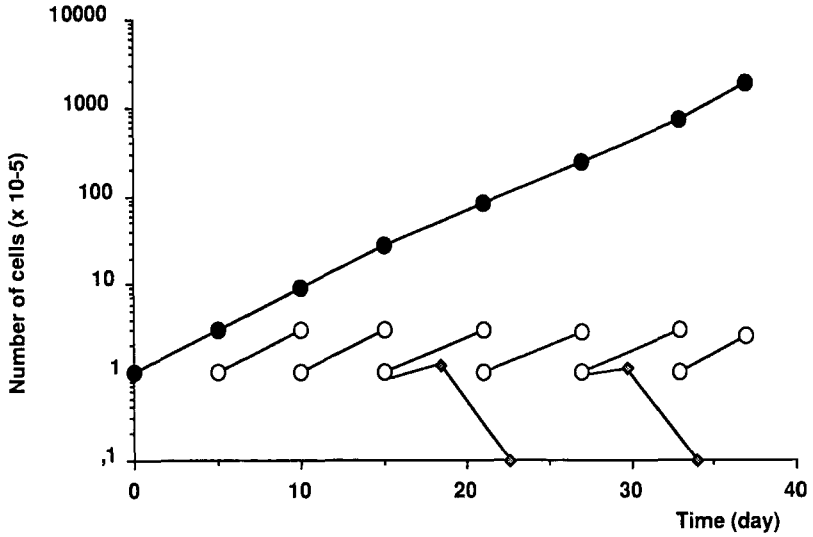


FIG. 13. Long-term proliferation of human B lymphocytes in the CD40 system. Purified B lymphocytes were cultured in the presence of irradiated CDw32 L cells, interleukin-4, and anti-CD40 monoclonal antibody 89. Cultures were divided every week (○). At days 18 and 24, a portion of cells were recultured without monoclonal antibody 89 and interleukin-4 (◆). ●—● , The theoretical number of generated B cells.

isolated from tonsils, spleen, blood, and cord blood. Naive $sIgD^+$, $sIgM^+$, isotype-committed $sIgD^-$, $CD5^+$, and $CD5^-$ B cells can all be induced to long-term growth. The generated B cell lines are strictly dependent on the presence of the anti-CD40 antibody and IL-4, as their removal halts cell proliferation and subsequently results in cell death. The combination of CDw32 L cells, anti-CD40, and IL-4 activates most B cells and induces them to enter into DNA synthesis. B cell clones can be generated that contain several hundred cells. Addition of anti-IgM antibody enhances the observed proliferation (Fig. 12).

Whereas IL-1 and IFN-γ enhance the DNA synthesis observed in the CD40 system, they do not enhance the recovery of viable B cells (Rousset *et al.*, 1991). IL-1 and, most notably, IFN-γ enhance the increase in viable B cells obtained in the CD40 system in the presence of IL-4. It is important to note that IL-2 does not modify B cell proliferation in the CD40 system, whether or not IL-4 is added to cultures. Cells cultured under these conditions express CD19, CD20, and high levels of CD23 and HLA class II antigens. Surprisingly, a quite significant proportion of cells cultured for 3 weeks still express sIgD, indicating that triggering of B cells with IL-4 and anti-CD40 is not sufficient to induce a downregulation of sIgD expression (Fig. 14). Addition of SAC particles, as a way to mimic antigen triggering, does not induce further downregulation of sIgD expression (our unpublished results); however, addition of agents increasing intracellular cAMP such as cholera toxin was found to downregulate sIgD expression, as well as enhance cell proliferation (P. Garrone and J. Banchereau, unpublished observations).

Both viral and human IL-10 enhance the proliferation of B cells cultured in the CD40 system, as determined by both tritiated thymidine incorporation and increased viable cell numbers (Table VII). It appears to be almost as efficient as IL-4 over a week's period, but proliferation slows down thereafter and eventually stops after 14 days. The combination of IL-4 and IL-10 is additive in terms of cell proliferation and results in a 60- to 100-fold expansion of viable B cells over a 2-week period. IL-10 was found to upregulate the expression of CD25/Tac on anti-CD40-activated B cells and, accordingly, addition of IL-2 enhances B cell proliferation (our unpublished results). B cell cultures in IL-10 differed microscopically from those in IL-4 in that loose aggregates were observed early, which decrease with time to yield cultures mostly composed of single large cells. At this stage, B cells have lost most of their B cell-specific antigens, such as CD19 and CD20, but express PCA-1 (Rousset *et al.*, 1992).

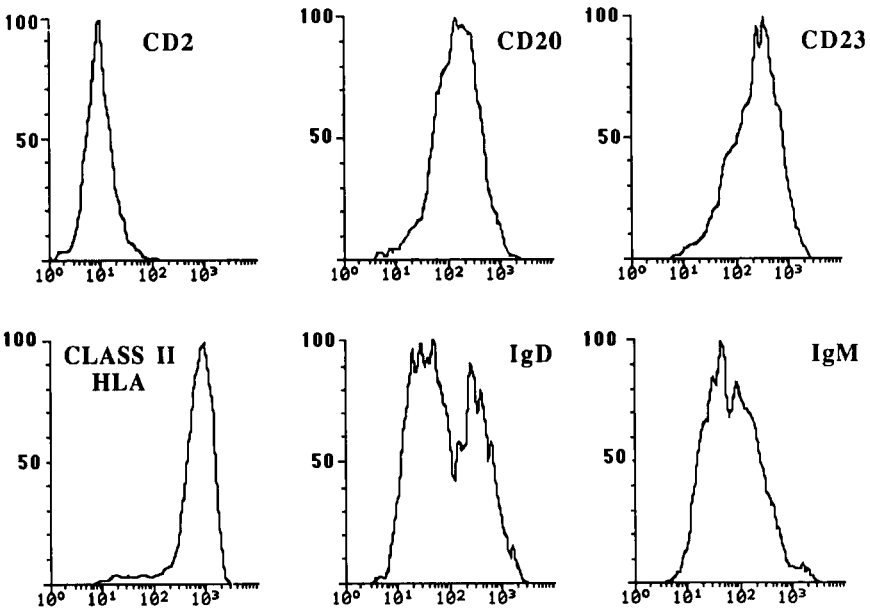


FIG. 14. Phenotype of human B lymphocytes grown in the CD40 system. Purified B lymphocytes were cultured in the presence of irradiated CDw32 L cells, interleukin-4, and anti-CD40 monoclonal antibody 89. Cultures were divided every week. At day 18, cells were stained with fluorescein isothiocyanate-labeled monoclonal antibodies specific for the shown antigens and fluorescence analysis was performed with a FACScan.

TABLE VII
GROWTH-PROMOTING ACTIVITY OF INTERLEUKIN-4 AND INTERLEUKIN-10 IN THE CD40 SYSTEM[a]

Cytokine	[^{3}H]Thymidine (cpm × 10^{-3})	Viable cell numbers (× 10^5)	
		Day 7	Day 15
None	18.6 ± 2.7	1.9	2.4
Interleukin-4 (IL-4), 50 U/ml	160.3 ± 4.4	5.7	18.2
Interleukin-10 (IL-10), 10 ng/ml	141.4 ± 4.7	5.5	8.1
IL-4 + IL-10	234.4 ± 9.6	6.8	68

[a] Purified B cells (5×10^4) were cocultured with 5×10^3 irradiated CDw32 L cells in complete medium with anti-CD40 mAb 89 with or without IL-4 and/or IL-10. Cells were pulsed for 16 hours with tritiated thymidine and harvested at day 6. Results are means ± SD of triplicate determinations. For enumeration of viable cells, cultures were initiated at 10^5 cells/well.

C. Activated T Cells

As discussed in detail earlier, activated T cells can induce a strong proliferation of resting B cells that is only weakly affected by addition of exogeneous cytokines; however, when activated T cell membranes are used to stimulate DNA synthesis of resting murine B cells, addition of cytokine-rich T cell supernatants enhances the observation B cell proliferation (Brian, 1988; Hodgkin *et al.*, 1990). Both TH_1 and TH_2 membranes induce similar levels of proliferation, and addition of IL-4 results in enhanced proliferation (Hodgkin *et al.*, 1990,1991; Noelle *et al.*, 1991). $CD4^+$ human T cell clones, preactivated for 24 hours with immobilized anti-CD3 and thoroughly washed to remove cytokines, can activate resting tonsillar B cells, and addition of IL-2 results in significant B cell proliferation (D. Blanchard and J. Banchereau, unpublished results).

D. Interleukin-6 and B Cell Growth

We have tested IL-6 for its proliferative activity in many different human B cell proliferation assays, such as anti-IgM and SAC costimulation, preactivation, and the CD40 system. These studies have failed to detect any effect of IL-6 on DNA synthesis. Similar results have been reported by several other groups (Splawski *et al.*, 1990; Tadmori *et al.*, 1989), although one group has recently observed that low levels of IL-6 display growth-promoting effects (Levy *et al.*, 1990). The lack of IL-6 activity on B cell growth may a priori suggest that IL-6 has no effect on human B cell proliferation; however, this lack of effect may also result from endogeneous production of IL-6, as high levels of IL-6 are found in the supernatants of B cells cultured in the presence of IL-4 (Smeland *et al.*, 1989; our unpublished results). (See discussion on autocrine growth in Section VIII.) This is particularly relevant as IL-6 has been described as a hybridoma/plasmacytoma growth factor for murine cells (Van Damme *et al.*, 1987; Van Snick *et al.*, 1986,1987) and stable expression of IL-6 gene in B cell lines has rendered them factor independent and tumorigenic (Scala *et al.*, 1990; Tohyama *et al.*, 1990). In human, the myeloma cell line U266 (Levy *et al.*, 1991; Schwab *et al.*, 1991), lymphoma B cell lines (Yee *et al.*, 1989), and EBV-transformed B cell lines (Yokoi *et al.*, 1990) have also been shown to depend on autocrine IL-6 for their growth, as neutralizing anti-IL-6 antibody or IL-6 antisense oligonucleotides inhibited cell proliferation. Furthermore, IL-6 has been shown to play a role in the development of myelomas either as an autocrine factor (Fielder *et al.*, 1990; Kawano *et al.*, 1988) or as a paracrine factor (Klein *et al.*, 1989; Portier *et al.*, 1991) produced by the bone marrow microenvironment.

In fact, increased serum IL-6 levels correlate with disease severity in multiple myelomas and plasma cell leukemias (Bataille *et al.*, 1989). The therapeutic administration of neutralizing anti-IL-6 monoclonal antibodies has transiently improved the clinical status of a patient with plasma cell leukemia (Klein *et al.*, 1991). The role of IL-6 in the development of myelomas is further demonstrated by the fact that mononuclear cells from patients with multiple myelomas cultured in the presence of IL-3 and IL-6 give rise to a population of actively proliferating B blasts which differentiate into monoclonal plasma cells expressing the Ig produced by the malignant plasma cells (Bergui *et al.*, 1989). This indicates that an IL-6-sensitive myeloma cell progenitor circulates in the blood of myeloma patients and that IL-6 may be a growth factor for plasmablasts.

VI. Cytokines and B Lymphocyte Differentiation

The role of cytokines in the differentiation of B lymphocytes was suspected when supernatants from lymphocytes activated either with polyclonal mitogens such as concanavalin A or in a mixed lymphocyte reaction were found to contain most of the B cell stimulatory activity previously ascribed to T cells (Dutton *et al.*, 1971; Schimpl and Wecker, 1972). The earlier studies on human B cell differentiation were performed using blood mononuclear cells and polyclonal activators such as pokeweed mitogen (Jelinek and Lipsky, 1987a) and antigens (Möller, 1979). The first studies on the role of cytokines in the differentiation of purified human B cells were performed in the early 1980s (Falkoff *et al.*, 1982; Saiki and Ralph, 1981).

A. Antigen Receptor-Dependent Activation

Early studies indicated that blood B cells stimulated by SAC and T cell supernatants produce IgM, IgG, and IgA (Harada *et al.*, 1982; Jelinek *et al.*, 1986). Separation of these cells into IgD^+ and IgD^- subsets has shown that the IgD^+ subset secretes predominantly IgM, whereas the IgD^- subset secretes predominantly IgG and IgA. This indicates that SAC stimulation is unlikely to give sufficient signals for B cells to undergo isotype switching, and, at the present time, no single recombinant cytokine has indeed been demonstrated to do so.

1. Interleukin-2

SAC-activated B cells secrete IgM, IgG, and IgA in response to IL-2 (Bertolini and Benson, 1990; Defrance *et al.*, 1988b; Emilie *et al.*, 1987; Muraguchi *et al.*, 1985; Nakagawa *et al.*, 1985,1986; Splawski *et*

al., 1989). Both IgG_1 and IgG_2 subclasses are stimulated and, according to Splawski, IL-2 induces SAC-activated B cells to produce IgE, but we have not been able to reproduce this latter finding. Several cytokines that cannot induce Ig production by themselves, such as IL-1, IL-3, IL-6, TNF-α/β, and IFN-α/γ, stimulate IL-2-induced Ig secretion (Emilie *et al.*, 1988; Jelinek and Lipsky, 1987b; Jelinek *et al.*, 1986; Kehrl *et al.*, 1987b; Nakagawa *et al.*, 1985; Tadmori *et al.*, 1989). TGF-β acts as a strong inhibitor of IL-2-induced Ig secretion by inhibiting the synthesis of Ig mRNA and inhibiting the switch from the membrane form to the secreted forms of μ and γ mRNA (Kehrl *et al.*, 1989, 1991).

IL-2 has also been shown to act as a potent T cell-replacing factor in influenza-specific antibody response of human B cells (Callard *et al.*, 1986; Smith *et al.*, 1989). Interestingly, IL-2 is the only recombinant cytokine able to induce this antibody response and it acts preferentially on low-density B cells. High-density B cells can also be induced to produce antigen-specific antibody, but only in the presence of the commercial low-molecular-weight BCGF and the responsible cytokine(s) has not been identified. IL-2 has also been shown to act as a T cell-replacing factor in the specific antitrinitrophenyl response of purified human B cells induced by TNP-PAA (Delfraissy *et al.*, 1988). An IL-2-free fraction containing T cell-replacing factor activity has also been isolated (Vazquez *et al.*, 1986) but the active component has not yet been molecularly identified. IFN-α was found to enhance strongly the IL-2-induced specific antibody response (Delfraissy *et al.*, 1988). In contrast, IL-4 inhibits the IL-2-induced specific antibody response (Llorente *et al.*, 1989) although it enhances antigen-induced B cell proliferation (Llorente *et al.*, 1990). The proliferation is entirely dependent on the antigen and can be inhibited by soluble hapten–protein conjugates and anti-IgM, suggesting that the activation is mediated through IgM antigen receptor. IL-2 also acts as a T cell-replacing factor for antigen-activated single murine B cells (Pike *et al.*, 1987).

2. *Interleukin-4*

IL-4 can induce SAC-preactivated B cells to produce IgG and IgM (Defrance *et al.*, 1988b; Jelinek and Lipsky, 1988). Unlike IL-2, it is unable to induce Ig production by B cells which are costimulated rather than preactivated with SAC. Moreover, it blocks IL-2-induced Ig secretion in SAC-costimulated B cells, and this inhibition is not observed with SAC-preactivated B cells. The mechanisms of the IL-4 inhibitory effects observed during costimulation of B cells with SAC

presently remain unexplained. IL-4 does not induce SAC-stimulated B cells to produce IgE, and IL-4-induced IgG and IgM secretion is not inhibited by IFN-γ. Finally, IL-4 enhances Ig secretion induced by a combination of lipopolysaccharide and IL-2 (Splawski *et al.*, 1989).

Inhibitory effects of IL-4 on antigen-specific Ig production have also been observed. In particular, the secondary response of B cells to influenza virus, which requires both antigen and IL-2, can be inhibited by IL-4 (Callard *et al.*, 1991). Likewise, the IL-2-dependent primary response to TNP-PAA is also inhibited by IL-4 (Llorente *et al.*, 1989); however, IL-4 essentially blocks the IL-2-dependent B cell differentiation whereas it stimulates the antigen-dependent specific B cell pro liferation (Llorente *et al.*, 1990). Studies in mice have also shown that IL-4 can induce the proliferation of hapten-specific B cells in response to T-independent antigen, while being a poor stimulator of B cell differentiation.

3. *Other Stimulatory Cytokines*

Both human and viral IL-10 are able to induce SAC-preactivated B lymphocytes to produce high levels of IgM, IgG, and IgA, but not IgE (Fig. 15). IL-10 appears to be more potent than either IL-2 or IL-4 (Rousset *et al.*, 1992).

Although our own unpublished studies and those of others (Splawski *et al.*, 1990; Tadmori *et al.*, 1989) failed to observe an effect of IL-6 on the proliferation and Ig secretion of SAC-stimulated human B cells, a recent study (Bertolini and Benson, 1990) indicated that IL-6 can induce SAC-preactivated B cells to produce IgM and IgG, but not IgA. Interestingly, pokeweed mitogen-stimulated B cells were found to produce IgM, IgG, and IgA in response to IL-6, thus suggesting that the lack of IgA in the SAC assay may be the result of the use of SAC rather than to IL-6 itself. IL-6 did not induce isotype switching of pokeweed mitogen-stimulated B cells as it induced $sIgM^+$ B cells to secrete only IgM. Whereas IL-2 and IL-4 enhanced the IL-6-induced Ig secretion, IFN-γ inhibited it. IL-3 induces Ig secretion by SAC-stimulated cells (Tadmori *et al.*, 1989) mostly as a consequence of increased B cell proliferation (Xia *et al.*, 1992). IL-3 also enhances IL-2-induced B cell proliferation and differentiation following enhanced IL-2R expression (Xia *et al.*, 1992).

B. CD40-Dependent Activation

Purified B cells cultured in the CD40 system produce very small amounts of IgM, IgG, and IgA and less than 5% of cultured cells secrete Igs, as detected by ELISPOT assays. Addition of IL-4, which

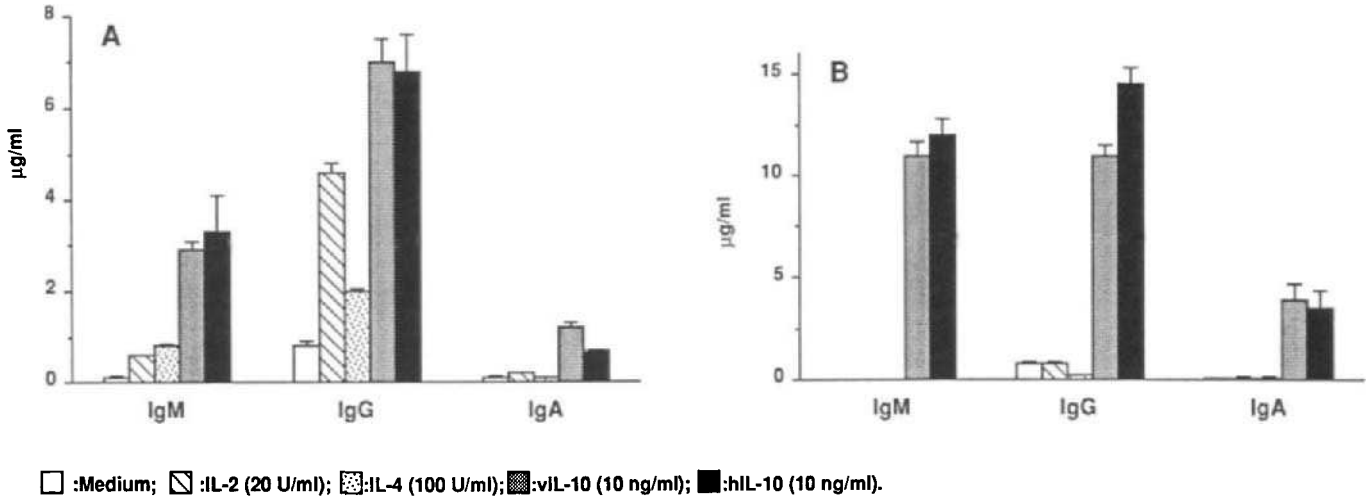


FIG. 15. Interleukin-10 is a potent B cell differentiation factor. (A) *Staphylococcus aureus* strain Cowan (SAC) activation: purified B lymphocytes (10^6/ml) are stimulated for 48 hours with 0.01% SAC; 5×10^4 blasts in 100 μl are cultured for 5 days with or without the recombinant cytokines. (B) CD40 system: 2.5×10^4 purified B lymphocytes are cultured in 100 μl for 10 days in the presence of 2.5×0^3 irradiated CDw32 L cells and 0.5 μg/ml anti-CD40 monoclonal antibody 89 with or without cytokines. Ig levels are measured by ELISA.

enhances proliferation, results in a slight increase in the production of IgM and IgG (Rousset *et al.*, 1991) and, more strikingly, in the secretion of large amounts of IgE. In fact, IgE production results from isotype switching as highly purified naive $sIgD^+$ B cells produce as much IgE as isotype-committed $sIgD^-$ B cells (Table VIII). In contrast to long-term B cell proliferation, the production of IgE does not require the presence of CDw32 L cells (Gascan *et al.*, 1991; Jabara *et al.*, 1990; Shapira *et al.*, 1992; K. Zhang *et al.*, 1991). Addition of IFN-γ or IFN-α to these cultures surprisingly fails to inhibit IL-4-induced IgE production, thus contrasting with earlier studies in which B cell costimulation was generated by either mononuclear non-B cells (Del Prete *et al.*, 1988; Pène *et al.*, 1988a; Sarfati and Delespesse, 1988; Vercelli *et al.*, 1990), T cell clones (Pène *et al.*, 1988b), and Epstein–Barr virus (Thyphronitis *et al.*, 1989). TGF-β, however, is able to block totally the production of IgE by CD40-activated B cells without much affecting the concomitant proliferation (de Waal Malefyt *et al.*, 1992; Gauchat *et al.*, 1992a; our unpublished observations). TNF-α strongly enhances IL-4-induced IgE production by anti-CD40 (and T cell)-activated B cells (Gauchat *et al.*, 1992a). Addition of IL-2 to B cells stimulated in the CD40 system together with IL-4 results in a significant increase in IgM and IgA secretion (Rousset *et al.*, 1991).

Addition of IL-10 to B lymphocytes cultured in the CD40 system results in the production of considerable amounts of IgM, IgG, and IgA

TABLE VIII
$sIgD^+$ NAIVE B CELLS PRODUCE IgE IN THE CD40 SYSTEM IN RESPONSE TO INTERLEUKIN-4[a]

B cell population	Cytokine		
	None	IL-10	IL-4
	IgE (ng/ml)		
Unseparated	<0.1	<0.1	33
$sIgD^+$	<0.1	<0.1	37
$sIgD^-$	<0.1	<0.1	18

[a] Highly purified unseparated, $sIgD^+$ and $sIgD^-$ B cells were isolated from tonsils as described in the legend to Fig. 4. B cells (2.5×10^4) were cocultured with 5×10^3 irradiated CDw32 L cells in complete medium with or without 50 U/ml IL-4. Supernatants were harvested after 10 days and IgE levels determined by enzyme-linked immunosorbent assay. Results are means of triplicate determinations.

without any IgE (Fig. 15). IL-10 induces virtually all B cells to secrete Ig, as determined in ELISPOT assays (Rousset *et al.*, 1992). In fact, cells cultured in the CD40 system in the presence of IL-10 differentiate into plasma cells expressing large amounts of intracytoplasmic immunoglobulin. Electron microscopy (Fig. 16) demonstrates the presence of extremely well-developed endoplasmic reticulum with cisternae which are the features characterizing plasma cells. Interestingly, most B cells cultured for 10 days in the presence of IL-10 float freely over the fibroblast layer. Addition of IL-6 to B cells cultured in the CD40 system with or without IL-10 has no effects on their growth, survival, or Ig production (Rousset, 1992).

Addition of IL-4 to B cells cultured in the CD40 system in the presence of IL-10 results in strong cell growth; the production of Igs is significantly reduced, most likely because IL-4 favors cell growth rather than cell differentiation. Nevertheless, in the long term, the combination of both cytokines permits the generation of large numbers of plasma cells. In fact, the production of IL-4 and IL-10 by TH_2 cell clones may account for the preferential effect of these cells on humoral responses. It has unfortunately not yet been established whether murine IL-10 also acts as a plasma cell differentiation factor *in vitro;* however, IL-10 is likely to play a role in B cell differentiation *in vivo* because administration of anti-IL-10 antibodies to mice results in decreased circulating IgM and IgA levels (M. Howard, personal communication). In keeping with this, lesions of lepromatous leprosy, the progressive form of the disease characterized by hypergammaglobulinemia, were found to contain mRNA for IL-4 and IL-10 (TH_2 type), whereas lesions from the self-healing tuberculoid leprosy display mRNA for IL-2 and IFN-γ (TH_1 type) (Yamamura *et al.*, 1991). It is possible that the IL-4 observed in these lesions may originate from either TH_2 $CD4^+$ T cells or suppressive $CD8^+$ T cell clones (Salgame *et al.*, 1991). Recent preliminary studies have indicated that addition of low concentrations of IL-2 to B cells cultured in the CD40 system enhances the IL-10-induced Ig secretion. This observation may not contrast with the TH_1, TH_2 cell classification as these two cell types produce IL-10 in humans (H. Yssel and D. Blanchard, personal communications). Furthermore, IL-10 is also produced in large amounts by antigen presenting cells during antigen presentation to T cells (de Waal Malefyt *et al.*, 1991b).

Addition of TGF-β to unseparated tonsillar B cells cultured in the CD40 system together with IL-10 inhibits the Ig production of the various isotypes; however, striking differences are observed when B cells are separated according to their sIgD. The naive $sIgD^+$ $sIgM^+$ B

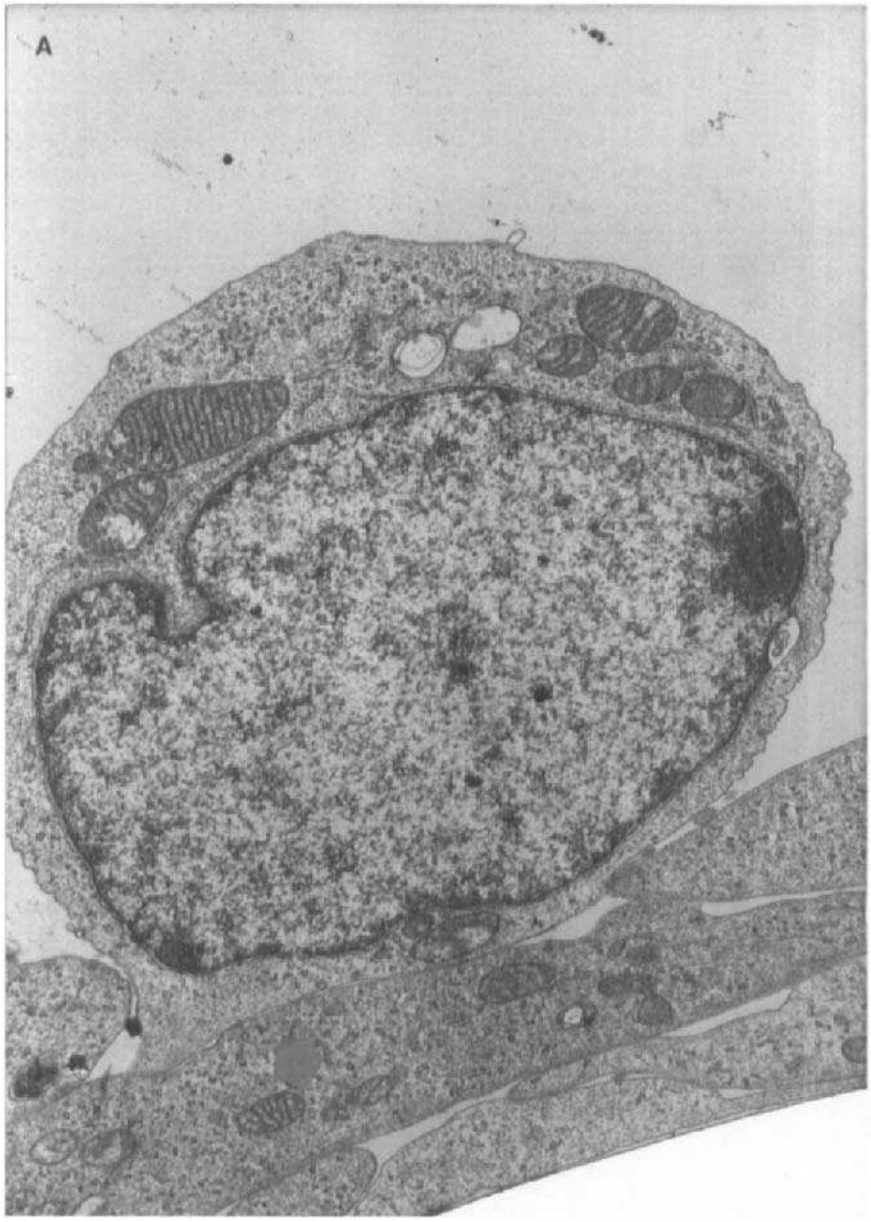


FIG. 16. Ultrastructure of purified tonsillar B lymphocytes cultured in the CD40 system. Cells have been grown for 7 days on fibroblastic L cells stably expressing human CDw32/FcγRII in the presence of anti-CD40 antibody and either interleukin (IL)-4 (A) or IL-10 (B). (A) IL-4-induced B cell blast with indented nuclei, few mitochondria, and many free ribosomes, but little endoplasmic reticulum. (B) IL-10-induced plasma cell with prominent rough-surfaced endoplasmic reticulum.

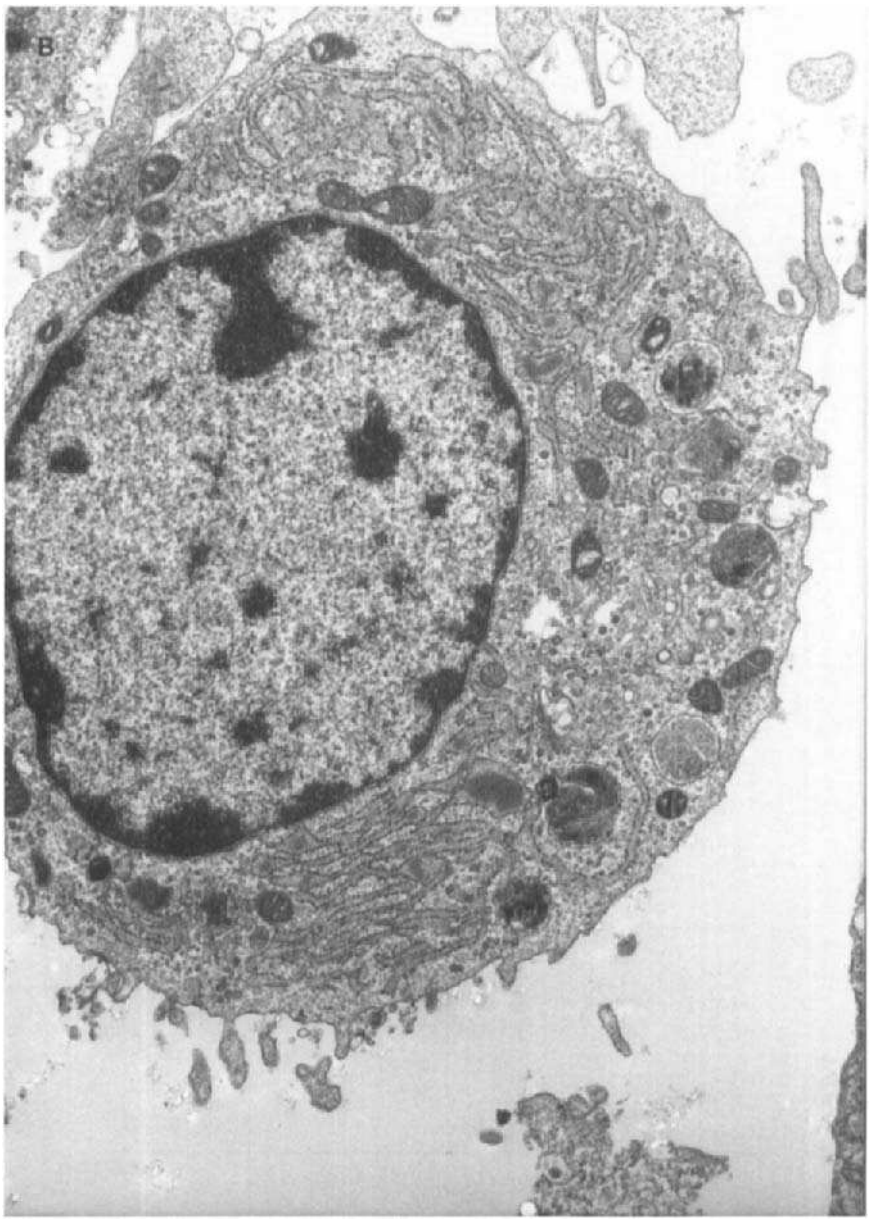


FIG. 16. Continued

cell population, which essentially produces IgM under these conditions, can be induced to produce quite large amounts of both IgA_1 and IgA_2 subtypes on addition of TGF-β, whereas IgM production is quite significantly inhibited (Defrance *et al.*, 1992b; Brière, 1992) (Table IX). In contrast, TGF-β inhibits the production of IgM, IgG, and IgA by isotype-committed $sIgD^-$ B cells stimulated by IL-10 in the CD40 system. This strongly suggests that TGF-β may represent the IgA switch factor for both human and mouse (Coffman *et al.*, 1989; Lebman *et al.*, 1990; Sonoda *et al.*, 1989). In line with our findings, recent studies have indicated that TGF-β is able to induce α_1 and α_2 germline transcripts in *Branhamella catarrhalis*-activated human B cells (Islam *et al.*, 1991; Nilsson *et al.*, 1991).

Therefore activating B cells through their CD40 antigen permits us to evaluate the switching capacity of both IL-4 and TGF-β and the plasma cell inducing activity of IL-10. No cytokine or cytokine combination has yet been found to induce switching toward IgG 1.3.

C. Activated T Cells

When cocultured with alloreactive and autoreactive T cell clones, normal human B lymphocytes can produce IgE and this activity is inhibited by anti-IL-4 antibodies (Del Prete *et al.*, 1988; Lanzavecchia, 1983; Pène *et al.*, 1988b; Ricci *et al.*, 1985; Umetsu *et al.*, 1985). Accordingly, addition of IL-4 to blood, tonsil, or spleen mononuclear cells results in the production of $CD4^+$ T cells (Chrétien *et al.*, 1990; Pène *et al.*, 1988a; Sarfati and Delespesse, 1988; Vercelli *et al.*, 1989). Addition of recom-

TABLE IX
$sIgD^+$ Naive B Cells Produce IgA in the CD40 System in Response to Interleukin-10 (IL-10) and Transforming Growth Factor β[a]

B cell population	Cytokine		
	None	IL-10	IL-10 + TGF-β
	IgA (μg/ml)		
Unseparated	<0.1	5	1
$sIgD^+$	<0.1	<0.1	6
$sIgD^-$	<0.1	2	1

[a] Highly purified unseparated, $sIgD^+$ and $sIgD^-$ cells were isolated from tonsils as described in the legend to Fig. 4. B cells (2.5×10^4) were cocultured with 5×10^3 irradiated CDw32 L cells in complete medium with or without 10 ng/ml IL-10 or 1 ng/ml TGF-β. Supernatants were harvested after 10 days and IgA levels determined by enzyme-linked immunosorbent assay. Results are means of triplicate determinations.

binant IL-4 to cocultures of B lymphocytes and peripheral blood T cells or the mouse thymoma EL-4 results in the production of IgG_4 and IgE (Lundgren *et al.*, 1989; Nüsslein and Spiegelberg, 1990). Single cell studies have shown that, under these conditions, IgE responses are generated frequently by either $sIgM^+$ or $sIgA^+$ B cells, but rarely by $sIgG^+$ B cells (X. Zhang *et al.*, 1991). (For more details, see Note Added in Proof, Section vi.) Comparable studies performed with activated $CD4^+$ human T cell clones, but not activated $CD8^+$ T cell clones, have also demonstrated that IL-4 can induce $sIgM^+$ B cells to secrete IgG_4 and IgE (Gascan *et al.*, 1991). B cells isolated from cord blood or from fetuses can also be induced to produce IgE and IgG_4 in response to T cell signaling and IL-4 (Pastorelli *et al.*, 1990; Punnonen *et al.*, 1992; Splawski and Lipsky, 1991). Interestingly, cocultivation of $sIgM^+$ cloned EBV-transformed cell lines or Burkitt lymphoma cells together with activated $CD4^+$ T cell clones and IL-4 results in the production of IgE. This production of IgE results from a recombination deletion event and is relatively stable even in the absence of IL-4 and $CD4^+$ T cells (Gauchat *et al.*, 1992). The induction of IgE production in these B cell lines is inhibited by IFN-α, IFN-γ, and TGF-β. Human $sIgA^-$ B cells activated by $CD4^+$ T cell clones and pokeweed mitogen can be induced to produce IgA following a pulse with TGF-β (Van Vlasselaer *et al.*, 1992).

Activated murine T cell membranes, which induce resting murine B cells to proliferate, are unable to induce them to secrete Igs. Addition of T cell supernatants results in significant Ig secretion (Hodgkin *et al.*, 1990,1991; Noelle *et al.*, 1991). The cytokine repertoire of the supernatants directs the level and quality of Ig production. TH_2 supernatants are much more efficient inducers of Ig secretion than TH_1 supernatants. Human B cells activated for 6 days with anti-CD3-activated T cells can produce Igs when recultured in the presence of cytokines, especially the combination of IL-2 and IL-6 (Vernino *et al.*, 1992a); however, addition of fresh anti-CD3-activated T cells appears to be more efficient, possibly because of the presence of IL-10 in these supernatants. When the secreted cytokines are removed from anti-CD3-preactivated human T cell clones, resting B cells can be induced to secrete large amounts of Igs in response to recombinant IL-2 and IL-10 (D. Blanchard and J. Banchereau, unpublished results).

D. *In Vivo* Activated Plasmablasts

In adult mammals, plasma cells capable of high-rate Ig secretion accumulate in the bone marrow, which represents the main source for antibodies in the secondary response. It has been estimated that up to two-thirds of daily production of IgG and IgA and 20–30% of daily IgM

secretion are derived from bone marrow (Benner *et al.*, 1981; Hibi and Dosch, 1986a,b). As will be discussed later, these high-rate Ig-secreting cells are not generated in the bone marrow but originate from stimulated secondary lymphoid organs. These cells, which represent 1–5% of bone marrow B cells (0.01–0.1% of bone marrow mononuclear cells), do not proliferate but secrete Igs at the rate of $0.5–1 \times 10^8$ Ig molecules per hour for up to 2 weeks *in vitro*. This rate is considerably larger than that of human plasma cells produced *in vitro* by pokeweed mitogen activation ($\approx 10^5$ Ig molecules/cell per hour) (Dosch *et al.*, 1985) or those reported for lipopolysaccharide-activated murine B cells (10^5 Ig molecules/cell per hour) (Andersson *et al.*, 1974). High-rate Ig-secreting cells are also observed in blood and tonsils, but the high-rate secreting cells found in the bone marrow display a more mature plasmablast-like phenotype ($CD20^-$, $CD19^{+/-}$, $CD38^+$) than those isolated from either blood ($CD20^-$, $CD19^+$, $CD38^{+/-}$) or tonsils ($CD20^+$, $CD19^+$, $CD38^{+/-}$) (Brieva *et al.*, 1991). Furthermore, bone marrow-derived high-rate IgG-secreting cells produce Ig in a linear fashion for as long as 2 weeks, whereas those isolated from either blood or tonsils lose this capacity after 3 days.

IL-6 was found to be essential for the maturation of high-rate Ig-secreting cells, as anti-IL-6 antibodies inhibit 75–90% of Ig production (Brieva *et al.*, 1991). Furthermore, when endogenous IL-6 synthesis is restricted by culture conditions, the inhibited Ig secretion can be restored by exogenous IL-6 (Roldan and Brieva, 1991). The IL-6 is needed early during the culture and probably induces B cell maturation, as proliferation is not affected. The IL-6 is secreted mostly by the microenvironment, as $CD38^+$ Ig-secreting B cells do not secrete IL-6 and as fibroblast stromal cell layers were found to support these high-rate Ig-secreting cells through the production of IL-6 (Roldan and Brieva, 1991). Antigen-activated antibody-producing B cells can be generated by systemic immunization with pneumococcal vaccine or diphtheria toxoid (Lue *et al.*, 1991) and can be recovered from the blood 7 to 9 days after vaccination. *In vitro* addition of IL-6 to these cells increases the frequency of antibody-secreting cells without modifying the isotype distribution. The amount of antibody detected in culture supernatants is increased in a parallel fashion.

Freshly isolated human appendix B cells respond to IL-6 by a three- to fivefold increase in the number of IgA-secreting cells, of which 60–70% are of the IgA_2 subclass (Fujihashi *et al.*, 1991). This and previous results obtained with murine Peyer's patch B cells (Beagley *et al.*, 1989,1991) indicate that IL-6 induces rapid differentiation of sIgA B cells into cells secreting IgA.

Taken together these different studies indicate that IL-6 acts as a differentiation factor for *in vivo* activated plasmablasts.

E. Interleukin-6 and Autoimmune Diseases

IL-6 has been described as a B cell differentiation factor, or BSF2 (Hirano *et al.*, 1985,1986), that enhances the immunoglobulin secretion by a B cell line. This *in vitro* observation has been confirmed by several *in vivo* studies. In particular, patients suffering from cardiac myxomas who present serum hypergammaglobulinemia, as well as multiple autoantibodies and increased acute-phase proteins, display elevated IL-6 levels of tumor origin (Hirano *et al.*, 1987; Jourdan *et al.*, 1990). Abnormal IL-6 production and polyclonal plasmacytosis have also been observed in patients with Castleman's disease, a chronic benign hyperplastic lymphadenopathy characterized by large follicles containing plasma cells (Yoshizaki *et al.*, 1989). In keeping with this, IL-6 transgenic mice also show plasmacytosis and a polyclonal increase in IgG_1 (Suematsu *et al.*, 1989). Likewise, mice reconstituted with IL-6 retrovirus-infected hematopoietic cells develop a syndrome that closely resembles Castleman's disease, with extensive plasma cell infiltration in many organs (Brandt *et al.*, 1990). Finally, rheumatoid arthritis, which displays polyclonal plasmacytosis, autoantibodies and increased acute-phase proteins, is also characterized by increased levels of IL-6 in joints and serum (Hirano *et al.*, 1988; Houssiau *et al.*, 1988b).

F. Isotype Switching of Human B Cells

1. General Considerations on Isotype Switching

The first Ig secreted by a naive B lymphocyte following antigen encounter is an IgM consisting of a functional variable-region gene (V_HDJ_H gene) associated with the $C\mu$ constant-region gene. In response to antigenic challenge, the cell can "switch" the constant-region gene expressing the same V_HDJ_H gene with another C_H gene. This mechanism contributes to the diversity of the immune response as the various classes mediate different effector functions on the antigenic target, such as agglutination, complement fixation, and transmembrane signaling after binding to different Fc receptors (Metzger, 1990).

Various mechanisms of isotype switching have been invoked. The studies in murine models have concluded that isotype switching is generally associated with a DNA recombination event in which the $C\mu$ gene is deleted together with all the other C_H genes originally lying

between Cμ and the newly expressed C_H gene (Esser and Radbruch, 1990). The recombination junction occurs within (or near) highly repetitive "switch sequences" that are located upstream of each C_H gene with the exception of Cδ. The recombination forms a hybrid switch region including an upstream segment of the μ switch region (Sμ) and a downstream segment from the S region of the targeted heavy chain. As a consequence of such a rearrangement, a circular piece of DNA is excised which has been named *switch circle* (Matsuoka *et al.*, 1990; Yoshida *et al.*, 1990).

The mechanisms involved in isotype switching are poorly defined. It is accepted that isotype switching is preceded by the expression of transcripts that initiate 5′ of the switch region specific for the constant region to be expressed and which encompass the downstream C_H gene (sterile transcript). A correlation between the transcriptional activation of a particular C_H gene and its subsequent participation in a switch recombination event has led to proposal of the "accessibility model" of heavy-chain switch (Stavnezer-Nordgren and Sirlin, 1986; Yancopoulos *et al.*, 1986). This model postulates that class switching in B cells occurs by modulating the accessibility of a selected C_H gene to a recombination machinery that would be common for all isotypes. The accessible state of a heavy-chain gene is thought to correlate with its transcriptional activation and the generation of transcripts that traverse the S region targeted for a switch recombination. Such transcripts, called germline transcripts, were first described for murine B cells coding for μ, γ_1, γ_{2b}, γ_3, α, and ε and, more recently, for human γ_1, γ_3, γ_4, α_1, ε, and α_2 (Gauchat *et al.*, 1990; Islam *et al.*, 1991; Kerr and Burrows, 1991; Nilsson *et al.*, 1991; Sideras *et al.*, 1989).These germline transcripts were all shown to display a similar structure. They initiate at multiple sites within regions called I (intron), located upstream of the corresponding S region, and they proceed through the S region and the constant chain region and terminate at normal sites downstream of the constant chain gene. The intervening sequences are spliced from the primary transcript to generate RNA molecules with sizes different from those of the productive V_H-containing transcripts of the same isotype. Germline transcripts coding for both the membrane and secreted forms of Cα have been detected in TGF-β-activated human B cells (Islam *et al.*, 1991).

Two other mechanisms have been proposed to explain isotype switching. In one case, cells may express two isotypes simultaneously by the alternative splicing of a long RNA transcript containing the VDJ_H gene segment and one or more C_H gene segments. Such an EBV-transformed B cell line, coexpressing IgM, IgD, and IgE, has

been described (Chan *et al.*, 1990; MacKenzie and Dosch, 1988). The studies with normal B lymphocytes have not yet convincingly addressed this possibility. Another possible mechanism for isotype switching and double isotype expression not requiring switch recombination is the transsplicing of the VDJ exon from functional Cμ transcripts to germline transcripts from unrearranged constant-region genes (Shimizu *et al.*, 1990, 1991).

2. *Interleukin-4 and Immunoglobulin E Switching*

Recent studies by MacKenzie and Dosch (1989) and Chan *et al.* (1990) have identified an EBV-transformed human B cell clone that produces IgE, IgD, and IgM without undergoing switch rearrangement. These observations have led to the suggestion that the IgE isotype may be unique in that IgE-producing cells may not require Cε gene rearrangement. In contrast, other studies (Gauchat *et al.*, 1992b; Shapira *et al.*, 1991; Thyphronitis *et al.*, 1991) have recently demonstrated that EBV-transformed B cell lines producing IgE generated in the presence of IL-4 display Cε rearrangements. Such results have also been found with normal B cells (Shapira *et al.*, 1992). Human IL-4, in a similar fashion to mouse IL-4, is able to induce purified resting B lymphocytes to express 1.8-kb germline Cε transcripts (Gauchat *et al.*, 1990, 1992a; Jabara *et al.*, 1990; Qiu *et al.*, 1990; Rothman *et al.*, 1988; Shapira *et al.*, 1992). IL-4 appears to be the sole cytokine able to induce the expression of germline Cε transcripts. TGF-β is the only cytokine able to block the IL-4-dependent expression of the germline Cε transcripts, thus explaining its inhibitory effects on IgE synthesis. In contrast, TNF-α is able to enhance the IL-4-dependent expression of Cε transcripts (de Waal Malefyt *et al.*, 1992; Gauchat *et al.*, 1992a). Interestingly, IFNs and IL-6, which respectively block and stimulate IL-4- and T cell-dependent IgE synthesis, do not modify the levels of Cε germline transcripts and thus must act at other regulatory levels of IgE synthesis; however, it should be noted that non-IL-4-producing $CD4^+$ T cell clones and their derived membranes are able to induce purified B cells to express germline Cε transcripts through a presently uncharacterized mechanism (Gauchat *et al.*, 1990).Further activation of B cells with anti-CD40 antibody strongly enhances the increase in germline Cε mRNA and results in the expression of a mature 2.0-kb Cε mRNA. This indicates that CD40 crosslinking may be an important event in the activation of the recombination machinery. DNA analysis of Sμ/Sε hybrid switch regions showed these fragments to result from the direct joining of Sμ to Sε. These results obtained after CD40 triggering are in agreement with those obtained with EBV, although in

another study with IL-4 and EBV fragments of Sγ were found interposed between Sμ in Sε (Mills *et al.*, 1992), as observed *in vivo* in parasite-infected mice (Yoshida *et al.*, 1990). These studies have indicated that the recombination sites in Sμ are clustered within 900 bp of the S- end of the μ switch region, with some sites ("hot spots") being preferentially used. In contrast, the Sε recombination sites appear to be scattered throughout this region (Mills *et al.*, 1992; Shapira *et al.*, 1992). The combination of hydrocortisone and IL-4 has also been shown to induce B cells to produce IgE as a consequence of isotype switching (Jabara *et al.*, 1991; Sarfati *et al.*, 1989; Wu *et al.*, 1991). The mechanisms of action of corticosteroids remain unclear and may involve the activation of transcription factors controlling Ig gene transcription.

VII. Role of Complement in B Cell Growth and Differentiation

The complement system is composed of 30 proteins. It participates in the elimination of foreign substances and organisms. It does so by enhancing phagocytosis (Rother and Till, 1988). The C3 component plays a key role, as it is activated through both the classical and the alternative pathways of complement activation (Lambris, 1990) and as it binds to many different proteins and receptors. Two of these receptors are expressed on B cells and three are expressed on follicular dendritic cells.

Genetic deficiencies of various complement proteins, in animals and humans, result in altered humoral immune responses and particularly defective isotype switching toward IgG. Mice depleted of C3 by treatment with cobra venom factor during priming fail to produce memory B cells (Klaus and Humphrey, 1977). Furthermore, a recombinant soluble form of CR2/CD21, made by fusing the C3 binding region of the receptor to IgG_1, competes with cellular CR2/CD21 for C3 binding and suppresses the antibody response to a T cell-dependent antigen when administered to mice at the time of immunization (Hebell *et al.*, 1991). Both the primary IgM response and the subsequent isotype switching are inhibited.

Early studies with human B cell lines have indicated that triggering CR2/CD21 can enhance their proliferation (Hatzfeld *et al.*, 1988; Pernegger *et al.*, 1988). Preincubation of resting human B lymphocytes with polymeric C3dg primes the B cell for subsequent stimulation through the antigen receptor (Carter and Fearon, 1989). Aggregated C3dg also costimulates with phorbol esters to induce DNA synthesis in resting B cells (Bohnsack and Cooper, 1988). With regard to the mecha-

nisms of action, polyvalent ligands of CR2/CD21 (including C3d and a synthetic peptide that represents the CR2 binding sequence) enhance the anti-Ig-induced increase in human B cells $[Ca^{2+}]_i$, whereas monovalent ligand appears inhibitory (Tsokos *et al.*, 1990). Such observations confirm the stimulation of B cell proliferation induced by antibodies to the CR2/CD21 (Frade *et al.*, 1985). Polymerized human C3 has also been found to sustain the proliferation of lipopolysaccharide-activated mouse B cell blasts (Erdei *et al.*, 1985; Melchers *et al.*, 1985).

The C3 component is not the only active complement protein on B cells, as the C1q subcomponent stimulates Ig production by human B lymphocytes costimulated with SAC (Young *et al.*, 1991). The Bb component, a product of the cleavage of factor B by factor D and C3b, enhances DNA synthesis of SAC-stimulated B cells (Peters *et al.*, 1988). Bb appears to be antigenically and functionally related to a high-molecular-weight BCGF, the molecular structure of which remains to be determined (Ambrus *et al.*, 1985). The human Ba component has also been shown to support the growth of activated murine lymphocytes (Praz and Ruuth, 1986).

Taken together several activated components of complement appear to activate B cells. These stimulatory effects of complement components on B cells benefit the immune reaction as activation of complement results from invasion of pathogens. The production of more antibody permits the immune system to combat the invasive agent, particularly as the formation of immune complexes depletes the pool of available antibodies. In fact, complement also participates in the elimination of these immune complexes (Schifferli *et al.*, 1986), as well as in their presentation on follicular dendritic cells.

VIII. Autocrine Aspects of B Cell Growth and Differentiation

A. Studies with B Cell Lines and Tumors

The conditioned medium of EBV-transformed B cell lines has revealed the presence of B cell stimulatory factors that could act in an autocrine fashion (Blazar *et al.*, 1983; Gordon *et al.*, 1984a,b). Various cytokines have been purified from these cell line supernatants and molecularly characterized: soluble CD23 (Gordon, 1991; Swendeman and Thorley-Lawson, 1987), thioredoxin (Wakasugi *et al.*, 1990), IL-1 (Scala *et al.*, 1984), IL-6 (Yee *et al.*, 1989; Yokoi *et al.*, 1990), TNF-β (Seregina *et al.*, 1989), IL-5 (Paul *et al.*, 1990); others remained uncharacterized (Ambrus and Fauci, 1985; Ambrus *et al.*, 1985; Buck *et al.*, 1987). Transformed B cell lines have also been found to produce

TNF-α but its autocrine growth effect has been ruled out (Pirisi *et al.*, 1990). In contrast, TNF-α has been claimed to act as an autocrine growth factor for chronic B cell malignancies (Cordingley *et al.*, 1988) and it has been further claimed that the therapeutic effect of IFN-α in such diseases may be the result of an interruption of such an autocrine growth loop (Heslop *et al.*, 1990). Some EBV-transformed B cell lines have been shown to use IL-5 as an autocrine growth factor as anti-IL-5 inhibits the proliferation of these cells (Baumann and Paul, 1992). This was, in some way, unexpected as IL-5 has repeatedly failed to display growth factor activity on normal activated human B cells (Clutterbuck *et al.*, 1987; our unpublished data). A 60-kDa B cell growth factor and its 14-kDa derivative have been isolated from B cell lymphomas and claimed to act as growth factors for tumor cells, as well as normal B cells (Sahasrabuddhe *et al.*, 1989). The low-molecular-weight entity may be the BCGF-12 kDa described earlier (Kumar *et al.*, 1990). Finally, recent studies have indicated that molecules such as retinol (Buck *et al.*, 1990) and lactic acid (Pike *et al.*, 1991) may play a role in the autocrine–paracrine growth of activated human B cells.

B. Studies with Normal B Cells

Several studies have now clearly indicated that normal B lymphocytes can produce cytokines in response to polyclonal activation. These include IL-6 (Horii *et al.*, 1988; Rieckmann *et al.*, 1991a; Smeland *et al.*, 1989; Xia *et al.*, 1989); TNF-α (Rieckmann *et al.*, 1991a; Sung *et al.*, 1988) ; TNF-β (Sung *et al.*, 1989); and IL-1α and IL-1β (Bonnefoy *et al.*, 1989; Matsushima *et al.*, 1985; Pistoia *et al.*, 1986). IL-4 has been shown to enhance strongly IL-6 production by such activated B cells (Hutchins *et al.*, 1990; Smeland *et al.*, 1989; our unpublished results), but it is not clear whether the autocrine IL-6 plays a role in IL-4-dependent proliferation and differentiation. In fact, the soluble CD23 produced by B cells in response to IL-4 (Bonnefoy *et al.*, 1988a; Cairns *et al.*, 1988) may participate in IL-4-induced proliferation, as suggested by the inhibitory effects of an antibody specifically recognizing soluble CD23 but not membrane CD23 (Gordon, 1990).

Several studies have focused on the role of autocrine IL-6 in the proliferation and differentiation of B cells costimulated with SAC and IL-2. Although IL-2 does not enhance SAC induced IL-6 production, addition of neutralizing anti-IL-6 antibodies to cultures results in a blocking of Ig secretion (Rieckmann *et al.*, 1991a; Xia *et al.*, 1989). Anti-TNF-α antibodies also inhibit Ig secretion and kinetic studies have demonstrated that TNF-α is produced earlier than IL-6. In fact, TNF-α acts in an autocrine fashion to trigger autocrine IL-6 production. The anti-IL-6 antibodies failed to inhibit B cell proliferation,

suggesting that IL-6 may not be acting as an autocrine B cell growth factor. Alternatively, the antibody may not neutralize all the produced IL-6 and the small amounts of available IL-6 may be sufficient to trigger B cell proliferation but not differentiation. This is worth considering as very small amounts of IL-6 (1–100 pg/ml) have been shown to induce the proliferation of activated human B cells, whereas larger amounts (1–10 ng/ml) were without effect (Levy *et al.*, 1990). Another study, however, has failed to demonstrate a role for IL-6 in IL-2-induced Ig secretion (Splawski *et al.*, 1990). Regarding IL-10, which in our hands is by far the most potent B cell differentiation factor, addition of exogeneous IL-6 has no effect on this final differentiation process (N. Burdin and F. Rousset, unpublished observations); however, we have not yet addressed whether blocking endogeneous IL-6 would result in inhibition of IL-10 induced plasma cell differentiation.

It is presently clear that the cytokines inducing activated B lymphocytes to secrete Igs (IL-2, IL-4, IL-10) deliver signals other than secretion of autocrine IL-6, as addition of IL-6 to either SAC or anti-CD40 activated B cells does not induce them to secrete Igs. These cytokines may thus induce the maturation of B cells to a point where they differentiate into Ig-secreting cells in response to their own IL-6.

IX. *In Vivo* Aspects of B Cell Growth and Differentiation

Immune responses take place in highly organized compartments of secondary lymphoid organs where T cells, B cells, and accessory cells are not randomly distributed. B cells are observed mainly in lymphoid follicles, whereas T cells constitute the paracortical and interfollicular areas (lymph node and tonsil) or periarteriolar lymphocyte sheath (spleen). The marginal zone is an additional B cell compartment of the spleen which may represent a distinct B cell lineage. *In vivo* studies in rodents have shown that resting virgin B cells enter the lymph nodes in the T cell-rich areas, then percolate through the follicles. These studies have also shown that primary and secondary responses differ in terms of anatomic modifications of lymph nodes. For detailed information on the topic, the reader is invited to consult several excellent recent reviews (Kroese *et al.*, 1990; Liu *et al.*, 1992; MacLennan and Gray, 1986; MacLennan *et al.*, 1990).

A. Extrafollicular B Cell Activation

The first phase of B cell activation occurs mainly in the T cell-rich area where the antigen is most likely taken up by interdigitating cells (dendritic cells of bone marrow origin) processed and presented to T cells and virgin B cells (Fig. 17A). Note that the antigen-loaded inter-

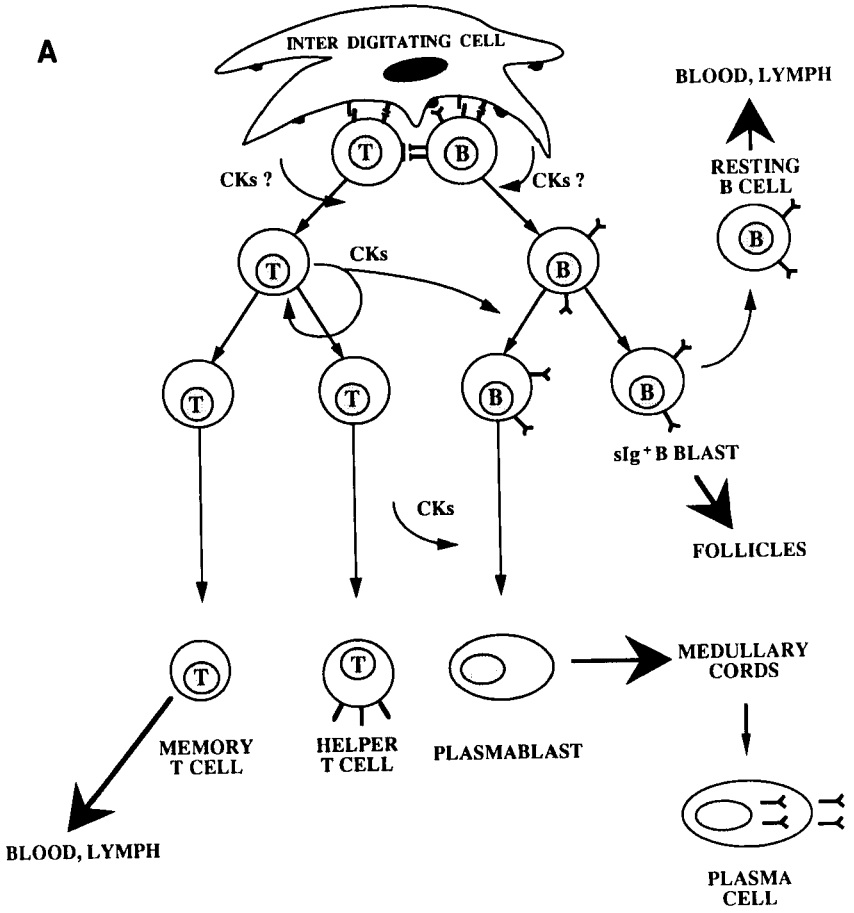
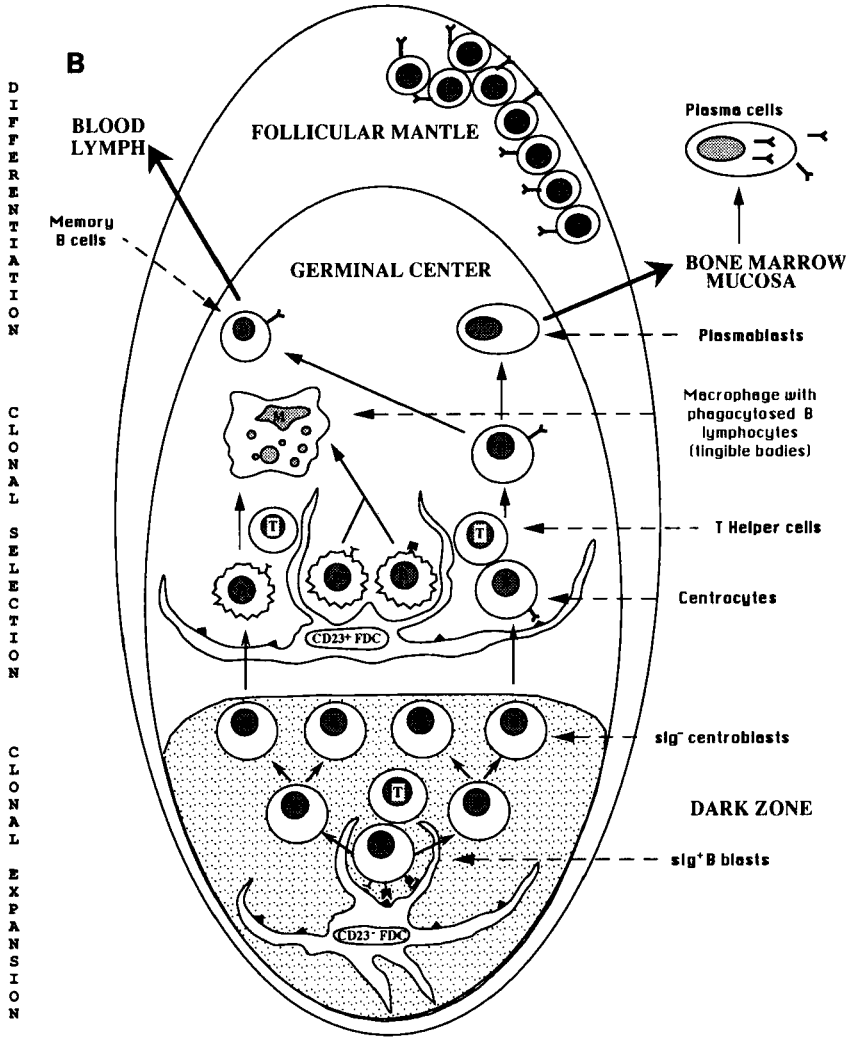


FIG. 17. Schematic representation of antigen-induced, T cell-dependent B cell immunopoiesis in secondary lymphoid organs. (A) Extrafollicular reaction: At the antigen/pathogen port of entry (mucosa, epidermis), antigen presenting cells, such as dendritic cells and Langerhans cells, engulf the antigen and then migrate via the afferent lymphatics into the T cell-rich areas of regional lymph nodes where they interact and interdigitate with T and B lymphocytes. Meanwhile the antigen is endocytosed, processed, and reexpressed in the form of peptides associated to class II molecules, complexes that will be further recognized by T cells. Antigen deposited on the dendritic cell surface possibly in the form of immune complexes is also presented to the B cells which can endocytose, process, and present it. Tight interactions occur between T cells, antigen presenting cells, and B cells that involve several surface antigens (see Fig. 18). High-affinity sIg molecules on B cells can also directly capture specific antigen and then B cells act as antigen presenting cells. The resting T cells are activated and release cytokines (CKs) which permit autocrine proliferation of T cells and their differentiation into effector helper cells or memory T cells. These resting memory T cells may later be able to directly interact with antigen-specific B cells without requiring dendritic cells. Furthermore, released cytokines also permit the proliferation of B cells and their differentiation into plasmablasts, migrating to medullary cords where they differentiate into short-lived plasma cells producing antigen-specific antibodies. These antibodies form, with free antigen, immune complexes that deposit on follicular dendritic cells. Some of the activated B cell blasts migrate into the follicles. It is possible that antigen-activated antigen



presenting cells secrete cytokines that participate in T cell and B cell proliferation and differentiation. (B) Germinal center reaction: The B blasts generated in the extrafollicular reaction recognize the antigen from the immune complexes deposited on the follicular dendritic cells and interact with these cells through various molecules. This leads to intense B cell proliferation, resulting in the generation within a few days of the germinal center's dark zone populated mostly by centroblasts lacking sIgs and $CD23^-$ follicular dendritic cells (FDCs). B cells are then supposed to undergo both isotype switching and somatic mutations. Centroblasts mature into centrocytes that reexpress sIg and fail to proliferate. These centrocytes displaying mutated sIg able to recognize antigen deposited on $CD23^+$ FDCs will survive, whereas those bearing sIg that mutations have reduced or abrogated antigen binding will die from apoptosis and be quickly phagocytosed by macrophages, and B cell remains will constitute the tingible bodies. Surviving B cells may differentiate into plasmablasts, which will emigrate from the germinal center to colonize either bone marrow or mucosal lamina propria, where they will terminally differentiate into long-lived plasma cells secreting IgG, IgA, or IgE. A fraction of centrocytes will also differentiate into resting memory B cells, which will colonize spleen marginal zone or recirculate in the whole body.

digitating cells may represent dendritic cells from other organs (e.g., Langerhans cells from skin) that have migrated to the nodes following antigen encounter (Steinman, 1991). This three-party setup appears necessary for an efficient B cell response, as a consequence of an inability of virgin B cells to present antigen to T cells. The extrafollicular B cell activation is confined to the first 3 days after antigen administration and the proliferating B cells will give rise to plasma cells and primary B blasts. The primary B blasts will colonize the lymphoid follicles and give rise to the germinal center reaction, whereas the plasma cells will migrate to the medullary cords. It is likely that the proliferating B cells will return to a resting state once the antigen is saturated/eliminated by the secreted antibodies.

B. Development of Germinal Centers

The second phase of B cell activation involves massive clonal expansion of primary B blasts within the follicles and the differentiation of these cells into centroblasts and centrocytes (Fig. 17B). This results in development of the germinal centers, many aspects of which are discussed in detail in the forum recently organized by Kosco (1991). It has been calculated that these blast cells divide every 6–7 hours (Liu *et al.*, 1991c; Zhang *et al.*, 1988) and that 2–5 cells would colonize one follicle (Kroese *et al.*, 1987), each of them producing 2000–10000 cells. The centroblasts form the dark zone of the germinal centers. They represent the differentiation stage where B cells undergo somatic mutations mostly within their complementarity-determining regions (CDRs) (Berek *et al.*, 1991; Jacob *et al.*, 1991). The mechanisms generating somatic mutations are presently unknown. The centroblasts are also undergoing isotype switching (Kraal *et al.*, 1982). Centroblasts further differentiate into centrocytes, forming the light zone of germinal centers (Fliedner *et al.*, 1964). The transition of centroblasts to centrocytes, which occurs in the basal "light" zone, is associated with the reexpression of surface immunoglobulins which will display the novel mutated amino acid sequences and altered affinity for antigens. The high-affinity centrocytes will receive a survival signal from the antigens deposited in the form of immune complexes on the surface of follicular dendritic cells and move into the apical light zone, where they undergo further differentiation to either memory B cells or plasmablasts (Liu *et al.*, 1992). The plasmablasts will migrate either to the bone marrow (Dilosa *et al.*, 1991) or to the mucosa where they will differentiate into plasma cells. The low-affinity centrocytes that fail to interact with the antigens on the follicular dendritic cells will die by apoptosis (see Box 3) (Liu *et al.*, 1989). This selection process is re-

flected by the presence of large numbers of tingible body macrophages which contain many apoptotic bodies. The signals that regulate the differentiation process may come from a distinct subpopulation of follicular dendritic cells and a distinct subpopulation of T cells in this area. The follicular dendritic cells express high levels of cytoplasmic CD23 antigen (Y.J. Liu, personal communication) and the T cells express CD4 and the NK cell-associated antigen CD57, but fail to express L-selectin/Leu 8 (Poppema *et al.*, 1983). These T cells are of the helper–inducer (CD45 RO^+) phenotype but produce virtually no IL-2, IL-4, IFN-γ, or TNF-α (Bowen *et al.*, 1991).

The germinal center reaction lasts for 3 weeks after a single antigen administration. The chronic B blast proliferation in the follicular center is maintained by long-term antigen retention on the follicular dendritic cells for many months. This maintains the long-term antibody titer and B cell memory during the established T cell-dependent antibody responses.

C. Follicular Dendritic Cells

Unlike other cells present in the germinal center, the follicular dendritic cell (FDC) is specific to lymphoid follicles. It is thus likely to play a critical role in germinal center function (Szakal, 1989; Tew *et al.*, 1980, 1990). These nonlymphoid cells present long slender processes that are closely intermingled with the surrounding lymphocytes (Heinen *et al.*, 1985; Parmentier *et al.*, 1991; Petrasch *et al.*, 1990; Schriever *et al.*, 1989, 1991). FDCs differ from macrophages and other dendritic cell types, such as the interdigitating cells found in T cell areas and the Langerhans cells of the skin, and, unlike the latter cells, FDCs may not even be of hematopoietic origin. FDCs have a complex phenotype (Dijkstra and Van den Berg, 1991), as they express antigens of various hematopoietic lineages (Fig. 18). They express some specific antigens such as those recognized by the antibodies R4/23 (Gerdes *et al.*, 1983) and KiM4 (Parwaresh *et al.*, 1983). As proposed by Rademakers (1991), they may represent a subpopulation of mesenchymal cells, myofibroblasts, that contain vimentin and desmin as intermediate filament proteins. Quite noteworthy, the bone marrow stromal cells, which play a key role in B lymphopoiesis, also appear to be of similar origin. FDCs are capable of trapping and retaining antigen–antibody complexes on their extended processes (Nossal *et al.*, 1968). These complexes are involved in the formation and regulation of immunological memory. They are particularly important for the continuation of the germinal center reaction rather than its initiation. The binding of immune complexes on the surface of FDCs is probably

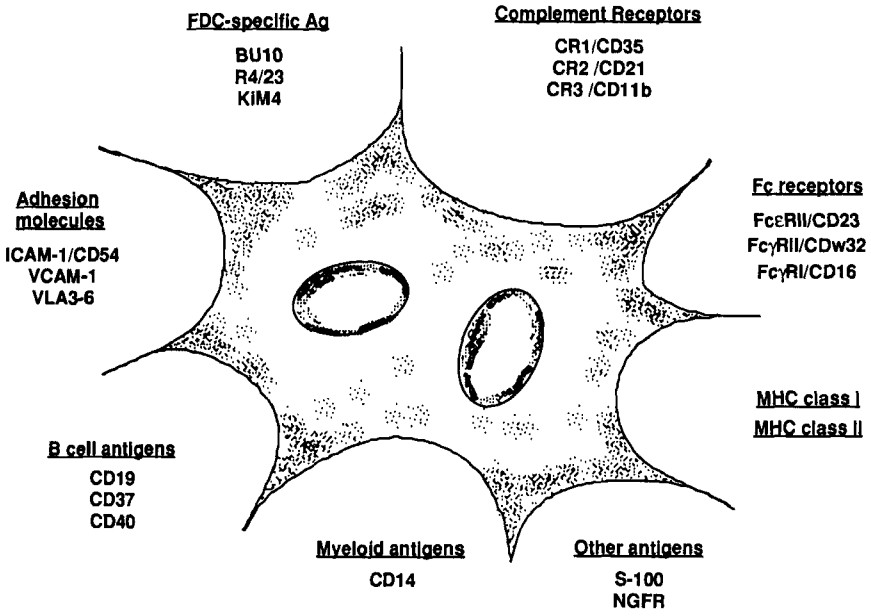


FIG. 18. Phenotype of follicular dendritic cells. NGF, nerve growth factor; MHC, major histocompatibility complex.

complement mediated, as these cells express CR1 (CD35), CR2 (CD21), CR3 (CD11b) (Reynes *et al.*, 1985). FDCs have recently been shown to express MHC class II molecules as a consequence of passive binding of molecules released by surrounding B cells (Gray *et al.*, 1991). How these molecules incorporate into the FDC plasma membrane remains to be established. Polymerase chain reactions on single FDCs have demonstrated the absence of mRNA for IFN-γ, TNF-α, IL-3, and IL-6 (Schriever *et al.*, 1991).

Recently, two different forms of FDCs have been identified by scanning electron microscopy: one with filiform dendrites and the other with immune complex-coated spherical bodies or iccosomes (Szakal and Tew, 1991). These structures would be more specifically produced during secondary responses and are endocytosed by germinal center B cells. The germinal center B cells process and present antigens to germinal center T cells. The activated T cells subsequently induce the differentiation of germinal center B cells into plasmablasts. As FDCs are very difficult to isolate, very few functional *in vitro* studies have been performed at the present time. Preliminary studies indicate that they can stimulate the survival and proliferation of human B lympho-

cytes (Cormann *et al.*, 1986; Petrasch *et al.*, 1991). Recently, *in vitro* studies with murine cells have indicated that the antigen deposited on FDC can be retrieved by specific B cells which process it and present it to T cells. *In vitro* cultures containing 5–10% FDCs, 1–5% T cells, and 85–94% B cells result in maintenance of germinal center B cell viability (Gray, 1991; Kosco, 1992). It is expected that the next few years will witness a much more detailed understanding of the FDC. Defining the origin of FDCs, being able to culture them as was recently claimed (Tsunoda *et al.*, 1990), and determining their cytokine repertoire will certainly represent important milestones for B lymphocyte biology.

D. Cytokines and B Cells: A Synthesis

In this review, we have described the effects of cytokines on B cells activated by three major means. First, antigen receptor-mediated activation was considered, the triggering of which represents the start of the immune response. Such triggering is likely to occur both at the level of the extrafollicular reaction, where the antigen is presented by the interdigitating cells, and at the level of the germinal center reaction, where the antigen is presented by the FDCs. Second, T cell-mediated activation was considered which is likely to occur at the level of both the extrafollicular and the germinal center reactions. These interactions between B cells and T cells or B cells and dendritic cells involve multiple sets of ligands and receptors (Fig. 19). Some of the interactions are restricted to a B cell and another cell type (e.g., LFA-3/CD2) whereas others are more ubiquitous (LFA-1/ICAM-1). The signals depend not only on the presence of the given molecule but also on its state of activation. The third means is CD40 activation, the physiological counterpart of which in immunopoiesis is not clarified yet. This now relatively well defined and powerful activation system may mimic the interaction of the B lymphocyte either with accessory cells (such as the interdigitating cell or the FDC) or with the T lymphocytes. During interactions of B lymphocytes with interdigitating cells, T cells, and FDCs, it is likely that the latter cells produce various cytokines. Recent *in situ* hybridization studies indicate that T cells containing intracytoplasmic IL-2 can be detected in T cell-rich areas following antigen administration (Bogen *et al.*, 1991), and that cells containing IL-4 mRNA (Heinen *et al.*, 1991) and IL-6 mRNA (Bosseloir *et al.*, 1989) can also be detected at the level of the extrafollicular reaction. Furthermore, cells containing IL-2 mRNA can be detected in the germinal centers (P. Galanaud, personal communication). Obviously, these

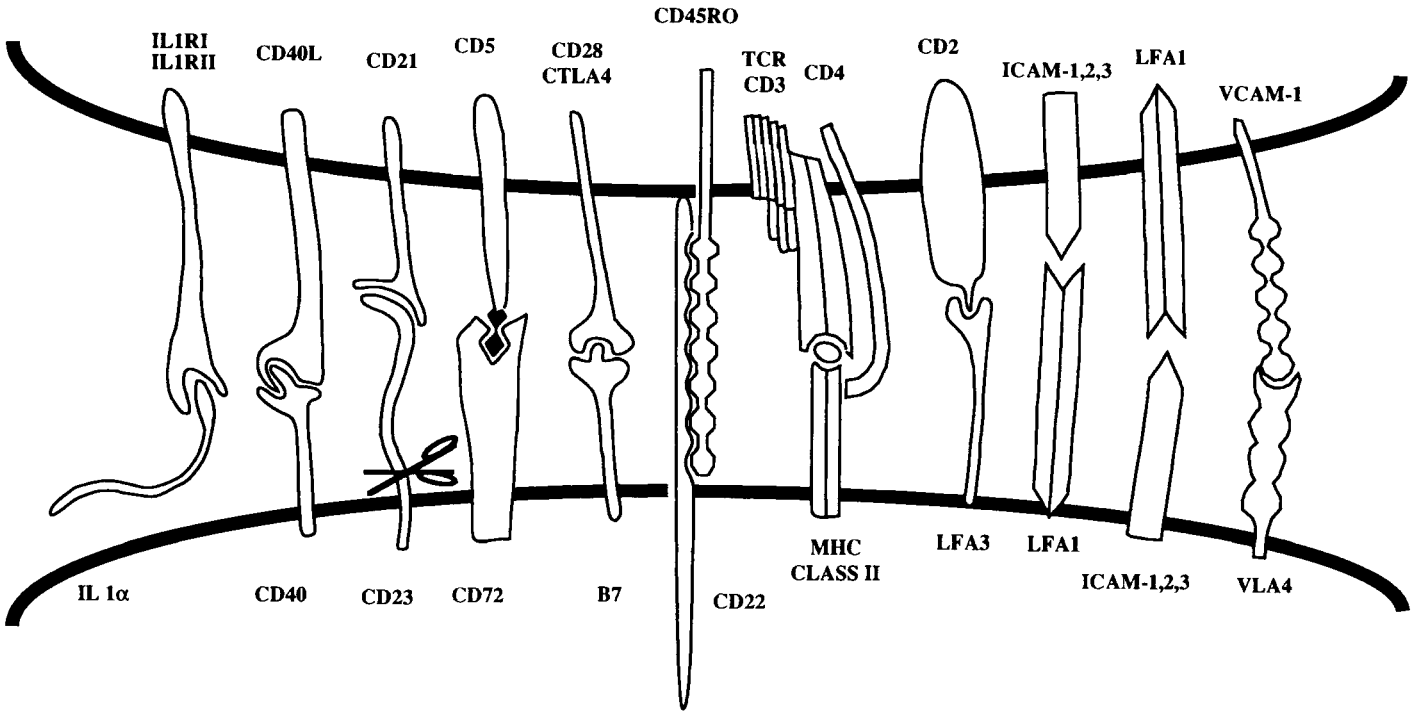


FIG. 19. Schematic representation of the molecular interactions occurring between B cells and helper $CD4^+$ T cells or other accessory cells. Sizes and shapes of interacting molecules are not drawn according to known structural features. The ligands of CD40 and CD23 are still hypothetical. IL, interleukin; R, receptor; MHC, major histocompatibility complex.

studies need to be confirmed and extended to other cytokines, such as IL-10.

The classical linear model of the cytokine-specific control of B cell activation, proliferation, and differentiation must now be reconsidered at various levels. First, it is now clear that there is no such molecule that is only for B cell growth or only for B cell differentiation. Furthermore, the model should be considered in three dimensions. Within the secondary lymphoid organs, two distinct areas have been identified where B cells undergo different events: proliferation and differentiation to generate large amounts of antibodies in the extrafollicular area, proliferation and differentiation into memory cells in the germinal centers to ensure improved further responses. Outside the secondary lymphoid organs, such as bone marrow and mucosa, plasmablasts interact with the microenvironment to become plasma cells. It is possible that different mechanisms of B cell proliferation and differentiation are turned on at different anatomic sites.

Nevertheless, we feel that the available results suggest a clear proliferative role of IL-4 in B cell development, although the isotype switching to IgE and IgG_4 represents another important property of this molecule (Fig. 20). IL-2 and IL-10 appear to play some role in the proliferation of B cells and, maybe more importantly, in their differentiation into Ig-secreting cells. Such a model may be consistent with the extrafollicular reaction. IL-6 of autocrine–paracrine origin may play an important role in the final differentiation of these cells into fully mature plasma cells in the lymph node medullary cords, bone marrow, and mucosa. In contrast, the differentiation in the germinal centers may involve different mechanisms. In particular, the intense B cell expansion observed in the dark zone may involve presently uncharacterized cytokines that are specific to the follicular dendritic cells, as IL-4 has not been detected there. We believe CD40 triggering may be particularly important in the observed proliferation. The differentiation of centrocytes of the light zone into plasma cells may be under the control of IL-2 or IL-10 or under the control of CD23 and IL-1α (Liu *et al.*, 1991a). A final model for the role of cytokines in B cell growth and differentiation will have to include the cytokines produced by activated B cells that may act on the microenvironment to turn interacting cells on or off.

We feel it is important at the present time to remain cautious in proposing models of the cytokine control of *in vivo* immunopoiesis, but we are confident that ongoing work in the field will render this feasible within the near future.

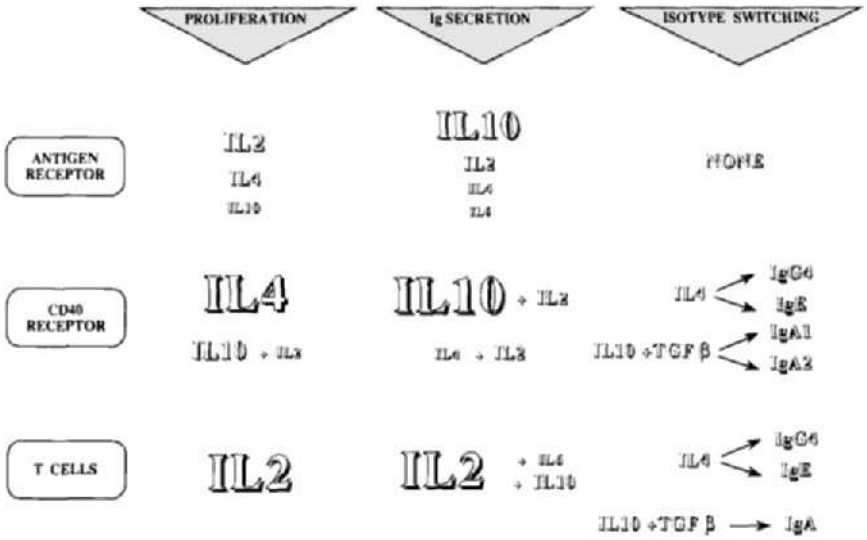


FIG. 20. Effect of cytokines on proliferation, Ig secretion, and isotype switching of human B lymphocytes; dependence on the activation step. The first column indicates the B cell activation system. The size of the cytokine attempts to indicate the relative level of the induced biological effect. IL, interleukin; TGF, transforming growth factor.

X. Concluding Remarks: Human B Cells in the Year 2000

The literature survey that the present review has forced and the subsequent related thoughts and discussions have helped us to identify areas of future investigation related to human B lymphocytes. Although this field is moving quite rapidly, as illustrated by the fact that the vast majority of the cited references are less than 5 years old (1987–1991), we believe the 1990s will witness the following achievements:

More cell surface molecules involved in the adhesion/recirculation and activation of B cells should be identified and their ligand/counterstructures on other cell types determined.

Many new cytokines acting on B cells should be identified. In particular, we believe that those possibly involved in the proliferation of dark zone blasts and in isotype switching toward IgG subclasses will be identified.

Studies dealing with the transformation of activated B cell blasts into resting (memory) B cells will be carried out. Whether specific signals are required or the process is passive after elimination of the eliciting antigen will be clarified.

The intracellular mechanisms involved during activation of resting B cells, as well as those controlling whether an activated B lymphocyte will either proliferate or differentiate into either a plasma cell or a memory B cell, will be a subject of active investigation.

The mechanisms controlling Ig gene expression should be fairly well determined. In fact, Ig transcriptional regulation is already one of the best studied systems because it represents an excellent model from which to gain an understanding of tissue-restricted gene control (Staudt, 1991).

The mechanisms controlling isotype switching should be deciphered. It is expected that a multiproteic complex controls this event. Such a complex may share properties with those involved in DNA replication and splicing of introns from immature mRNA. The components of these complexes will be identified.

The mechanisms controlling somatic mutations should be unraveled. The putative molecular complex generating the mutations should at least be partially characterized.

An increased understanding of the aforementioned events will permit definition of the etiology of such diseases as autoimmunity, allergy, B cell neoplasias, IgA deficiencies, and common variable immunodeficiencies. This will allow the design of specific therapies.

Finally, these studies will allow us to establish specific *in vitro* culture methods that duplicate both extrafollicular and germinal center reactions. Such culture methods should ultimately allow us to shape the Ig repertoire of naive human B cells; that is, these methods will facilitate *in vitro* immunization of human B cells. Such an asset, coupled to the presently available highly efficient molecular biology of Ig genes (Winter and Milstein, 1991), will permit us to generate large amounts of human monoclonal antibodies of desired specificity. Such molecules should represent safe and efficient pharmacological agents covering virtually all therapeutic areas.

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Note Added in Proof

i. Coligation of CD19 with the B cell antigen receptor was found to decrease the threshold for antigen receptor-dependent stimulation by 100-fold [R. H. Carter and D. T. Fearon, (1992). *Science* **256,** 105–107].

ii. Another member of the nerve growth factor receptor family was recently cloned, CD30. This is an activation antigen of T and B lymphocytes which is expressed on the Reed–Sternberg cells specific of Hodgkin's disease. The CD30 is composed of 595 AAs, including a 18-AA leader peptide, an extracellular domain of 365 AAs (and six cysteine-rich motifs), a transmembrane domain of 24 AAs, and a cytoplasmic domain of 188 AAs [Dürkop *et al.*, (1992). *Cell (Cambridge)* **68,** 421–427]. The Ipr mice, which develop lymphadenopathy and suffer from a systemic lupus erythematosus-like autoimmune disease, have defects in the Fas/Apo antigen. This suggests a role for this antigen in the negative selection of autoreactive T cells in the thymus [Watanabe-Fukunaga, (1992). *Nature (London)* **356,** 314–317].

iii. A murine ligand to CD40 has been molecularly and biologically identified on activated murine T lymphocytes. It is a type II glycoprotein of 260 AAs, including a 24-AA signal peptide, a 22-AA intracytoplasmic domain, and a 214-AA extracellular domain which contains a single potential N-linked glycosylation site and four cysteine residues. The cell surface molecule is 33 kDa and binds CD40 with an association constant $K_D \approx 2 \times 10^9\ M^{-1}$. When expressed on fibroblasts, it induces B cell proliferation and production of IgE together with IL-4 [Armitage *et al.*, (1992). *Nature (London)* **357,** 80–82]. This CD40 ligand is probably the 33-kDa T cell surface activation antigen mediating the contact dependent element of the helper function of $CD4^+$ T lymphocytes [Lederman *et al.*, (1992). *J. Exp. Med.* **175,** 1091–1101].

iv. The gp130 subunit of the IL-6 receptor is also part of the receptor for leukemia inhibitory factor (LIF) and Oncostatin M (OSM). The gp130 confers high affinity binding of both LIF and OSM when expressed with the low affinity LIF receptor. Interestingly, the gp130 binds OSM with low affinity [D. P. Gearing *et al.*, (1992). *Science* **255,** 1434–1437].

v. A cDNA encoding the human TGF-β type II receptor protein has been isolated which encodes for a 565-AA glycoprotein. It is composed of a 23-AA signal peptide, a 137-AA cysteine-rich extracellular domain containing three N-linked glycosylation sites, a 30-AA transmembrane domain, and a 375-AA intracellular domain containing a serine/threonine kinase domain, and is similar to the activin receptor. Interestingly, the extracellular domain of the TGF-β type II and type III receptors do not display any homology [Lin *et al.*, (1992). *Cell (Cambridge)* **68**, 775–785].

vi. The role of cytokines in the proliferation and differentiation of B lymphocytes activated with IL-4 cells has been studied in greater detail [A. Tucci *et al.*, (1992). *J. Immunol.* **148**, 2778–2784]. IL-1, TNF, and IL-2 costimulated B cell proliferation and IgM, IgA, and IgG secretion. Interestingly, a strict hierarchy of cytokine interactions was found where IL-1 was required to induce TNF responsiveness and TNF was required for responsiveness. IL-4 together with IL-1 induced IgE secretion. The striking role of IL-1 and TNF in this assay may be accounted for by the low number of B cells set up into cultures (300/well), which minimize the autocrine B cell factors.

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Cytokine Gene Regulation: Regulatory *cis*-Elements and DNA Binding Factors Involved in the Interferon System

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I. Introduction

Cytokines constitute a class of soluble mediators involved in cell-to-cell communication and play a crucial role in the regulation of cell growth and differentiation. The expression of cytokines is tightly regulated temporally and spatially at the transcriptional level, and their biological effects are transmitted to many target cells. The transcription of many cytokine genes is transiently induced by such extracellular stimuli as antigens, viruses, and cytokine themselves (Taniguchi, 1988; DeMaeyar and DeMaeyar-Guignard, 1988; Crabtree, 1989). These newly synthesized cytokines subsequently exert their biological effects, interacting with specific receptors expressed on the surface of target cells. Following interaction, cytokine signals are transmitted to the nucleus, resulting in the induction of many effecter genes. The mechanisms of the induction of cytokine genes and cytokine-inducible genes, in particularly for the interferon system, have been extensively studied in the 1980s (Pestka *et al.*, 1987; Taniguchi, 1988; DeMaeyar and DeMaeyar-Guignard, 1988; Vilček, 1990).

Generally, a relatively limited number of transcription factors appear to be responsible for the regulation of a large number of genes, and, in turn, individual genes are regulated by various combinations of these regulatory factors. In addition, many transcription factors regulate their own and each other's expression, resulting in a fairly complex network of regulatory interactions (Johnson and McKnight, 1989). Such feedback interactions presumably serve to establish and maintain the appropriate levels of the various transcription factors. Signals induced by extracellular stimuli alter this balance and, thus, alter the pattern of target gene expression and cytokine production in the cell. These biological effects may be transient or may result in stable change in the cell, such as differentiation.

To understand cytokine gene expression and its consequent effects, it is necessary to elucidate the system of cytokine gene regulatory factors and their actions. In this review, we focus on the gene regula-

tion of the type I interferon (IFN) system, a well-characterized model of cytokine gene regulation, and recapitulate the complex regulation mechanism of the cytokine system.

II. The Interferon System

Interferons are a heterogeneous family of multifunctional cytokines that were originally identified as proteins responsible for the induction of cellular resistance to viral infection. In fact, IFNs were discovered through the study of viral interference phenomena in which infection with an avirulent virus protects the host from subsequent infection by a virulent virus (Isaacs and Lindenmann, 1957). Subsequently much evidence has accumulated with regard to their roles in cell growth, differentiation, and immunomodulation (Stewart, 1979; Lengyel, 1982; Pestka *et al.*, 1987; Vilček, 1990). In fact, IFNs are regarded as important "negative growth factors," which may be important to the control of cell growth in a variety of cell types (Clemens and McNurlan, 1985; Tamm *et al.*, 1987; Gresser, 1989; Vilček, 1990). The sensitivity of cells in culture to the antiproliferative action of IFNs varies greatly, and the mechanisms mediating this antiproliferative action are still unclear. The action of IFNs on various stages of the cell cycle has been studied, especially in murine 3T3 fibroblast cell lines. In G_0-arrested cells, IFN treatment can inhibit serum- or growth factor-induced entry into G_1 and transition to S phase (Sokawa *et al.*, 1977; Balkwill and Taylor-Papadimitriou, 1978); however, other stages of the cell cycle can also be affected by IFNs, and prolongation of the G_1, S, and M phases has been reported in many types of cells (Clemens and McNurlan, 1985; Tamm *et al.*, 1987). Recent reports showing that IFN genes are deleted in some tumor cells further underline their potential importance in regulating cell growth (Diaz *et al.*, 1990; Miyakoshi *et al.*, 1990).

Three types of IFNs have been identified in humans, and their genes have been characterized in detail. IFN-α and IFN-β are classified as type I IFNs; IFN-γ is classified as a type II IFN. All animal species examined possess large IFN-α gene families, but most have only one gene for IFN-β (the exception is the ungulates, which have multiple IFN-β families) (Weissman and Weber, 1986). In humans, at least 24 genes coding for the different IFN-α proteins are located on a single cluster (the short arm of chromosome 9), and it is generally thought that this superfamily evolved from a single ancestor by gene duplication and spontaneous mutation. Human IFN-β is encoded by a single gene also located on the short arm of chromosome 9, presumably near the IFN-α loci.

Many different types of cells can produce IFN-α, IFN-β, or both. The predominant form of IFN produced by white blood cells (especially by monocytes/macrophages and B lymphocytes is often IFN-α ("leukocyte" IFN). Nonhemopoietic cells often produce IFN-β ("fibroblast" IFN); however, it is very common for a single cell population to produce IFN-α and IFN-β (Weissman and Weber, 1986; DeMaeyer and DeMaeyer-Guignard, 1988; Vilček, 1990). The production of IFN-α and IFN-β is not detectable in normally growing cells unless they are infected by virus or exposed to double-stranded RNAs such as poly(rI):poly(rC). Induction of IFN in response to viral infection or double-stranded RNAs is a transient process; transcription of the IFN genes is induced rapidly and then declines despite the continuous presence of the inducer. In addition, some evidence suggests that IFNs are also produced in cells responding to other cytokines, implying the existence of complex cytokine networks (Van Damme *et al.*, 1987; Kohase *et al.*, 1987; Onozaki *et al.*, 1988). As with other cytokines, IFNs transmit their signal to target cells through specific receptors, and modulate a wide range of gene expression (Revel and Chebath, 1986). Many of the biological actions of the IFNs can be attributed to the induction of specific genes, the "IFN-inducible genes." Typically, extracellular stimuli initially induce the expression of the IFN genes themselves. The synthesized IFNs then stimulate the expression of the larger set of IFN-inducible genes. We try to summarize our current knowledge of the DNA elements and transcription factors that operate in the regulation of IFN system.

III. Regulatory DNA Sequences in Type I Interferon Genes

The induction of type I IFN genes by virus or double-stranded RNA is due primarily to transcriptional activation requiring sequences in the 5′ region of IFN-α and IFN-β genes (Ragg and Weissmann, 1983; Ohno and Taniguchi, 1983; Zinn *et al.*, 1983). These promoter sequences show some degree of homology between IFN-α and IFN-β (Fig. 1). Through analysis of various deletion mutations in these promoter regions, several groups have identified *cis*-acting elements responsible for induction by virus in various host cells (Ryals *et al.*, 1985; Fujita *et al.*, 1985; Goodbourn *et al.*, 1985).

By introduction of deletion mutants stably into mouse L929 cells, it was that the boundaries of the human IFN-β gene promoter region required for full inducibility by virus lie between -117 and -105 relative to the transcriptional start site (Fujita *et al.*, 1985). Fujita *et al.* (1985) originally argued that virus-induced transcriptional activation is mediated by seven repeated hexanucleotide sequences contained

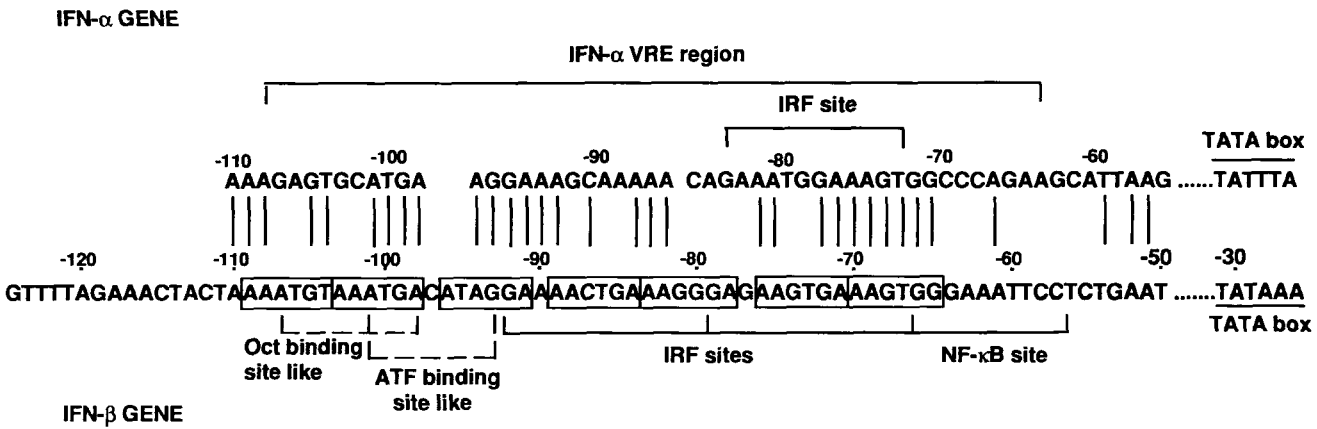

FIG. 1. Regulatory DNA sequences and factor binding sites within the human interferon (IFN)-α and IFN-β genes: The TATA box and each transcription factor's binding sites are indicated; numbers refer to distance in nucleotides from the start site of transcription. The repetitive hexameric sequences are framed. Gaps are introduced to maximize the sequence homology between the two genes.

within this region. The consensus hexanucleotide motif was deduced to be AA(A/G)(T/G)GA. In fact, when variations of this consensus sequence were multimerized most were shown to function as virus-inducible enhancers (Fujita *et al.*, 1987; Kuhl *et al.*, 1987). The sequence AAGTGA was found to be insufficient to activate an enhancer-less IFN-β promoter when repeated two times, but was sufficient to induce low-level activation when repeated three times and resulted in dramatically increased induction when repeated four times (Fujita *et al.*, 1987). The molecular basis of this phenomenon is discussed later.

Functional studies on hexamer repeats revealed other interesting properties of the repeats. Fujita *et al.* (1987, 1988) and Kuhl *et al.* (1987) found that viral enhancers placed just upstream of the repeated hexameric sequence, e.g., $(AAGTGA)_4$, failed to exert their effect on distal chloramphenicol acetyltransferase (CAT) gene expression unless the recipient cells were virus induced: the "silencing" effect (Kuhl *et al.*, 1987). Thus, the question has been raised as to whether a single factor acts as both "activator" and "silencer" in virus-induced and uninduced cells, respectively (see later).

Another sequence motif found between −66 and −57 of the IFN-β promoter, GGGAAATTCC, was found be the consensus sequence for the binding site of the transcription factor NF-κB (Lenardo and Baltimore, 1989). In fact, the importance of NF-κB in the maximal induction of IFN-β promoter has been fully documented by factor binding studies and mutational analyses (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989; Fujita *et al.*, 1989a; Hiscott *et al.*, 1989).

The regulatory regions of the human IFN-α_1 gene required for full inducibility by virus have been determined by Ryals *et al.* (1985). These results showed that a 46-bp DNA segment (between −109 and −64) could confer maximal inducibility. This 46-bp element, termed VRE (virus responsible element), shows some degree of homology to sequences in the IFN-β gene promoter and contains the "TG sequence," GAAATGGAAAGT (see Fig. 1) (MacDonald *et al.*, 1990).

IV. Transcription Factors That Bind to Regulatory Elements of Type I Interferon Genes

A. Interferon Regulatory Factors 1 and 2

Fujita *et al.* (1988) identified interferon regulatory factor 1 (IRF-1), a factor that binds to the aforementioned hexamer repeats or to the IFN-β gene promoter in mouse L929 cell nuclear extracts. Miyamoto *et al.* (1988) cloned a cDNA encoding the mouse IRF-1 from a λgt11 cDNA library using a DNA probe containing multiple copies of the

AAGTGA hexamer sequence. Subsequently, Harada *et al.* (1989) cloned a related factor, IRF-2, by cross-hybridization with the IRF-1 cDNA. The deduced protein structures of IRF-1 and IRF-2 are 62% identical in their amino-terminal regions, spanning the first 154 residues, whereas the rest of molecules are only 25% related (Fig. 2). These two factors bind to the IFN-β promoter with almost the same affinity (Harada *et al.*, 1989). Mutational analysis of IRF-1 and IRF-2 has shown that DNA binding activity resides in amino-terminal regions (the first 154 residues) (Harada *et al.*, 1989), although these regions contain none of the motifs common to many other DNA bind-

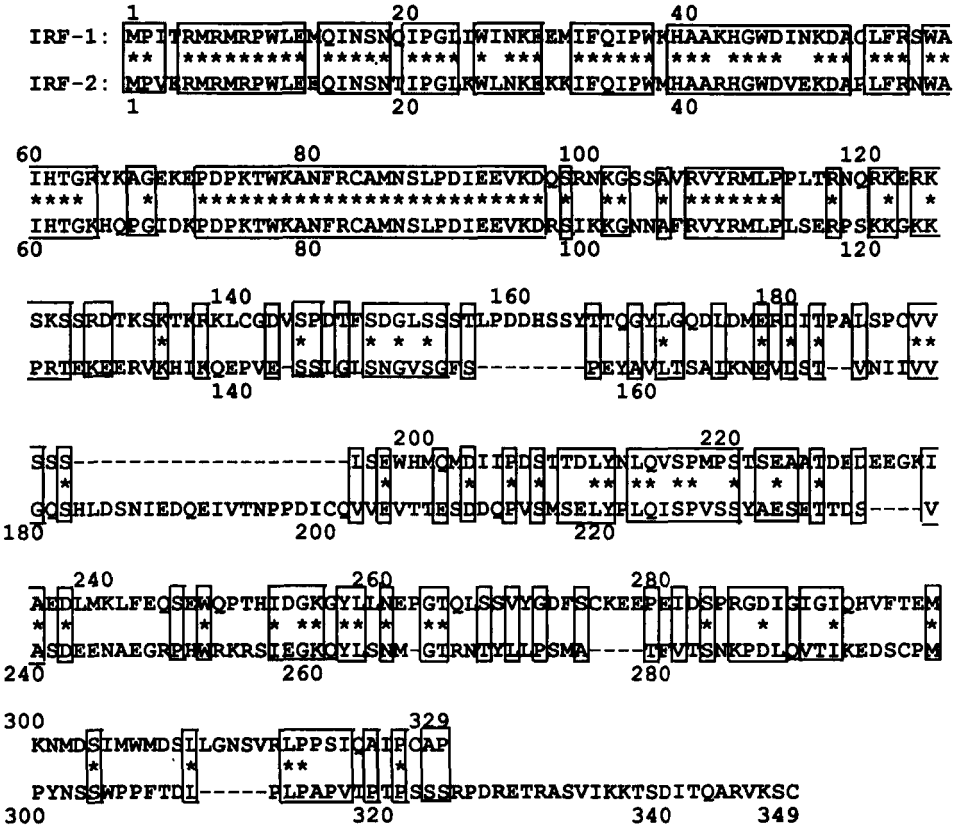

FIG. 2. Comparison of the amino acid sequences deduced for mouse interferon regulatory factors 1 and 2 (IRF-1 and IRF-2). Identical amino acids are marked by asterisks. Regions occupied by identical and chemically similar amino acids are boxed. (From Harada *et al.*, 1989).

ing proteins, such as helix–turn–helix, helix–loop–helix, zinc finger, or leucine zipper motifs (Johnson and McKnight, 1989). In contrast, the carboxy-terminal sequences of the two factors are quite different. The carboxy-terminal region of IRF-1 is characterized by an abundance of acidic amino acids and serine–threonine residues, whereas the same region of IRF-2 is relatively rich in basic amino acids (Harada *et al.*, 1989). These two factors have different effects on the expression of the IFN-β gene as described later.

A series of cDNA transfection experiments have shown that IRF-1 can activate a CAT reporter gene containing four AAGTGA repeats as well as the IFN-β promoter (Harada *et al.*, 1989, 1990). Furthermore, high-level expression of IRF-1 cDNA in monkey COS cells results in activation of the endogenous IFN-α and IFN-β genes, although the level of IFN production is low compared with that induced by viral infection (Fujita *et al.*, 1989b). Unlike IRF-1, IRF-2 has no effect on the transcription of cotransfected reporter genes containing four AAGTGA repeats and, in fact, represses IRF-1-dependent transcriptional activation from the same sites (Harada *et al.*, 1989). These effects are more prominent in the embryonal carcinoma (EC) cell line P19. In EC cells, neither IFN-α nor IFN-β is induced by virus (Burke *et al.*, 1978; Barlow *et al.*, 1984), and expression of endogenous IRF-1 and IRF-2 genes is not observed (Harada *et al.*, 1990). Expression of IRF-1 results in the effective activation of a cotransfected IFN-α and IFN-β or endogenous IFN-α promoter, and this activation is repressed by the coexpression of IRF-2 (Harada *et al.*, 1990). These results suggest that IRF-1 functions as a transcriptional activator, whereas IRF-2 functions as a repressor. Interestingly, IRF-1 protein is very unstable (half-life ~ 30 minutes) whereas IRF-2 protein is apparently stable with a half-life of more than 8 hours, as monitored in virus-infected L929 cells (Watanabe *et al.*, 1991). In this respect, in a variety of differentiated cells, both IRF-1 and IRF-2 genes are constitutively expressed at low levels, resulting in accumulation of the stable IRF-2 protein (Harada *et al.*, 1990). This may explain the observation that transfected IRF-1 and IRF-2 cDNAs do not affect IFN gene expression in differentiated cells to the extent seen in undifferentiated cells. The observation also suggests that the "silencing" of viral enhancers is caused by IRF-2 but not IRF-1 (Fujita *et al.*, 1987; Kuhl *et al.*, 1987).

The DNA binding properties of IRF-1 and IRF-2 were determined by Harada *et al.* (1989) using methylation interference analysis. From these data, it was determined that both IRFs recognize the same G residues of the sequence GAAGTGAAAG in the IFN-β gene and of GAAATGGAAAG in the IFN-α gene. Therefore, a complete IRF-

binding site would require three or four repeats of the hexamer AAGTGA sequence, which may explain the earlier observation that a promoter containing at least three repeats can be induced by virus whereas a promoter containing two cannot.

Expression of IRF-1 (and IRF-2) mRNA is induced by viral infection (Miyamoto *et al.*, 1988; Harada *et al.*, 1989). The induction kinetics for IRF-1 mRNA revealed that the induction levels peak at the same time that IFN-β mRNA levels peak and subsequently decrease in a similar coordinate manner (Harada *et al.*, 1989). This observation suggests that viral infection initially induces IRF-1 expression which is followed by induction of the IFN genes.

Reis *et al.* (1992) reported that human fibroblast cells (GM637) stably expressing a sense IRF-1 mRNA showed significantly higher levels of IFN-β production than control cells when induced by viral infection or poly(rI):poly(rC), whereas cells expressing antisense IRF-1 mRNA showed little or no IFN-β production. These results indicate that IRF-1 is in fact critical for IFN-β gene expression.

On the other hand, how IRF-1 is able to activate IFN genes efficiently in virus-induced cells despite the existence of the repressor IRF-2 is puzzling. Earlier results showed that efficient expression of the IFN-β gene in response to virus or poly(rI):poly(rC) could be inhibited by the protein kinase inhibitor 2-aminopurine (Zinn *et al.*, 1988; Marcus and Sekellic, 1988). Similarly, IRF-1-mediated activation of gene expression has been shown to require an additional post-translational event to achieve full virus- or poly(rI):poly(rC)-induced levels (Watanabe *et al.*, 1991). In the latter study it was found that transfection of IRF-1 cDNA into mouse L929 cells was not sufficient in the absence of virus to activate expression of a cotransfected reporter containing the AAGTGA repeats; however, gene activation was observed on stimulation of the cells by virus in the presence of cycloheximide (Watanabe *et al.*, 1991). These results suggested that activation by IRF-1 requires a posttranslational event in addition to IRF-1 synthesis. The nature of this posttranslational event is still unknown, but some possibilities include the modification of IRF-1 by a protein kinase or association with some other factor(s). It is well known that in most cell types the IFN-β gene is induced to low levels in the presence of cycloheximide (Lengyel, 1982; Pestka *et al.*, 1987; DeMaeyer and DeMaeyer-Guignard, 1988). This phenomenon could be due to the posttranslational modification of existing low levels of IRF-1 protein present in uninduced cells; however, activation is transient because the induction of IRF-1 itself is transient and the half-life of the IRF-1

protein is shorter (about 30 minutes) than that of IRF-2 protein (more than 8 hours: Watanabe *et al.*, 1991).

B. NF-κB

NF-κB was first identified as a B cell-specific nuclear protein that binds to the κB regulatory element within the immunoglobulin κ-chain (Igκ) gene enhancer (Sen and Baltimore, 1986a,b). In uninduced cells, NF-κB is found in the cytoplasm where it is bound to the negative factor IκB (Baeuerle and Baltimore, 1988). Stimulation by phorbol esters, cAMP, lipopolysaccharide, or other stimuli causes phosphorylation of IκB, dissociation of the NF-κB/IκB complex, and subsequent migration of NF-κB to the nucleus.

Purified NF-κB consists of two subunits, p50 and p65 (Kawakami *et al.*, 1988; Baeuerle and Baltimore, 1989), the cDNAs of which have been recently cloned (Ghosh *et al.*, 1990; Kieren *et al.*, 1990; Nolan *et al.*, 1991). The p50 cDNA clone was found to encode a 105-kDa precursor form of p50. The amino-terminal 300 amino acids exhibit a remarkable homology to the *c-rel* (Wilhelmsen *et al.*, 1984; Brownell *et al.*, 1989) and *Drosophila dorsal* gene products (Steward, 1989). This 105-kDa precursor does not exhibit any DNA binding ability until processed to the smaller p50 form by removal of its carboxy-terminal region. p50 forms a homodimer or heterodimer with p65 that binds to DNA. Although p65 can form a DNA binding heterodimer complex with p50, it is unable to form a homodimer because of a carboxy-terminal "inhibitory domain," but it can form a DNA binding complex with p50. The DNA binding activity of this complex is inhibited by the binding of IκB to p65.

Many groups have reported that NF-κB may play an important role in viral induction of the IFN-β gene (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989; Fujita *et al.*, 1989a; Hiscott *et al.*, 1989; Cohen *et al.*, 1991). NF-κB binds specifically to the −66 to −57 region of the human IFN-β gene promoter, and mutations in this region result in decreased inducibility by virus. A promoter containing multimerized NF-κB binding sequence was shown to be efficiently activated following viral infection. Furthermore, nuclear localization of NF-κB is activated by virus or poly (rI):poly(rC) induction. These results indicate that both NF-κB and IRF-1 are important for induction of the IFN-β gene; however, it has been observed that stimulation by tumor necrosis factor α (TNF-α) (Osborn *et al.*, 1989; Watanabe *et al.*, 1991), although it causes both IRF-1 and also NF-κB to be synthesized, does not result in significant induction of the IFN-β gene. Therefore, effi-

cient induction of the IFN-β gene requires not only induction of IRF-1 and NF-κB but also some modification signal, as mentioned earlier.

C. Other Factors

The transcription factors that bind further upstream in the -125 to -93 region of the human IFN-β gene are still unclear. This region contains some IRF binding hexamer repeats, but their affinity for IRFs is weak. DNA sequence analysis suggests that this region may contain elements that recognize members of the octamer binding factor (Garcia-Blanco *et al.*, 1989) and ATF families (Hai *et al.*, 1988, 1989). Because mutations downstream of IRF binding sites abolish inducibility by virus, the role of these upstream binding factors might be more to augment the action of "modified" IRF-1 in inducing IFN-β.

V. Transcriptional Regulation of Interferon-Inducible Genes: Potential Role of IRF-1 and IRF-2

Expression of many genes is increased on IFN treatment of cells. Many of the biological actions of IFNs can be attributed to the induction of these IFN-inducible genes. The biological functions of only a few these IFN-inducible proteins are characterized. The enzyme 2′,5′-oligoadenylate synthetase converts ATP to oligonucleotides ppp $(A2'p)_n$ (Hovanessian *et al.*, 1977). The 2′,5′-oligoadenylate produced binds and activates a latent endonuclease, RNase L (Lengyel, 1982). An activated 2′,5′-oligoadenylate synthetase/RNase L system is sufficient to influence the viral replication (Chebath *et al.*, 1987). Another IFN-inducible enzyme is a double-stranded RNA-dependent serine and threonine protein kinase (Lebleu *et al.*, 1976; Roberts *et al.*, 1976; Zilberstein *et al.*, 1976; Meurs *et al.*,1990). The activated double-stranded RNA-dependent protein kinase can phosphorylate itself and other substrates such as the α subunit of translational initiation factor 2 (eIF2) (Galabru and Hovanessian, 1985). Phosphorylation of eIF2 leads to inhibition of protein synthesis. Some other IFN-inducible proteins were reviewed by Revel and Chebath (1986), Vilček (1990), and Senes (1991).

Synthesis of IRF-1 and IRF-2 is induced by virus, as well as by many cytokines such as IFNs (α, β, and γ), TNF-α, interleukin-1 (IL-1), IL-6 and leukemia inhibitory factor (Fujita *et al.*, 1989c; Pine *et al.*, 1990; Yan-Lee *et al.*, 1990; Abdollahi *et al.*, 1991). In addition, IRF binding motifs similar to that of the IFN-β gene are found in many IFN-inducible genes such as 2′,5′-oligoadenylate synthetase gene, MHC class I gene, and other genes within the IFN response sequences

(Friedman and Stark, 1985; Revel and Chebath, 1986) (Table I). DNA binding analysis of *Escherichia coli*-produced IRF-1 or IRF-2 proteins has shown that IRFs bind to sequences found in the major histocompatibility complex class I gene, the 2′,5′-oligoadenylate synthetase gene, and the IRF-2 gene (Harada *et al.*, 1989; N. Tanaka, unpublished data). Furthermore, each of these genes is activated by IRF-1 *in vivo* (Harada *et al.*, 1989; H. Harada, unpublished data). Recently, it has been observed that there are clear differences in stably transfectant cells constitutively expressing sense or antisense IRF-1 mRNAs in the level of expression of two IFN-inducible genes (2′,5′-oligoadenylate synthetase and class I HLA) (Reis *et al.*, 1992). These results indicate an important role for IRF-1 and IRF-2 in the expression of IFN-inducible genes.

The transcription factors IRF-1 and IRF-2 regulate both IFN and IFN-inducible genes coordinately. In cells infected by virus, IRF-1 is produced and modified by a yet unknown signal. This "modified" IRF-1 activates the IFN-β gene with the cooperation of NF-κB and other factors. Synthesized IFN-β subsequently binds to the receptors of its own and surrounding cells, resulting in the further induction of IRF-1 and other transcription factors by the signals transmitted to the nucleus. Ultimately, the expression of these IFN-inducible genes results in the establishment of an antiviral state and produces other biological reactions. IRF-1 is also induced by many cytokines, for

TABLE I
PUTATIVE INTERFERON REGULATORY FACTOR-BINDING SITES IN INTERFERON-INDUCIBLE GENES

Gene	Sequence	Reference
Mouse H-2Dd	−137 CAGAAGTGAAACT −151	Israël *et al.* (1986)
Human 2′,5′-OAS[a]	−98 G AAA CGAAACC −88	Benech *et al.* (1987) (1987)
Human ISG 15	−106 G AAACCGAAACT −95	Reich *et al.* (1987)
Human ISG 54	−92 G AAAGTGAAACT −103	Reich *et al.* (1987)
Mouse *Mx*	−121 G AAA CGAAACT −131	Hug *et al.* (1988)
Human 6-16	−151 GAAAA TGAAACC −140 −110 G AAA TGAAACT −99	Porter *et al.* (1987)
Human IP-10	−220 G AAAGTGAAACC −208	Luster and Ravetch (1987)
Mouse β_2-microglobulin	−139 G AAACTGAAAATC −127	Israël *et al.* (1987)
Mouse IL-7 receptor	−262 G AAATGGAAGAGT−249	Pleiman *et al.* (1991)
Consensus	G(A/—)(A/G)AA(G/C/—)(C/T)GAAA(G/C)(T/C)	

[a] Human 2′,5′-oligo-adenosine synthetase gene.

example, TNF-α and IL-1 (Fujita *et al.*, 1989c). In this regard, it is interesting that both cytokines were found to have IFN-like actions. Both TNF-α (Kohase *et al.*, 1986; Mestan *et al.*, 1986; Reis *et al.*, 1988) and IL-1 (Content *et al.*, 1985; Van Damme *et al.*, 1987) in some cells induce an IFN-like antiviral action. In addition, TNF-α and IL-1 were shown to induce the synthesis of some proteins that are also induced by IFNs (Pfizenmaier *et al.*, 1987; Beresini *et al.*, 1988; Rubin *et al.*, 1988). Finally, TNF-α and IL-1 appear to modulate IFN-β production; i.e., under certain conditions both cytokines, like IFNs, can prime cells to produce increased levels of IFN-β (Kohase *et al.*, 1988), and in some cells TNF-α (Onozaki *et al.*, 1988) or IL-1 (Van Damme *et al.*, 1985) induces IFN-β. Thus, it is possible that many, if not all, of these phenomena are also mediated by IRF-1.

In view of the fact that the IRF-1 gene is induced by IFNs and the observation that IRF-1 may mediate many of the biological actions of IFNs, it is intriguing that IRF-1 may be the nuclear factor that regulates the cell growth. In this regard, Yamada *et al.* (1990) generated transgenic mice carrying the IRF-1 gene linked to the immunoglobulin heavy-chain enhancer. The resulting mice showed a drastic reduction of B lymphocytes, suggesting that IRF-1 has growth inhibitory action. Recently, Abdollahi *et al.* (1991) reported that IRF-1 is an early response gene in myeloid precursor cells (M1 cells) following induction for terminal differentiation and growth arrest by IL-6 or leukemia inhibitory factor. They also showed that this growth inhibition could be partially abrogated by the use of IRF-1 antisense oligomers or IFN-β antiserum. Interestingly, the human IRF-1 gene is located on chromosome 5q23–31 (Itoh *et al.*, 1991). Interstitial deletion of this region is the most common structural rearrangement in acute myelocytic leukemia and myelodysplastic syndromes (Van den Berghe *et al.*, 1985). Although the molecular mechanisms of these myeloid disorders are still unclear, it is thought that loss of a gene(s) that negatively regulates cell growth such as an anti-oncogene may play an important part in the pathogenesis of 5q-related disorders. It is intriguing that the IRF-1 gene may correspond to one such gene that directly affects cell growth. The role of IRF-1 in cell growth control awaits further elucidation.

Other factors that bind to IFN-responsible elements have also been identified. Interferon-stimulated gene factor 3 (ISGF-3) is a multicomponent cytoplasmic factor that is assembled and transported to the nucleus in the absence of new protein synthesis following IFN-α stimulation (Larner *et al.*, 1986; Levy *et al.*, 1988, 1989, 1990). When cells are treated with IFN-α, ISGF-3 can be detected in the cytoplasmic

fraction within 1 minute, and in the nuclear fraction after 3 to 4 minutes. ISGF-3 is composed of two fractions, ISGF-α and ISGF-γ. The ISGF-γ component (48-kDa) possesses a DNA binding activity that specifically recognizes interferon-stimulated response element (ISRE). ISGF-3 has been shown to activate an ISRE-containing promoter in an in vitro transcription assay (Fu *et al.*, 1990). This result shows that ISGF-3 may also act as an activator of IFN-inducible genes. Interestingly, these ISREs contain IRF binding sites (GAAAGTGAAAGT in ISG 54 gene, GAAACCGAAAGT in ISG 15 gene) (Levy *et al.*, 1988) and ISGF-3 have been shown to bind specifically to these motifs; however, ISGF-3 does not bind to the IRF binding sites found within the IFN-β gene. Although these two factors recognize overlapping sequence motifs, the binding affinities for these various sequences have yet to be determined. Such analysis may lead to a better understanding of how IRF-1 and ISGF-3 can function either independently or in a cooperative manner for various IFN-inducible genes.

Other factors that bind to IRF binding sites in the IFN-β gene and IFN-inducible genes include PRD1-BF1 (Keller and Maniatis, 1991), ICSBP (Driggers *et al.*, 1990), and IREBF-1 (Yan and Tamm, 1991). These three factors have also been cloned by probing a λgt 11 cDNA library with DNA containing repeated elements, including IRF-1 recognition motifs. The amino-terminal region of ICSBP shows significant sequence homology to the corresponding regions of IRF-1 and IRF-2, and is thus a new member of the IRF family; however, the activity of these factors and their requirement in the expression of IFN genes and IFN-inducible genes have not been determined.

VI. Conclusions

Studies on the regulation of IFN-β and IFN-inducible genes have led to significant advances in the understanding of the mechanisms of cytokine gene regulation. In this review, we have described mainly the IFN and IRF regulatory network (Fig. 3). IRFs appear to be key factors in the regulation of the IFN-β gene and other IFN-inducible genes. Viral infection of cells causes the generation of a specific signal(s) that induces the expression and modification of the transcriptional activator IRF-1. Subsequently, this modified IRF-1, in cooperation with other transcription factors, acts to activate many IFN-inducible genes and to activate efficiently the IFN-β gene. This IRF-1-mediated transcriptional activation is transient, reflecting the fact that IRF-1 itself is both transiently expressed and rapidly degraded. IFN-β,

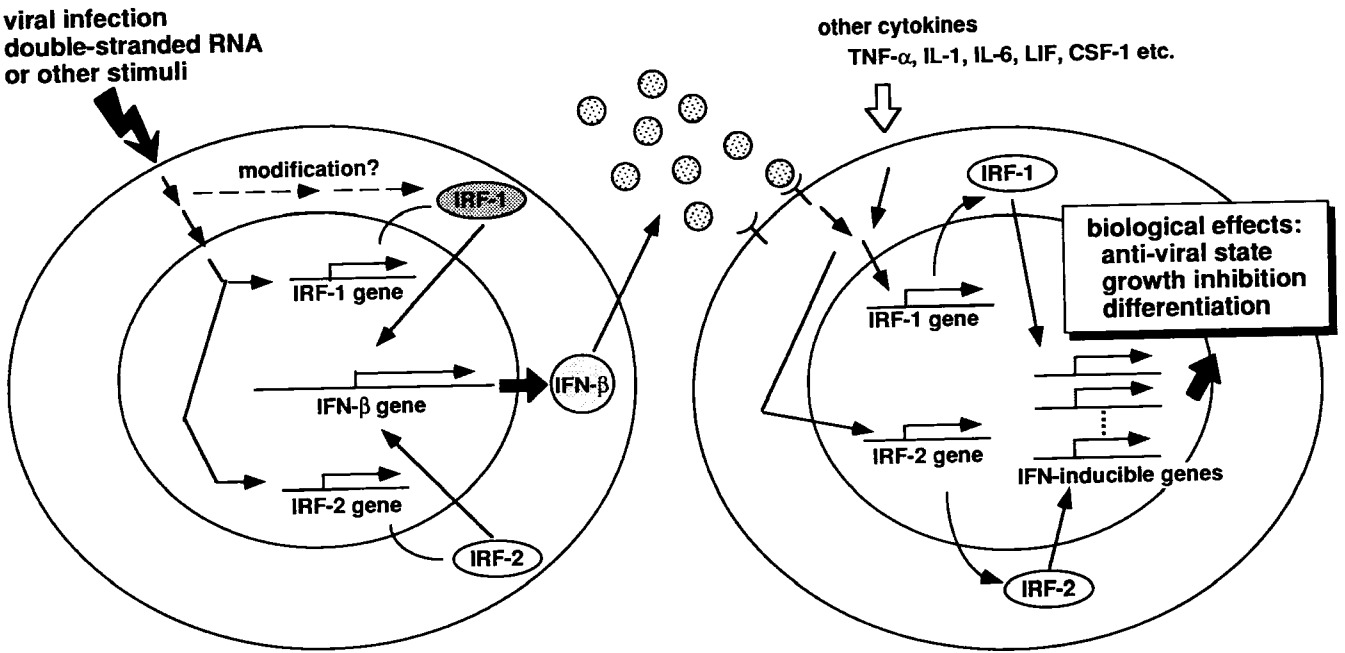

FIG. 3. Model for the interferon regulatory factor (IRF)-mediated regulation of the interferon-β (IFN-β) gene and interferon-inducible genes. See text for details.

synthesized by virally infected cells, is secreted and binds to the receptors of the target cells. This IFN-β signal is transduced to the nucleus, leading to the induction of IRF-1 expression and activation of IFN-inducible genes, but does not cause the synthesis of IFN-β because of the repressor IRF-2, unless cells receive another signal(s). This is presumably because in the absence of a signal(s) from virus, IRF-1 in these cells is not modified and therefore not able to overcome the IRF-2-mediated repression of IFN-β. Thus, in this system, IRF-2 acts as "safety valve" to prevent the overexpression of IFN-β. Therefore, although changes in IRF-1 levels may regulate expression of many IFN-inducible genes, the requirement for a virally induced modification event causes the same IRF-1 to regulate IFN-β in a more tightly controlled manner.

The physiological significance of such a regulatory system may be critical in the case of stimuli such as viruses. As a first line of defense, a viral stimulus activates the IFN system, which in turn induces IFN production to impart an antiviral state to the surrounding cells. Concomitantly, IFN and other IFN-inducing cytokines can upregulate the IRF system, thus accelerating further IFN production (e.g., priming effect) to limit the extent of the viral infection; this may constitute a second line of defense. Thus, one may envisage intercellular amplification of the IFN system as mediated by the product (IFN)-mediated induction of the IRF-1 gene. Finally, it should be pointed out that cytokine-induced IFNs, the level of which is much lower than that of virus-induced IFNs, may be of greater physiological importance in the control of cell growth and differentiation.

As IRF-1 is induced by many cytokines some of the biological responses to these cytokines may also be mediated by genes regulated by IRF-1. These cytokines regulate many biological activities such as cell growth and differentiation. It might be very interesting to investigate the possible role of IRF-1 in also mediating cell growth and differentiation.

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Cellular and Molecular Mechanisms of B Lymphocyte Tolerance

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I. Introduction

It is somewhat sobering to realize that this review on B lymphocyte tolerance will appear exactly 30 years after my first review for "Advances in Immunology," Volume 2 (Nossal, 1962). Although we have learned a monumental amount about the immune system in the intervening period, cellular immunology displays a curious habit of revisiting its old haunts, one of the most important of which is the mechanism of immunological tolerance. Academic immunology has succeeded brilliantly in its reductionist mode. The definition of the molecules, cells, and organs constituting the immune apparatus represents one of the true triumphs of modern bioscience. Nevertheless, there are some real problems in defining the regulatory rules that govern the relationships between these component parts. These chiefly come from one unique feature of immunological receptor molecules. Most macromolecular interactions, such as enzyme action, hormonal stimulation, viral adherence, and neurotransmission, depend on ligand–receptor binding where evolution has shaped the reaction to have a precise, defined affinity. In the immune system, receptors have evolved based on the need to recognize all foreign substances, more or less well. Immune recognition traverses a 10 million-fold span in terms of precision, and cellular events, particularly toward the lower end of this spectrum, may require ancillary mechanisms of binding. This being so, cellular immunology has a much larger operational component than most disciplines. For example, a monoclonal immunoglobulin may be an antibody to a particular protein if it is an IgM and the antigen is presented as a closely spaced matrix on an enzyme-linked immunosorbent assay (ELISA) plate, whereas the same antibody may score negative as a F(ab) monomer. A T cell may proliferate when confronted with a particular major histocompatibility complex (MHC) haplotype *in vitro* in the presence of interleukin-2 (IL-2), yet be of such low affinity as to prove incapable of skin graft rejection. Almost inherently, an academic subject based on such recognition will

spawn experimental results in apparent conflict with one another when different protocols with various thresholds are used. This is what makes regulatory immunology so impenetrable to the outsider.

Of all the facets of immunoregulation, the most important is self-nonself discrimination, manifesting itself as self tolerance and frequently studied as experimental tolerance. Given the dramatic nature of tolerance (what could be more striking than the contrast between fierce rejection of an allograft and the indifference of the same lymphocyte population to the autologous equivalent), it is understandable that scientists should seek an equally dramatic explanation for it, as Burnet (1957), Lederberg (1959), and Bretscher and Cohn (1970) sought to do. We now know that all these pioneers were right, but explained only part of the total phenomenology. Over the past 4 years, tolerance research has been revolutionized by the imaginative use of transgenic mouse technology. This has confirmed the existence of deletional *and* nondeletional mechanisms of repertoire purging in both T *and* B lymphocyte compartments. The purpose of this review is to highlight recent B cell tolerance work, placing the findings from transgenic experiments into a framework developed in normal animals. I also hope to establish that tolerance within the primary and secondary B lymphocyte repertoires is a useful ancillary bulwark against autoimmunity, even though T cell tolerance exists and probably represents the most important safeguard.

II. Early Tolerance Studies Affecting Antibody Formation

A. Tolerance and Antibody Formation before the T and B Cell Era

Shortly after the experimental induction of immunological tolerance by Billingham *et al.* (1953), numerous attempts were made to use defined, nonliving antigens as tolerogens. At that time, effort was concentrated on the perinatal period, tolerance being seen as a phenomenon tied to the immaturity of the immune system. It was quickly shown that pure protein antigens could abrogate the newborn animal's capacity to respond to later immunogenic challenge by antibody formation (Hanan and Oyama, 1954; Cinader and Dubert, 1955; Dixon and Maurer, 1955); however, the story was different for particulate antigens and various microbial products. Burnet himself failed to induce tolerance in chick embryos toward influenza virus, and I was able to show that this was, at least in part, caused by failure of antigen administered as a single dose to persist long enough within the body;

regular injections of foreign erythrocytes, starting at birth, could induce tolerance (Nossal, 1957), and even a very powerful antigen of microbial origin, *Salmonella* flagellin, could do so at surprisingly low doses, provided it was given daily over a substantial period (Shellam and Nossal, 1968). All of this seemed in line with Burnet–Lederberg notions that saw the essence of tolerance as repertoire purging, that is, an elimination of cells with reactivity to self. At that time, no one placed emphasis on the fact that tolerance toward allogeneic skin grafts and tolerance after a challenge designed to elicit antibody formation represented two different phenomena. It was clearly recognized that graft rejection was more akin to delayed-type hypersensitivity reactions, but the full significance of the difference between primary and secondary lymphoid organs (Glick *et al.*, 1956; Miller, 1961; Jankovic *et al.*, 1962; Good *et al.*, 1964) and the importance of distinguishing between the two great families of lymphocytes (Szenberg and Warner, 1962) in terms of mechanisms of activation or silencing took some years to come into focus. Therefore, it is in retrospect not surprising that the striking finding of Dresser (1962), where freshly deaggregated xenogeneic serum proteins caused tolerance in *adult* mice, made most workers in the field uncomfortable. It did not fit into the paradigm of tolerance representing repertoire purging among *immature* immunocytes.

Two developments in the late 1960s ensured that the tolerance debate no longer progressed separately from the T cell versus B cell debate. The first was the realization that thymus-derived cells and bone marrow-derived cells collaborated in antibody formation (Claman *et al.*, 1966; Miller and Mitchell, 1968; Rajewsky *et al.*, 1969; Mitchison, 1971). The second was the invention (Raff, 1970) and soon widespread use of immunofluorescence and other techniques to distinguish T and B cells, so that the differences between them could no longer be ignored. When it was formally proven (e.g., Nossal *et al.*, 1968) that B cells were the exclusive formers of antibody, it soon became apparent that workers using antibody formation as a readout of tolerance needed to determine whether the tolerance observed represented a property of the B cell population or of the T cells, in the absence of which only a restricted, T-independent B cell response could ensue.

B. Dissection of T Cell versus B Cell Tolerance

Three endeavors of the early 1970s shaped thinking about tolerance mechanisms during the first phase of the T cell–B cell revolution. The first was the theoretical contribution of Bretscher and Cohn (1970).

Realizing that immune activation required not only the union of antigen with lymphocyte receptor ("signal 1") but also some other signal ("signal 2"), which was normally delivered by a helper T cell and which involved recognition of a linked epitope on the antigen, they argued that signal 1 only was followed not by a null response but rather by a negative signal, i.e., tolerance. Signal 1 plus signal 2 led to immune activation, e.g., antibody formation. If an antigen could not engage the interest of the T cell, e.g., a foreign serum protein free of aggregates that was not palatable to macrophages and thus did not activate T cells, it was likely to lead to tolerance. This formulation, with little emphasis on the maturity of the system, made the Dresser (1962) finding less impenetrable and continues to inform current views of clonal anergy.

The second major development was the classical study of Chiller *et al.* (1970). Mice were rendered tolerant to deaggregated human gamma globulin and, to determine the cellular sites of tolerance induction, adoptive transfer experiments were conducted using thymus as a source of T cells and bone marrow as a source of B cells. It was shown that both T and B cells could be rendered tolerant, but that unresponsiveness in either cell type was sufficient for a severe impedance of antibody formation. Furthermore, B cell tolerance required a much higher dose of antigen, and tolerance was lost more rapidly in the B cell compartment.

The third advance was the demonstration (McCullagh, 1970; Gershon and Kondo, 1971) that some models of tolerance involved the development of T lymphocytes capable of suppressing immune responses. Although the exact nature of this last phenomenon remains controversial, all three contributions highlight the importance of the T cell in self recognition. As this subject is outside the scope of the present review, the reader is directed to other recent efforts that give access to the rich T cell tolerance literature (Miller *et al.*, 1989; Ferrick *et al.*, 1990; Nossal, 1991). As had been tacitly assumed for many years, the thymus itself is critically involved in shaping the T cell repertoire. It ensures that MHC restriction is learned through positive selection and tolerance is learned through negative selection, though the detailed signaling pathways differentiating these two processes are not yet clear. Nevertheless, there are self antigens not expressed in the thymus to which it is important to maintain tolerance. It is clear that there are ancillary mechanisms operative peripherally that ensure this, including induction of T cell anergy and perhaps induction of T cell suppression in some cases. Other models appear to rely on exhaustive differentiation of T cells which ends up with the phenotype of T cell

deletion. Only further work will clarify which of these peripheral mechanisms are the most physiologically relevant.

As the rest of this review concentrates on B cell tolerance, it is important to recall that this aspect of tolerance is rarely the full story.

III. Antigen Polymerization and B Cell Tolerance

A. Felton's Paralysis and Other Early Results with Polymers

Even before the notion of self-tolerance was conceived, it was clear that highly polymeric antigens could inhibit antibody formation. Felton's (1949) work on pneumococcal polysaccharide had shown that supra-immunogenic but still quite small amounts of this antigen could inhibit antibody formation, causing "immunologic paralysis," and subsequently workers tended to put this into a separate conceptual basket than self tolerance. The matter took on a different complexion when it was realized that quite low concentrations of soluble immune complexes formed in the zone of antigen excess could be powerfully inhibitory of B cell function (e.g., Feldmann and Nossal, 1972; Sinclair, 1990). This raises the issue of whether a B cell that might, for any reason, begin to form antibody to some monomeric self antigen, such as a serum protein, would immediately be silenced because the antibody formed would complex with antigen providing a profound negative feedback effect. The plausibility of such a process is increased through the discovery of effector cell blockade (Schrader and Nossal, 1974). This surprising finding revealed that an actual fully differentiated antibody-forming cell could have its secretory capacity markedly downregulated through brief incubation with highly multivalent antigen. For example, dinitrophenyl (DNP)-specific plaque-forming cells incubated for 30 minutes with 100 μg of DNP-polymerized flagellin showed a marked suppression of antibody secretion rate at the single cell level. Similar results were obtained independently by Klaus and Humphrey (1974), and much of the early literature on negative signaling of B cells by polymeric antigens has been previously summarized (Nossal and Schrader, 1975). It revealed that although most studies, such as those just mentioned, showed a direct effect on B cells, some models highlighted the absorption of antibody by persisting, nondegradable antigen ("antibody formation on a treadmill") and others appeared to involve suppressor T cell effects. Doubtless, in some cases, more than one of these mechanisms operated in the same animal. For example, the profoundly tolerogenic DNP coupled to a

copolymer of D-glutamic acid and D-lysine undoubtedly exerted effects directly on the B cell (Nossal *et al.*, 1973), but the nondegradability of this antigen would have contributed to its *in vivo* efficacy. A persistent theme in this early work was that impedance of B cell function depended on the differentiation state of the B cell. Antibody-forming cells required stronger surface Ig receptor crosslinking than mature, immunocompetent B cells to be silenced. The latter, in turn, were less readily turned off than immature B cells, a question to which we shall return later.

B. Size-Fractionated Polymers: The Dintzis Model

In more recent years, a penetrating quantitative study of the effects of polymeric antigens on the B cell has been carried out by Dintzis' group (Dintzis and Dintzis, 1990). This work was initiated on the basis of the original observation (Dintzis *et al.*, 1976) that the immunological effects of injecting DNP-substituted, linear polymers of acrylamide depended on both the size of the polymer and its degree of substitution. Polymer molecules that bore 10 to 20 effectively spaced DNP groups were immunogenic. Preparations with fewer than 8 DNP groups were totally nonimmunogenic. This led to the concept of the "immunon," namely, the idea that a cluster of 10–20 surface Ig receptors, all crosslinked to one another, were required for T-independent triggering of the B cell. Moreover, nonimmunogenic conjugates could impede the response to immunogenic conjugates given simultaneously or 5 days later. For each immunogenic type of polymer there was a bell-shaped dose–response curve, excess amounts failing to induce antibody formation. Fluorescein, a substantially larger and more complex hapten than DNP, has been used in more recent experiments (e.g., Dintzis and Dintzis, 1988). Both *in vivo* and *in vitro*, all of a variety of chemically diverse fluoresceinated polymers with molecular weights greater than 100 kDa and a hapten valency higher than 20 were immunogenic, whereas polymers less than 100 kDa were nonimmunogenic. The rules applied also to natural polymers such as dextran and pneumococcal polysaccharide type 3 as routinely used in vaccines. The latter preparations were quite heterogeneous in size, consisting of a mixture of immunogenic and nonimmunogenic molecules. If these findings can be generalized, they have substantial implications for vaccine design.

The aforementioned studies relate to primary IgM responses, evoked by these polymeric antigens acting as T-independent stimuli of IgM responses. In the latest work of Dintzis and Dintzis (1992), attention has been focused on fluorescein-derivatized dextran as an im-

munosuppressive agent for T-dependent responses. Preparations of 80 kDa with approximately 30 fluorescein haptens per molecule were injected in saline to a total dose of 2 mg. Immune responses of mice were evoked by two or three spaced injections of fluorescein–ovalbumin. Even when the boosted serum antifluorescein response was well established, treatment with the suppressive polymer reduced the serum anti-hapten IgM level by 70%, and repeated injections lowered levels still further. There was no effect on the antiovalbumin response. Interestingly, IgG levels were inhibited much more profoundly, especially high-affinity antifluorescein antibodies. The results were not due to a simple quenching of antibody by antigen, as suppressive polymer could not be found in the serum at the time of assay, and as the number of splenocytes producing specific antibody was reduced to the same degree as serum antibody. A single injection of fluorescein–dextran reduced the fluorescein-specific IgE response 10-fold, and a second injection, 1000-fold. Again, antiovalbumin IgE levels were not reduced. This specific suppression of established T-dependent immune responses, admittedly by relatively large doses of tolerogen, is impressive. It is not clear whether the antifluorescein B cell population is eliminated or rendered anergic. The authors stress the therapeutic implications of their findings for both autoimmunity and allergy. Whether this form of B cell inhibition is relevant to self tolerance or not (and some self antigens are certainly present in highly polymeric form), it does illustrate in a powerful way the capacity for polymeric antigens in the absence of T cell help to deliver negative signals to the B cell.

C. Polymeric Antigens and IgE Responses

Though the field of carbohydrate antigens and T cell effects is not well developed, there are hints in both the pneumococcal polysaccharide and dextran models mentioned earlier of T cell effects, especially suppression. From that viewpoint, a body of work on the effects of soluble polymeric antigens on the IgE response of mice initiated by Sehon (e.g., Lee and Sehon, 1978) is of particular interest, as it led to the conclusion that such conjugates suppress IgE production via a suppressor T cell mechanism. Antihapten IgE responses were induced by the injection of haptens, e.g., DNP, coupled to ovalbumin (OA) adsorbed into aluminum hydroxide. Among a range of conjugates that abrogated the anti-DNP IgE response was the carrier, OA, coupled to monomethoxypolyethylene gylcol (PEG). Indeed, a wide variety of PEGylated antigens, including proteins, pollen allergens, helminth allergens and bacterial allergens, were found to be nonantigenic and

specifically immunosuppressive, with adoptive transfer studies implicating suppressor T cells (Sehon, 1982). Similarly, PEGylated monoclonal immunoglobulins caused tolerance transferable by T cells (Wilkinson *et al.*, 1987).

This line of work has been extended in an interesting way by Hay-Glass and Stefura (1990, 1991). They used glutaraldehyde-polymerized OA as a soluble polymer of relative molecular mass (M_r) about 3.5×10^7, and found specific abrogation of the murine IgE response but a marked *increase* in the IgG_{2a} response. Unmodified OA does neither of these two things but does lead to increases in OA-specific IgG_1 responses. When a monoclonal antibody to interferon-γ was injected with polymerized OA, both the suppression of IgE and the enhancement of IgG_{2a} were inhibited. The results suggest that the polymerized form of the antigen preferentially induces interferon-γ-producing T cells which negate the function of the IL-4 produced when alum-adsorbed OA is injected and therefore prevent the IL-4-dependent IgE formation. In other words, the form in which the antigen is presented profoundly influences the cytokine pattern induced. The study also shows how subtle influences on the T cell system may be. It is clear that each polymeric antigen believed to be a B cell tolerogen requires to be examined for its effects on T cells as well, and in a way that allows differential effects in B cell help to be recognized.

It is important to recognize that not all polymeric antigens are tolerogenic. For example, the T-independent antigen fluorescein–Ficoll has been shown to *reduce* the tolerance susceptibility of B cells during a brief 48-hour incubation period (Scott *et al.*, 1992). It is clear that molecular weight, epitope spacing, and chemical composition of the carrier are all important variables. In a contact between a B cell and a polymeric antigen there will always be a strong signal 1, i.e., receptor crosslinking, but certainly some carriers have the capacity to induce signal 2 as well, thus converting a tolerogenic signal into an activating one.

IV. B Cell Maturity and Tolerance

Many of the studies just discussed used adult mice, and indeed since the studies of Dresser (1962) and Chiller *et al.* (1970), no one has questioned the fact that the capacity for antibody production can be switched off in the adult animal. Nevertheless, the question of whether there is a special tolerance susceptibility in immature B cells is still worth asking, because many self antigens are present at concentrations far lower than required for adult B cell tolerance induction by experi-

mental injection. During the 1970s and early 1980s, that is, before the transgenic era, three groups were chiefly involved in pursuing this issue with normal B cells, namely, our own (Nossal, 1983), that of Klinman (Metcalf and Klinman, 1976, 1977), and that of Scott (Scott *et al.*, 1987a). Other groups, in work reviewed in Section V, have used B cell lines to address biochemical aspects of tolerance susceptibility.

A. Clonal Abortion and Clonal Anergy

Following several years of work on the tolerogenic potential of flagellin and flagellin–antibody conjugates, which showed that remarkably low concentrations could signal the B cell negatively, we decided to switch our attention to models using more "self-like" carrier proteins, i.e., antigens that could persist for some time in the extracellular fluids. We were struck by the model of Borel (e.g., Borel and Kilham, 1974) in which anti-hapten B cells were rendered tolerant by hapten–immunoglobulin conjugates. Much of our work was done using fluorescein (FLU) coupled to human gamma globulin (HGG) and freshly deaggregated before injection as a tolerogen. Our approach had the following strategic features: (1) careful dose–response studies of tolerogenesis both *in vivo* and *in vitro;* (2) capacity to collect and culture antigen-specific B cells by antigen-affinity fractionation procedures for splenocytes from tolerized or uninjected mice; (3) accurate enumeration of antibody-forming cell precursors (AFCP) by T-independent and T-dependent *in vitro* cloning techniques; and (4) comparative analysis of fetal, newborn, and adult situations and of splenic versus bone marrow-derived B cells.

The results of over a decade's research have been reviewed elsewhere (Nossal, 1983; Pike *et al.*, 1987), and so need only be briefly recapitulated here:

1. Adult, newborn, and fetal mice can all be rendered tolerant in the B cell compartment by FLU–HGG, but the required threshold concentration is considerably lower for newborn than adult mice, and also for fetal than newborn mice.
2. Tolerance can be induced among hapten-specific cells *in vitro* and the sequence of sensitivities is as follows: greatest sensitivity for cells maturing from pre-B to B status; next highest level of sensitivity for immature B cells harvested from newborn spleen or adult bone marrow; highest concentrations required for adult splenic immunocompetent B cells.
3. Hapten density was an important variable in tolerogenicity as conjugates incapable of crosslinking the B cell's Ig receptor failed to

tolerize, and highly multivalent conjugates were most active. As the conjugation rates increased, the degree to which immature cells were more sensitive than mature cells decreased, a possible reason for some authors failing to notice the differential susceptibility.

4. Simultaneously crosslinking the Fc receptor and the Ig receptor increased the strength of the tolerance signal but was not obligate for it, as adequate concentrations of FLU–F(ab) of HGG were tolerogenic.

5. When tolerance was induced either *in vivo* in very young life or *in vitro* in circumstances where pre-B cells matured into B cells, the cellular basis of tolerance rested on one of two phenomena. With high concentrations of tolerogen, antigen-specific B cells simply failed to appear. There was a maturation arrest at the pre-B/B cell transition, possibly involving death of the newly formed B cell. We termed this *clonal abortion* to distinguish the phenomenon from deletion of already formed B cells, be these immature or mature. On the other hand, when much lower concentrations of tolerogen acted either *in vitro* or *in vivo*, B cells capable of binding the antigen in question were formed and these exhibited a normal number of Ig receptors, but the antigen-specific B cells were incapable of generating antibody-forming clones *in vitro* or of responding *in vivo* to either T-independent or T-dependent stimuli. This surprising finding clearly showed the capacity to induce B cell tolerance *without* clonal abortion or deletion. For this new phenomenon, we coined the phrase *clonal anergy* (Nossal and Pike, 1980) to describe a state in a B cell induced by a tolerogen which, without killing the cell, conferred a negative or downregulatory signal that rendered the cell refractory to later normally adequate immunostimulatory signals. This concept of clonal anergy has since been demonstrated for the T cell as well (e.g., Lamb *et al.*, 1983; Jenkins and Schwartz, 1987) and, as we shall see, has formed the centerpoint of much of the transgenic work on B cell tolerance.

The work of Metcalf and Klinman (1976, 1977), pursued independently of our own, has used a B cell cloning technique, the splenic focus assay, whereby B cells are first adoptively transferred into a lethally irradiated mouse in limitingly small numbers and then cultured within tiny fragments prepared from the spleen of the host mouse. Preinjection of the host mouse with a carrier antigen to create a radioresistant population of helper T cells, or actual transfer of carrier-primed T cells after irradiation (Linton *et al.*, 1989, 1991), ensures optimal T cell help for the hapten-specific B cells under study. *In vitro* tolerogenesis is induced by the hapten in question coupled to an

irrelevant carrier, and T-dependent immunization is induced by the addition of the hapten coupled to the carrier for which T cell help is available. The chief conclusion regarding tolerogenesis within the primary B cell repertoire is that tolerance is readily induced within cells harvested from newborn spleen or adult bone marrow, but that cells from adult spleen are resistant to tolerogenesis. Furthermore, the hapten must be multivalently coupled to a protein to induce tolerance, the process of tolerogenesis taking at least several hours. If tolerogen and immunogenic hapten–carrier act simultaneously on the B cells in the splenic focus, immunity "wins" over tolerance. The effects of toleragens on the secondary B cell repertoire are discussed separately in Section VII.

The work of Scott's group, like ours, makes extensive use of fractionated, hapten-specific B cells. Their experience also highlights the ability of "signal 2" to interfere with tolerogenesis, as lipopolysaccharide (LPS) completely or a graft-versus-host reaction partially blocks tolerance induction (Ornellas *et al.*, 1974). The work also supports clonal anergy as an important mechanism, but shows that anergy can sometimes be partial, as manifested by a reduced burst size of antibody-forming cells (AFC) from a single hapten-specific AFCP (Pillai and Scott, 1983) and the capacity of living macrophages to reverse partially the inhibition of mitogen responses in anergic B cells (Pillai and Scott, 1981). This study is one of a number (Pike *et al.*, 1987) showing that the anergic B cell displays a greater capacity for responding to mitogens than it does to antigen-specific stimuli. The biochemical events of anergy are discussed in Section V where more recent work of the Scott group is covered.

This older work therefore shows a good consensus on the main points, supports the reality of B cell tolerance, and substantiates the existence of a state of clonal anergy. Given the much greater susceptibility of immature B cells to tolerogenic signals, it leads naturally to the question of whether "signal 1" is transmitted differently in an immature versus a mature B cell. As our knowledge of signaling pathways in B cells is still fragmentary, unanimity has not yet been established on this point, but significant differences are emerging which we must now consider.

A wonderful new tool for B cell tolerance research has recently become available, namely, a system that allows the progression of pre-B cell lines and clones to mitogen-reactive B cells either *in vitro* or *in vivo* (Rolink *et al.*, 1991). Extensions of this technology actually allow the clonal enumeration of pre-B cells in fetal and newborn

animals. This system lends itself to the intentional addition of surrogate self antigens *in vitro* and to a new look at the cellular mechanisms underlying deletion or anergy.

V. Biochemical Basis of B Cell Tolerance

A. Constraints to Biochemical Study of Antigenic Signaling

One of the abiding fascinations of the immune system is also the chief reason making detailed biochemical study of lymphocyte signaling difficult. This is the extreme heterogeneity of lymphocytes. As regards the B cell, one must consider the ontogenetic stage (pro-B cell to early and late pre-B cell to immature B cell to immunocompetent virgin B cell); the possibility of separate lineages (Hayakawa *et al.*, 1984; Linton *et al.*, 1989) such as Ly-1 or B1 lymphocytes, conventional virgin B cells, and secondary B cells, viz., the precursors of memory cells; clear heterogeneities in activation status and geographical location (extrafollicular and intrafollicular B blasts, germinal center blasts, and centrocytes; plasmablasts, immature and mature plasma cells); heterogeneities in surface isotype; and difference in circulation pathways and life span. Even more important than this, a selective immune system mandates extreme heterogeneity in the specificity of the surface Ig receptors. The unique minigene assembly process ensures that one B cell will express only one VH and VL gene pair. It is probable that once the VDJ-μ constant-region and VJ-light chain constant-region gene translocations have been completed, the number of divisions among pre-B cells is quite limited (for fuller discussion, see Nossal, 1990). Thus only a few identical exemplars of each new B cell exist in the primary repertoire; to that degree every virgin B cell is virtually unique. This means a unique capacity to bind particular epitopes more or less well and, thus, a unique response to a particular antigenic signal. Even sorting out antigen-specific B cells prior to mixing with antigen does not completely beat this problem.

Accordingly, scientists have sought a number of ways around this most awkward of all the heterogeneities. An early and still popular one is the use of anti-immunoglobulin antibodies as surrogate antigens. Polyclonal B cell activators such as LPS have also been used to study signaling, but suffer from the defect that they bind to an unknown and possibly heterogeneous set of receptors, whereas anti-immunoglobulins crosslink the authentic receptors for antigen, though not in a way that occupies the receptor's physiological binding site. Another possibility is to study a cloned B cell line believed to be representative

of a particular differentiation stage. Valuable knowledge has been gained through this approach although, being derived from B cell malignancies, such lines are hardly sure pointers to normal cellular behavior. A further worthwhile approach has been to study the receptor apparatus of authentic immature versus mature B cells to infer possible differences in signaling mechanisms. The final strategy, in which populations of B cells with identical, transgenically imposed receptors are studied, is dealt with separately in Section VI.

B. Anti-Immunoglobulins as Surrogate Antigens: Differential Effects on Mature and Immature Cells

Conventional virgin B cells emerge from the bone marrow bearing IgM and IgD receptors of identical specificity. It has been known for a long time (Lawton and Cooper, 1974) that anti-immunoglobulin μ heavy-chain antibodies administered to mice repeatedly from the day of birth can have profound effects on B cell differentiation. With appropriate doses, such animals are rendered agammaglobulinemic; i.e., they possess essentially no B cells, though their pre-B cell pool is normal. *In vitro* studies on the action of anti-immunoglobulins revealed part of the reason for this dramatic effect (Raff *et al.*, 1975; Sidman and Unanue, 1975), as the removal of surface Ig by anti-Ig through the patching, capping, and endocytosis mechanism was readily reversible in the case of adult B cells, but irreversible in the case of immature B cells. It soon became evident (Cambier *et al.*, 1977) that neonatal cells were much more susceptible to tolerance induction *in vitro*. A further and more subtle finding (Pike *et al.*, 1982) was that very low concentrations of anti-Ig, which failed to cause Ig receptor modulation in mature B cells, and which actually permitted the emergence of a normal Ig receptor complement in B cells maturing from pre-B precursors in short-term tissue culture, nevertheless negatively signaled the new B cells and induced a state of clonal anergy within them. In a certain sense such anti-Ig-treated B cells could be thought of as universally anergic. Scott *et al.* (1992) have picturesquely described this model as the "poor man's" transgenic (see Section VI).

As tolerance can readily be induced *in vitro* in immature cells, one can ask what biochemical steps take place during tolerance induction. This is certainly an active process, requiring some hours (Nossal *et al.*, 1979) and both protein and RNA synthesis (Teale and Klinman, 1980, 1984). Mature B cells react to anti-Ig with a cascade of early events that lead to the hydrolysis of phosphatidylinositol (PI) phospholipids and increases in intracellular calcium ions (reviewed in Cambier *et al.*, 1987). Activation of protein kinase C (PKC) also follows, and it is likely

that both increased calcium ions and PKC activation are involved in initiating physiological B cell activation and such events as movement of the cell from G_0 and G_1, increased expression of class II MHC genes, increased transcription of *c-fos* and *c-myc* (Monroe, 1990), and transcription of the immediate early gene *egr-1* (Seyfert *et al.*, 1989). It is therefore of considerable interest to compare the responses of mature versus immature B cells to anti-Ig stimulation with respect to all of these parameters. Yellen *et al.*, (1991) have investigated this question using adult or neonatal splenic B cells. Increase of Ca^{2+} ions after anti-Ig stimulation was observed in both populations and to a similar degree; however, the cells did not progress into cell cycle or display any of the typical phenotypic changes of activation. Moreover, the immature B cells did not undergo detectable PI hydrolysis, showing an unexpected uncoupling of Ca^{2+} accumulation and phospholipase C (PLC)-mediated hydrolysis.

When the need for such coupling was bypassed through the combined use of the artificial calcium mobilizer ionomycin and the artificial activator of PKC, phorbol myristate acetate (PMA), immature B cells could be activated, so clearly possessing the reactive machinery. This raises the interesting prospect that the uncoupled Ca^{2+} pathway is responsible for the negative signal. It also raises the suggestion (Monroe *et al.*, 1992) that the defect in the capacity to be positively signaled by anti-Ig lies at some point before PLC involvement. In seeking a molecular explanation of the uncoupling, these workers found an association between surface IgM and a constitutively tyrosine phosphorylated protein of 56 kDa in adult but not neonatal B cells. This protein, which migrates as a dimer under nonreducing conditions, may be a tyrosine kinase of the *src* family, but does not appear to be *lyn*, a kinase associated with surface IgM in a B cell line (Yamanishi *et al.*, 1991). So far, there is no suggestion that the other recently described CD3-like 28- to 38-kDa molecules associated with surface IgM (Campbell and Cambier, 1990; Hombach *et al.*, 1990) are absent from the Ig receptor complex of the immature cell. Nevertheless, the recent work of the Monroe group (Monroe *et al.*, 1992; Yellen *et al.*, 1991, and as yet unpublished studies referred to in their review) mounts a compelling case for the hypothesis that differences in the signal transduction machinery of immature versus mature B cells account, at least in part, for the greater tolerance susceptibility of the former. Both the nature of the 56-kDa homodimer associated with the sIgM of mature but not immature B cells and the nature of the PI hydrolysis-independent Ca^{2+} pathway in immature cells are deserving of much further study; however, the clear finding (e.g., Pike *et al.*,

1981) that mature B cells *can* be tolerized *in vitro* provided that the sIg crosslinking signal is strong enough indicates that differences in the receptor complex structure between mature and immature B cells are not the only factor at work. For example, a great deal of work has been done by Scott and colleagues (e.g., Chace and Scott, 1988; Scott *et al.*, 1989) on the biochemistry of tolerance induction in *mature* hapten-specific B cells using hapten–protein tolerogens. These cells, which possess an intact and normal Ig receptor complex at least before tolerization, exhibit most of the early biochemical events typical of activation when appropriately challenged by antigen or mitogen, yet fail to progress from G_1 to S phase of the mitotic cycle and fail to initiate large-scale immunoglobulin transcription. This suggests the existence of regulatory mechanisms, downstream of those initiating the G_0 to G_1 transition, capable of leading to tolerance. Of course, the relevance of such mechanisms to physiological self tolerance is not clear.

A complete analysis of the biochemical events underlying the "choice" between immune activation and anergy induction would require much more knowledge of the biochemistry of the signaling pathways used in activation which are adjunct to crosslinking of Ig receptors. These are essentially cytokine-mediated signals and other, less well defined, signals. In T cell stimulation, a great deal of work has been done on accessory cell-derived costimulator activity (Lafferty *et al.*, 1983). It is probable that this uses a distinct pathway, as costimulatory signals do not increase PI hydrolysis, Ca^{2+} mobilization, or PKC activation (Jenkins, 1992). In some way, the cell must integrate these various signaling pathways, and clearly a second messenger that may contribute to positive signaling when acting in concert with others could be a negative influence when acting alone.

One area has opened up in a serious way only recently that could have implications for the assembly of the signal-transducing apparatus. It concerns the surrogate light and heavy chains that appear at the surface of the pre-B cell, the function of which is presently unknown (Kudo *et al.*, 1989; Karasuyama *et al.*, 1990). This is a very rapidly moving area which, if combined with the area of CD3-like molecules for the B cell, well deserves a review of its own. What are VpreB, $\lambda 5$, and μ heavy chains recognizing and what consequences flow from such recognition?

C. Respective Roles of Surface IgM and IgD

One of the features of the B cell maturation process is that IgM appears on the surface earlier than IgD (Uhr and Vitetta, 1975; Kearney *et al.*, 1977; Lala *et al.*, 1979), and it was thus natural that the hypothe-

sis would emerge that IgM might be involved in suppressive signaling and IgD in B cell activation. Some early findings seemed to lend a degree of support to this view, in that papain-mediated cleavage of cell surface IgD rendered mature B cells more susceptible to *in vitro* tolerogenesis (Cambier *et al.*, 1977), and the concomitant presence of anti-δ chain antibody and antigen facilitated tolerance induction in adult B cells (Scott *et al.*, 1977). Moreover, cells taken from mice repeatedly injected with anti-δ, and thus possessing B cells lacking sIgD, are readily rendered tolerant (Layton *et al.*, 1978); however, careful quantitative cloning studies on $sIgM^+$ $sIgD^-$ versus $sIgM^+$ $sIgD^+$ cells harvested from mice aged 16–18 days showed that this simple view was not correct (Nossal *et al.*, 1979). At this age, about half the B cells are $sIgD^+$ and half $sIgD^-$, but when these two populations are FACS-sorted, the latter population is equally efficient as AFCPs in a T-independent antibody-forming cell cloning system. Moreover, $sIgM^+$ $sIgD^-$ cells actually perform slightly better in the T-dependent Klinman splenic microfocus assay. Finally, the timing of the acquisition of IgD by the majority of B cells in ontogeny does not correlate with the timing of loss of ready tolerizability. It should also be noted that signal transduction via sIgM and sIgD has identical effects on membrane depolarization, Ca^{2+} fluxes, PI hydrolysis, PKC activation, Ia upregulation, and entry into cell cycle (Isakson *et al.*, 1980; Cambier and Ransom, 1987), making it somewhat unlikely that the two molecules transmit entirely different signals.

In recent years, research on the question of sIgM versus sIgD-mediated signaling has concentrated on cloned cell lines and on transgenic approaches, subjects that are discussed further in the following sections.

D. Cloned B Cell Lines and the Biochemistry of Negative Signaling

In contrast to T cells, where a significant proportion of normal cells are capable of being grown *in vitro* as long-term cell lines, B cells, though yielding short-term clones with high efficiency, have been rather resistant to long-term culture. In the human, transformation with the Epstein–Barr virus has been used and in the mouse a few successes have been noted in creating lines from Ly-1 or B1 B cells, but in the main, workers interested in the use of homogeneous populations of B cells for biochemical or other studies have turned to malignant cells. These have undergone transformation, be this spontaneous or induced by Abelson virus, by transgenic imposition of oncogenes, through the use of peritoneal irritants, through fusion to established malignant

lines, or other means. The clonotypic and, where required, secretory uniformity of such cells is a distinct advantage, though the fact that the cells under study are malignant and thus not under full regulatory control is a counterbalancing disadvantage. In particular, most critical steps in immune activation involve moving a resting, G_0 small B lymphocyte into active cell cycle, and this cannot be mimicked with a cloned, tissue cultured population that is dividing anyway. A simple microscopic examination of any B cell line shows the contrast between these large cells and a small lymphocyte.

Nevertheless a great deal of valuable work has been done with B cell lines, which in many cases represent semifrozen examples of a particular differentiation stage. Frequently, the cell line can respond to a degree to appropriate signals, be this by movement to a further stage of differentiation, by an alteration in division rate, by biochemical and phenotypic changes, or by a switch in secreted isotype. In those circumstances, at least a partial analysis of the biochemical events underlying these changes is possible, though their relevance to physiological events still always requires to be checked.

Our laboratory first became involved in this field, as it relates to tolerance, through the use of a murine B cell lymphoma line known as WEHI 231 (Boyd and Schrader, 1981). This line is positive for sIgM and not IgD, in that respect bearing some similarity to an immature B cell. WEHI 231 cells do respond to stimulation with LPS by a 6-fold increase in Ig secretion, and the most striking finding we made was that a low concentration (0.1 μg/ml) of antiglobulin antibody inhibited cell proliferation and led to death of the cells, in other words, an immature cell-like clear negative response to signal 1 only. These findings were rapidly confirmed by de Franco *et al.*(1982), since when the cell has been widely used as a model of an immature B cell for the biochemical analysis of negative signaling (e.g., Scott *et al.*, 1985; Monroe, 1988). Its immature status is also supported by the absence of C3 receptors and the low level of Ia (Lanier *et al.*, 1981); however, sIgM crosslinking is *not* followed by the uncoupling of Ca^{2+} flux and PI hydrolysis noted in Section V.B for authentic immature B cells, and all the early signaling events seem to be the same as those of mature B cells (Monroe and Haldar, 1989). It is therefore clear that WEHI 231 represents a stage in B cell differentiation beyond the very immature B cell that has just inserted the first IgM receptors. One of the most interesting lines of research in WEHI 231 concerns the immediate early gene *egr-1*. This gene is important in the B cell activation cascade because it is switched on following B cell stimulation (Seyfert *et al.*, 1990a) and because antisense oligonucleotides neutralizing transla-

tion inhibit B cell activation (Monroe *et al.*, 1992). When WEHI 231 cells were treated with anti-μ heavy-chain antibody, *egr-1* was not induced (Seyfert *et al.*, 1989). The cause of this unresponsiveness was investigated, and it was found that the *egr-1* gene was hypermethylated, particularly in its promoter region (Seyfert *et al.*, 1990b). When WEHI 231 cells were cultured in the presence of the methylation inhibitor 5-azacytidine, the resultant cells *did* express *egr-1* following anti-Ig treatment, building a strong case for the involvement of hypermethylation in the failure of responsiveness. The favored hypothesis is that stimulation of the B cell at this differentiation stage is followed by early signaling events including Ca^{2+} accumulation and PKC induction. The signaling cascade stops when it reaches the point of *egr-1* induction. In the absence of being able to proceed further, the second messengers mediate a negative signal. The implications of these results for future work are imaginatively explored by Monroe *et al.* (1992).

Research on the biochemistry of negative signaling of immature B cells is at far too early a stage for a consensus to have been achieved. Furthermore, the existence of multiple negative signaling pathways is by no means excluded. For example, Beckwith *et al.* (1991) have recently reported results that are biologically rather similar to the aforementioned results using a human B cell lymphoma line. Again, the parameter being examined was the inhibition of growth by anti-μ chain antibody. This negative signaling appeared to be dependent on tyrosine phosphorylation but independent of Ca^{2+} upregulation or PI hydrolysis, in other words, seemingly quite different biochemically from the WEH 231 negative signaling pathway.

Scott and colleagues (1985, 1986; Pennell and Scott, 1986) have broadened this line of research to include a panel of phenotypically immature B cell tumors that all share the characteristic of growth inhibition by nanogram/milliliter concentrations of anti-Ig. These include WEHI-231, CH31, and CH33. Evidence is presented that the block is not in the movement of the cell from G_0 to G_1 but rather in the progression from G_1 to S phase. The fate of these partially activated cells is death by apoptosis; however, supernatants from activated T cells could partially reverse the cell death induced (Scott *et al.*, 1987b) and at least some of this protective effect was due to IL-4. These findings are consistent with the tenets of the Bretscher–Cohn (1970) model of signal 1 leading to tolerance but signal 2 addition converting the result to induction of immunity. The neutralization of tolerogenesis by helper T cells is also consistent with the experimental results of

Metcalf and Klinman (1976). In the lines WEHI-231 and CH33, anti-Ig treatment elicited Ca^{2+} flux and PI hydrolysis, confirming the findings of the Monroe–Cambier groups; but in the case of CH31, Ca^{2+} mobilization did not occur. Indeed, negative signaling appeared to be independent of the classical signal pathway because it could not be inhibited by either culture in Ca^{2+}-free medium or protein kinase inhibitors such as H-7 and H-8 (Warner and Scott, 1988). To complicate matters still further, cholera toxin, an agent that raises cAMP levels, has no effects on negative signaling in WEHI-231, CH31, and CH33, but completely blocks the entry of normal B cells into cycle, again suggesting that positive and negative stimuli follow different signaling pathways (Warner *et al.*, 1989). In the latest paper from this group (Ales-Martinez *et al.*, 1991), complete protection from negative signaling was provided by activated D10 or BK3 cells, cloned T cell lines of so-called TH_2 type, but not by activated A.E7 or BK2.43 cells, TH_1 T cells. The protective effect of TH_2 cells could not be reversed by anti-IL-4, suggesting that it was a more complex phenomenon than just the secretion of this one lymphokine.

Still further evidence that positive and negative signaling follow different pathways comes from another approach (Ales-Martinez *et al.*, 1990). CH33 cells were transfected with a δ heavy-chain construct such that the cells express both IgM and IgD. Both anti-μ- and anti-δ-chain antibodies rapidly mobilize Ca^{2+} from intracellular stores in these cells; however, anti-δ antibody does not transmit the negative signal. It "desensitizes" the cell such that anti-μ added shortly thereafter cannot induce a calcium flux; however, these same cells still register the negative signal imposed by anti-μ. Furthermore, PMA treatment also renders the cells temporarily incapable of Ca^{2+} mobilization, yet does not affect anti-μ-induced growth inhibition. Tisch *et al.* (1988) also find that in IgD-transfected B cell lymphoma lines, both sIgM and sIgD crosslinking can induce Ca^{2+} mobilization, but only sIgM crosslinking can induce growth inhibition. This line of work seems to negate definitively the notion that IgD appearance on the cell surface heralds the end of tolerance susceptibility in the B cell ontogenetic sequence. We consider this issue again in Section VI, where it will become evident that IgD *can* transmit negative signals in some circumstances.

We are left, then, with two contrasting notions about the transduction of negative signals to the B cell. Experiments of the Monroe group suggest that in the early immature B cell, i.e., a fetal liver or bone marrow B cell that has *just* acquired sIgM, there is uncoupling of the Ca^{2+} and PI hydrolysis pathway, and that crosslinking of the sIgM

receptor, with a critical 56-kDa molecule not yet associated, leads to a negative signal. On the one hand, this negative signal may be Ca^{2+}-dependent, and the calcium flux itself, in the absence of other stimuli, may lead to apoptotic cell death or anergy. On the other, the C33 experiments of the Scott group suggest that the tolerance signal may involve quite a separate and as yet unknown signaling pathway which somehow dominates when the full Ca^{2+}–PI–PKC route is not yet open. Although the function of the 56-kDa molecule is not established, its coupling to IgM in mature but not immature cells represents one of the few differences between the two, and its entry into the receptor complex may play a role in differential signal transduction. The hypermethylation-induced silencing of *egr-1* may represent an additional safeguard against the premature activation of early immature B cells by self antigen. Somewhat later in ontogeny, the B cells demethylate *egr-1* and the B cell is seeded out, e.g., to the spleen, as a late immature B cell, but with the transmembrane signaling apparatus still lacking p56 IgM association and not permitting PI hydrolysis following receptor crosslinking. This cell can still be silenced by the negative sIgM-crosslinking signal but can potentially be activated by artificial stimuli such as PMA plus ionomycin. Maturity is achieved when p56 and IgM become associated in the membrane receptor complex and when, perhaps coincidentally or perhaps consequentially, the Ca^{2+} pathway becomes linked to the PI hydrolysis and PKC activation pathways. The insertion of IgD into the membrane is essentially irrelevant to the transition from a cell that is tolerance susceptible to a cell that is tolerance resistant on IgM crosslinking.

How the mature B cell, with its fully competent sIgM and sIgD signaling complexes, manages to be switched off by excessive receptor crosslinkage is entirely obscure at the moment. Biochemical analysis so far has not explained the mechanisms accounting for the observation that sIg crosslinking alone cannot induce full activation. Ancillary signals, such as those coming from authentic activated T cells, and/or from lymphokines, or from molecules with the peculiar properties of polyclonal B cell activators, are required, but how these coordinate with the sIg-derived signals is unknown. In this review I have not even addressed the further substantial literature that exists on yet other lymphocyte signaling pathways, such as those mediated via the Fc receptor or molecules such as CD2, CD20, CD23, CD28, CD40, and adhesion molecules. It is to be hoped that the valuable transgenic models will soon begin to contribute to the solution of what is clearly a knotty biochemical problem.

VI. Transgenic Approaches to B Cell Tolerance

A. Advantages and Disadvantages of Transgenic Models

Why did the Medawar group (Billingham *et al.*, 1953) succeed in tolerance induction when Burnet failed? One of the answers to that question is persistence of antigen. To achieve tolerance to allografts it is necessary that enough cells of the tolerizing inoculum survive in the host to constitute a pool of foreign antigen capable of "catching" new immunocytes that arise throughout life. Single or even repeated injections of nonliving antigens achieve this only if enough is given to maintain a pool of tolerizing material, and many models show waning of tolerance following antigen elimination. Of course, most self antigens are made within the body of the animal before the immune system has matured, and are synthesized continuously as part of normal bodily commerce. As cells senesce and die, and as serum or other molecules are catabolized, they are replaced through the normal homeostatic mechanisms of the body.

We owe a substantial debt to Adams *et al.* (1987) and Arnold *et al.* (1988) for first introducing transgenic antigens to experimental immunology, although even earlier than that, Georges Köhler (1986) promoted the view that transgenic technology would solve many of the key problems in tolerance research. Adams *et al.*, (1987) placed the gene for a foreign antigen, the SV40 large T antigen (Tag), under the control of the rat insulin promoter (RIP) and introduced this construct as a transgene. Several founder lines of transgenic RIP-Tag mice were produced. In these lines, the foreign antigen was expressed only in the insulin-secreting β cells of the pancreatic islets of Langerhans. In that location, transgene-encoded antigen was constantly synthesized in a manner simulating the way an authentic β cell-specific differentiation antigen would have been. An unanticipated and not yet fully explained finding was that some founder lines expressed Tag early in ontogeny, e.g., in embryonic life, whereas others showed developmentally delayed expression. When the immune status of the mice was examined, a very Burnetian result was observed. Mice of the former sort were solidly tolerant of Tag, whereas the latter mice not only made anti-Tag antibody, but also developed an autoimmune lymphocytic infiltrate or insulitis, which in some cases led to diabetes. The RIP promoter has been used by others to create a variety of tolerance models, particularly for T cell tolerance work.

The work of Arnold *et al.* (1988) led to a conclusion that, in the first instance, seemed disappointing. Their transgenic construct was a class

I MHC gene, under its own promoter, so designed as to lack the transmembrane portion of the class I heavy chain. As a result, a soluble form of H-2 antigen was present constantly in the serum of transgenic mice; however, no impairment of antibody formation was noted, arguing against either T or B cell tolerance in such transgenic mice. This experiment was interpreted to show that soluble monomeric antigens at relatively low molarity are poor tolerogens *in vivo*.

A variety of other transgenic antigens have been used since, and we shall encounter many of these in the following text. What should already be apparent are the two main advantages of transgene-encoded antigens in tolerance research. First, they are produced by the mouse itself as a part of its metabolism, rather than being injected by the investigator, with inevitable subsequent decline in concentration. Second, judicious selection of gene regulatory sequences can target transgene expression to particular locations in the body. There are some disadvantages to this approach as well. First, it is possible that the expressed transgene can alter the function of the cell in which it is expressed. For example, Miller *et al.* (1989) found that the overexpression even of syngeneic class I MHC under the control of the RIP gradually destroyed the β cells of the pancreas by clearly nonimmunological processes. Second, promoters can be "leaky." For example, in the RIP-H-2 model just mentioned, although conventional techniques fail to detect any transgene expression in the thymus, polymerase chain reaction analysis shows clear positivity. It is wise, therefore, to check key conclusions coming from transgenic models also in a variety of conventional tolerance models.

The transgenic approach has been even more helpful with respect to the heterogeneity of the B cell repertoire. It is possible to introduce constructs of fully rearranged immunoglobulin genes into fertilized oocytes and so to derive mice with high frequencies of B cells with the transgene-imposed specificity (Grosschedl *et al.*, 1984; Rusconi and Köhler, 1985; Storb *et al.*, 1986). The technique is particularly powerful when both heavy- and light-chain gene products are coordinately expressed, ie., when the full receptor is transgenically imposed. Use of the heavy-chain enhancer ensures targeting to B cells, and the best constructs ensure that B cells make both IgM and IgD of a single, defined specificity (Goodnow *et al.*, 1988). Such antibody-transgenic mice are, as Köhler (1986) predicted, wonderful tools but a most sophisticated further variation represents a special triumph. This was the idea (Goodnow *et al.*, 1988) of the doubly transgenic mouse. Antigen-transgenic mice are mated with antibody-transgenic mice to create a situation in which, for tolerance to occur, essentially every immuno-

cyte has to be purged or modified. This elegant design has since been taken up by others, in both a B and a T cell context.

The antibody-transgene approach has some disadvantages also. First, the normal ontogeny of the B cell may be perturbed through earlier than normal expression of the receptor and suppression of the normal order of Ig minigene translocations. Second, it has not yet been possible to create constructs that permit the inclusion of VH constant regions other than the first two elements of the Honjo sequence, namely μ and δ. This means that T cell-dependent isotype switch mechanisms cannot yet be studied in transgenic models. Third, it transpires that antibody-transgenic mice show a degree of spontaneous secretion of the antibody in question, probably because of environmental priming, lymphokine release, and some bystander activation. This leakage of antibody may alter the way injected antigen is handled, and may even have effects on endogenous or transgene-encoded antigen. Fourth, although B cells with transgenic receptors may dominate the primary repertoire, cells with endogenously encoded receptors do sneak through and may be expanded in the secondary lymphoid organs by antigenic exposure, thus taking away from the otherwise homogeneous picture. Finally, as the fully rearranged gene set frequently comes from a hybridoma chosen for specificity to a particular antigen, it may be of high affinity. In contrast, many virgin B cells that react with an antigen introduced for the first time display only low-affinity receptors for that antigen. Awareness of these constraints will help interpretation of antibody-transgenic experiments. Although it will not be possible to do justice to every experiment on tolerance performed using the aforementioned concepts, I shall attempt to summarize in turn the achievements of each major group active in the field, following which a reconciliation of experimental differences is attempted.

B. The Hen Egg Lysozyme Model: Clonal Anergy Vindicated

The Goodnow–Basten group has developed a series of models using a transgenic antigen, hen egg lysozyme (HEL), and a transgenic B cell receptor, anti-HEL. Several key aspects of experimental design were already revealed in their first paper (Goodnow *et al.*, 1988). The antigen, HEL, was placed under the control of the metallothionein promoter. Several founder lines were established that varied in the degree of expression of HEL in the absence of intentional zinc feeding. This constitutive HEL production resulted in relatively constant serum levels of HEL within members of a given founder line, but widely varying levels between the different lines, ranging from a mean of 58 ng/ml to <0.5 ng/ml (Adelstein *et al.*, 1991). When low-producer

lines were given 25 m*M* zinc sulfate in the drinking water very marked elevations of serum HEL levels could be achieved; e.g., the line ML4 had its serum level raised from <0.5 ng/ml to >100 ng/ml. The experimental design therefore offered a wide choice of baseline HEL levels and the possibility of varying the level at different stages of ontogeny. The anti-HEL transgenic line was also designed with great care. The hybridoma HyHEL10 (Smith-Gill *et al.*, 1984) was selected as producing a high-affinity anti lysozyme antibody, the K_a of $2 \times 10^9\ M^{-1}$ being very much greater than those of most primary response antibodies. Genomic clones were used for the transgenic construct such that the $\gamma 1$ C region of the hybridoma's heavy-chain gene was replaced by a gene segment comprising the $C\mu$ and $C\delta$ genes from Balb/c mice which had a heavy-chain allotype different from that of the endogenous C57Bl/6 heavy chains, allowing ready discrimination between transgene-encoded and endogenous antibody. Light-chain gene constructs from HyHEL 10 were injected into fertilized oocytes together with the heavy-chain construct, and in six newborn mice both transgenic heavy and light chains were expressed. In the first line chosen for detailed study, MD3, more than 90 percent of splenic B cells displayed anti-HEL IgM and IgD on the cell surface. Endogenous receptors were almost completely suppressed.

The single-transgenic lines were already full of interest. Lines expressing substantial amounts of HEL were tolerant to HEL challenge in adjuvant. Plaque-forming cell assays showed that this was not simply due to neutralization of formed anti-HEL antibody by HEL. Analysis of T cell proliferation following HEL recall stimulation *in vitro* showed T cell tolerance. Lack of antibody formation when challenge involved HEL coupled to a different carrier showed B cell tolerance, with a suggestion that high-affinity anti-HEL B cells were preferentially affected. The anti-HEL transgenic lines, in contrast, showed substantial spontaneous anti-HEL secretion and hyperresponsiveness to HEL challenge, neither of which were unexpected findings.

When HEL–anti-HEL double-transgenic mice were produced, the key initial findings (Goodnow *et al.*, 1988) were as follows: (1) The mice were profoundly tolerant on antigenic challenge even with adequate T cell help, (2) The B cells were not clonally deleted, but persisted in the spleen, initially in normal numbers, (3) A surprising phenotype of B cell predominated, with normal levels of expression of surface IgD, B220, JIID, and Ia and normal numbers of Ly-1- or Mac-1-positive cells, but with a dramatic selective downmodulation of IgM; later, B cell numbers declined, (4) The tolerant phenotype persisted on

adoptive transfer into a HEL-free host, suggesting that the state of clonal anergy did not rapidly reverse spontaneously.

The next series of studies addressed the issue of whether B cell immaturity was obligate for tolerance induction (Goodnow *et al.*, 1989a). This was addressed in two ways. Double-transgenic mice displaying low levels of HEL and with demonstrably low occupany of anti-HEL receptors by antigen showed little or no B cell tolerance, even though the low levels of HEL were encountered by immature B cells. Such mice were fed zinc, and HEL levels rose nearly 100-fold in the next 4 days. Over this period, the phenotype of the B cells changed to $SIgM^{low}$ $sIgD^{normal}$ and to a functionally anergized state. As this suggested the capacity to anergize mature cells, a second set of experiments involved transfer of mature B cells from antibody-transgenic mice into the high-HEL environment of X-irradiated high-HEL transgenic recipients. Downmodulation of IgM followed, and within 2 days the transferred cells were anergic. The two changes appear to be linked, as a particular double-transgenic line which, for some reason, fails to show the usual IgM downmodulation also fails to show clonal anergy of B cells.

The cells of IgM^{low} IgD^{high} phenotype resemble cells normally seen in the mantle zone of lymphoid follicles, and that indeed is where the majority of lysozyme-binding B cells in double-transgenic, tolerant mice are located (Goodnow *et al.*, 1989b). In fact, careful analysis of immunohistological preparations from spleens of doubly transgenic mice (Mason *et al.*, 1992) suggested that maturation and migration of B cells to the follicular mantle zone occurred after the cells had been phenotypically and functionally tolerized in the bone marrow, where they first encounter antigen. In singly anti-HEL transgenic mice, not only mantle zone but also marginal zone HEL-binding B cells, as well as small numbers of red pulp plasma cells, could be found. In contrast, the tolerant doubly transgenic mice showed virtually no HEL-binding B cells anywhere except in the mantle zone. It is not clear what processes in singly HEL transgenic mice drive the activation process that creates marginal zone B cells and antibody-forming plasma cells. This could be the result of fortuitous cross-reactions between environmental antigens and the HEL-specific B cells; bystander activation of B cells through lymphokine fluxes arising from stimulation with irrelevant antigens; or perhaps preprogrammed antigen-independent maturation events. In the doubly transgenic mice, maturation to the mantle zone, presumably the recirculating differentiation stage, is permitted but further development, perhaps requiring direct B cell activation, is not permitted because of the anergic state.

The striking phenotypic change of sIgM downmodulation with no change in sIgD level could lead to the impression that sIgM crosslinking is absolutely essential for anergy induction. This question was investigated through the use of anti-HEL transgenics where the constructs were such that B cells expressed only sIgM or sIgD, not both (Basten *et al.*, 1989). When doubly HEL–anti-HEL transgenics of the sIgM-only type were prepared, the same 10- to 20-fold reduction in sIgM levels on B cells was seen as in the sIgM and sIgD double transgenics already described. When sIgD-only transgenics were prepared and mated with HEL transgenics, downregulation of sIgD was only 3- to 4-fold, and the IgD-only B cells were rendered anergic, though to a lesser degree than the IgM-only double transgenics. Nevertheless, the point is clearly made that sIgD can transmit the negative signal.

The nature of the anergy in the standard high HEL-sIgM an HEL-sIgD double transgenic mice was explored by using LPS as a B cell activator (Adams *et al.*, 1990). The double-transgenic cells made 5- to 20- fold less antibody, though, interestingly, limiting dilution analysis showed only a 2-fold reduction in responding B cells compared with single anti-HEL transgenic splenocytes. This defect in responsiveness was less than the 50- to 100-fold lower *in vivo* responsiveness. The results suggest that, at the single cell level, anergy is not an all-or-none concept, but is partially reversible through LPS, though with a much reduced burst size of antibody-forming cells per clone. This agrees with our fluorescein–HGG model in normal mice, where LPS could stimulate division but only limited differentiation in fluorescein-specific cells from tolerant mice (Pike *et al.*, 1987). Double-transgenic B cells also responded poorly to antigen specific stimuli *in vitro*. Adoptive transfer experiments failed to show any evidence for a role of suppressor T cells in the induction of B cell tolerance. Further work has indeed shown that anergy can be reversed (Goodnow *et al.*, 1991). B cells from doubly transgenic mice were adoptively transferred into normal mice. In this new, soluble HEL-free environment, gradual recovery of the sIgM was noted, full reversal taking about 10 days; however, these cells were still anergic. When they were stimulated by two injections of HEL coupled to sheep erythrocytes, adequate helper T cells having been cotransferred, serum anti-HEL concentrations only twofold lower than those of controls were noted. In other words, the strong immunogenic stimulation, coupled with the absence of soluble tolerogen, was needed for partial reversal of anergy. A period of rest from antigen alone was not enough. In these studies, transfer into a HEL-transgenic environment prevented tolerance breakdown.

Obviously, the question needs to be asked as to why nature would bother to keep anergic cells within the lymphoid system. Certainly, the mantle zone of normal lymphoid follicles contains cells of similar phenotype that show a lower cloning efficiency *in vitro* (Lalor and Morahan, 1990) than typical sIgM^{high} virgin splenocytes, and these could represent anergized self-reactive B cells. Two speculations (Basten *et al.*, 1991) are that such anergic cells could, if the anergy were to be reversed, act as a reservoir of B cells for the hypermutation process, or that anergic B cells using their antigen processing and presenting function manage to present self T cell epitopes in a tolerogenic manner to the recirculating T cell pool, a concept discussed more fully later.

I have argued repeatedly that B cell tolerance can be only part of the story of self recognition. This is well illustrated by a close analysis of tolerance mechanisms in the various single HEL-only transgenic lines (Adelstein *et al.*, 1991). This showed that cell lines expressing any transgenically imposed HEL, even lines in which the serum level of HEL is less than 10^{-10} *M*, demonstrated T cell tolerance; however, if serum HEL levels were below 10^{-9} *M*, there was no B cell tolerance. As progressively higher HEL levels were examined, it was noted that B cells of high affinity were embraced in the tolerance first, and at the higher HEL levels (around 10^{-8} *M*,), the median affinity of anti/HEL B cells resulting was decreased by 95–99 percent, relative to B cells from nontransgenic littermates.

As we shall see in Section VI.C, other transgenic models have yielded a deletional type of B cell tolerance mechanism. Challenged by these findings, Hartley *et al.* (1991) altered the HEL gene construct to include (1) a cDNA fragment encoding the extracellular spacer sequence, transmembrane segment, and cytoplasmic tail of the H-2K^b class I MHC gene, and (2) the promoter from the H-2K^b gene replacing the metallothionein promoter. This maneuver ensured that HEL would be expressed as an integral membrane protein on most nucleated cells including cells in the bone marrow. Doubly transgenic mice were produced by mating with anti-HEL transgenics of two types, namely, sIgM + sIgD-positive or sIgD-positive. Tolerance to HEL was noted in both resulting lines, but on this occasion it was of a different type. There was a virtually complete absence of mature HEL-reactive B cells, with retention of a somewhat increased population of immature, B220-low bone marrow B cells, with a markedly decreased sIgM level. The results suggested that peripheral deletion arose from a failure or arrest of maturation of the antigen-affected immature bone marrow B cells. We postpone discussion of this provocative finding until Section VI.

One apparent discrepancy between the Goodnow–Basten transgenic model of tolerance and the prior studies of Klinman's group and my own, referred to in Section IV, relates to the epitope valency of tolerogens. In normal mice, the latter studies suggest a requirement for some degree of sIgM receptor crosslinking for the delivery of the negative signal to B cells both *in vitro* and *in vivo*. At first glance, HEL might be expected to interact univalently with each B cell receptor and thus not be able to induce crosslinking. The question therefore arises whether, in HEL-transgenic mice, some polymerization of HEL might occur after its original synthesis. This has recently been studied (Basten *et al.*, 1991; P. Peake *et al.*, unpublished material kindly sent to me by Professor A. Basten with permission to quote) by gel filtration and antigenic analysis of fractions derived from serum of HEL transgenic mice. It appears that endogenous HEL is present in the serum in multimeric form, probably complexed to other serum proteins, and these high-molecular-weight forms are multivalent for a single epitope recognized by a monoclonal antibody, HY-HEL5. So this discrepancy has proven to be illusory. It remains to be determined whether authentic self antigens that exist exclusively as monomers in the serum can induce B cell tolerance. One can imagine a variety of epitope matrix-generating mechanisms *in vivo*, including attachment to cell surfaces (even of B cells recognizing some epitope other than the tested one), complexing to carrier proteins, and even a low rate of endogenous thermal or enzymatic denaturation leading to aggregation prior to elimination.

A second and more puzzling discrepancy is the clear and incontrovertible evidence that HEL can tolerize mature B cells *in vivo*. There simply is no suggestion in the double-transgenic model that, for the soluble rather than the membrane form of HEL, immature cells are more susceptible to anergy induction than mature ones. In fact, the induction of mature sIg transgenic B cells into the sIgM-downregulated, nonresponsive phenotype seems to parallel the continuous events in double-transgenic, tolerant mice. Neither Basten's group nor we have come up with a truly satisfactory explanation for this important difference. Somewhat lamely, I might suggest that the apparently mature anti-HEL transgenic B cell retains some immature features of its signal transduction mechanism such that it retains undue sensitivity to the "signal-1 only" stimulus of sIg crosslinking in the absence of T cell help. We do not know what the premature activation of the transgenic, assembled Ig genes does to the assembly of the membrane signal transduction complex. Alternatively, we noted in Section IV.B that very high epitope valency tends to compress the

sensitivity difference to negative signaling between immature and mature B cells. Perhaps this *in vivo* polymerization of transgenic HEL converts it to molecular forms functionally similar to highly haptenated serum proteins.

Next we consider some transgenic models in which B cell tolerance was not induced. At that time, a further operational difference between our studies and the Goodnow–Basten studies is taken up, namely, that our virgin hapten-specific B cells exhibited mainly low- to medium-affinity receptors but the anti-HEL transgenic mice exhibited very high affinity receptors for the antigen in question. The selective down regulation of sIgM is difficult to explain. Both receptor isotypes exist as dimeric, four-chain integral membrane proteins, and both are of identical binding specificity, possessing the same Fv, as in normal cells. Essential differences in the patching–capping–endocytosis receptor resynthesis pathway have not been described for sIgM versus sIgD in normal mature B cells. It must be admitted that the receptor modulation pathway has not been able to be observed before for a majority B cell population under constant receptor stimulation by low levels of a multimeric antigen. We can only await with interest whether this differential treatment of sIgM versus sIgD proves to be a consistent feature in B cell anergy models.

The HEL system leaves in no doubt the reality of B cell clonal anergy as an important and robust component of tolerance. It highlights the possibility of anergizing mature cells, shows the incompleteness and reversibility of the anergic state, describes a novel phenotype for the anergic cell, and raises some speculative possibilities for the function of anti-self anergic B cells.

C. The Anti-H-2K^k Transgenic Model

Another very interesting transgenic tolerance model is the Nemazee–Buerki model (Nemazee and Buerki, 1989a, b). Here, mice were rendered transgenic for a construct comprising the μ and κ forms of an originally IgG_{2a} antibody, 3-83 (Ozato *et al.*, 1980), which exhibited a high affinity for the H-2 antigen H-2K^k and a 100-fold lower affinity for H-2K^b. In transgenic mice of H-2^d background, i.e., exhibiting an irrelevant class I genotype, the great majority of B cells express the transgene-encoded specificity. Such mice could then be mated to H-2^k or H-2^b mice to create a situation akin in some respects to the aforementioned double-transgenic situation, in that B cells were forced to differentiate in a "sea" of the antigen against which they were specific. The key difference was that this time the antigen of interest was not a serum molecule but a cell surface transmembrane protein.

The attentive reader will already have guessed the result, which, of course, antedated the membrane-HEL results summarized in Section VI.B. In this model, a deletional form of tolerance resulted. There was profound and permanent tolerance and an absence of transgenic B cells in the secondary lymphoid organs. In the bone marrow, a large population of immature cells with very low IgM levels of the transgenic specificity was noted. This population represented cells that had just emerged from pre-B status. In the presence of cells expressing "their" antigen, be it the one for which the receptor had high affinity or low affinity, the immature B cells simply could not progress further and certainly could not exit the bone marrow to reach the spleen or lymph nodes. The secondary lymphoid organs were populated by the small number of B cells that expressed endogenous Ig genes which were then suitably expanded by environmental antigenic stimulation. It is a matter of semantics whether this process is termed *maturation arrest* at the very immature B cell stage or *clonal abortion,* but anergic B cells are certainly *not* produced in the Nemazee–Buerki model.

Bone marrow cells from antibody-transgenic mice of H-2^d background were adoptively transferred into irradiated recipients of H-2^k background. This experiment was performed to still the criticism that B cells committed clonal abortion only because they synthesized receptors specific for an antigen present within their own endoplasmic reticulum. After transfer, B cells bearing a very low level of sIgM of the anti-H-$2K^k$ idiotype were found in the bone marrow of the recipients, but these could not progress and no peripheral transgenic B cells appeared. This clearly showed that the events of clonal abortion did not require the self antigen to be associated with the actual cell undergoing censorship.

The next question addressed in this model was whether deletion was confined to immature self-reactive cells, or whether B cells that had emerged from the marrow could be silenced or eliminated if they encountered antigen peripherally (Russell *et al.*, 1991). This question was solved through collaboration between Nemazee's group and the Hall Institute. Doubly transgenic mice were produced by mating Nemazee–Buerki anti-MHC antibody transgenic mice with mice (Morahan *et al.*, 1989) that were transgenic for the H-$2K^b$ MHC antigen under the control of the metallothionein promoter which, in the absence of zinc feeding, targets the MHC gene expression chiefly to the liver. No mRNA or surface protein expression of the K^b transgene is detectable in lymphoid tissues. In the double-transgenic situation, there was no deletion of anti-MHC transgenic B cells in the bone

marrow. So we must presume that the B cells left the marrow at a normal rate. Yet there was no accumulation of the autoreactive B cells in the target organ, the liver, and a virtually complete deletion of transgenic B cells from the lymph nodes and spleen. Furthermore, adoptive transfer of B cells from singly antibody-transgenic mice into irradiated singly antigen-transgenic mice resulted in deletion. As this set of experiments involved new constructs in which both IgM and IgD were present at the B cell surface, this deletional phenomenon showed that IgD is not protective for deletion. So, clearly, this particular membrane antigen can cause deletion if encountered either in the primary lymphoid organs or peripherally. Moreover, peripheral deletion occurs even with K^b rather than K^k, showing that affinity is not, by itself, a major concern.

An exciting recent finding in this system (Tiegs *et al.*, 1992) relates to the molecular events occurring in the bone marrow of mice experiencing the central repertoire purging or deletional phenomenon. Two unexpected findings alerted Tiegs *et al.* (1992) to look more deeply at this question. Centrally deleting mice ended up with more lymph node B cells than peripherally deleting mice, a curious finding as one might have expected an equal number of endogenous ("escape") Ig gene-expressing cells in both cases. Second, a surprisingly large proportion of peripheral B cells in centrally deleting mice bore λ light chains. This raised the intriguing possibility that auto-specific highly immature B cells that encounter antigen in the bone marrow do *not* in fact undergo clonal abortion but, having been found to be unwanted, "try again" by undergoing further immunoglobulin gene rearrangement. This would presumably generate variant B cells, including those previously expressing κ now having a chance to "try" λ. Two genes are known to form part of the unique recombinase system that assembles Ig minigenes into the finally expressed gene: RAG1 and RAG2 (Schatz *et al.*, 1989; Oettinger *et al.*, 1990). In antibody-only transgenic mice RAG-1 and RAG-2 expression is depressed, presumably because of some homeostatic mechanism; the pre-B cells do not "need" the enzymes as the cell already possesses fully rearranged Ig genes. Centrally but not peripherally deleting mice had relatively far greater and essentially normal RAG-1 and RAG-2 levels. One of several possible explanations of this finding is "receptor editing": the recombinases swing into action to rescue cells that would otherwise be of no use to the body by reactivating the recombination process and silencing the existing fully rearranged genes. This is perhaps a somewhat exotic notion, but one certainly worthy of further study in different models.

D. The Anti-DNA Transgenic Model

Some autoimmune diseases arise through pathogenetic mechanisms that appear reasonably transparent. In acquired hemolytic anemia, antibodies develop against certain erythrocyte surface antigens leading to the premature autophagocytosis of those red cells and to anemia and splenomegaly. It stands to reason that a person *should* be tolerant of his or her own erythrocyte surface antigens and equally that erythrocytes would be opsonized by antibody coating if that tolerance were to break down for any reason. In contrast, other autoimmune diseases are more puzzling. In the case of systemic lupus erythematosus, where (among other things) antibodies are made to DNA, it is by no means apparent that an animal or person should be tolerant of DNA, given that there is no signature that demarcates one individual's DNA from another's immunologically, and that DNA is not normally present extracellularly or at the cell surface. Nor is it clear how antibodies or T cells directed against DNA as such could be damaging, and blaming immune complexes alone does not solve the similar conceptual puzzle of antimitochondrial antibodies in primary biliary cirrhosis, anticentromeric antibodies in scleroderma, and so on. It is therefore important to have models of immunological tolerance that probe mechanisms of silencing of lymphocytes directed not only to cell surface or serum molecules but also to internal cellular constituents.

From that viewpoint, the work of Erikson *et al.* (1991) is of great interest. Mice were rendered transgenic for the VH gene of a monoclonal antibody, 3H9, which specifies anti-DNA when associated with many different light chains; some combinations react with native, double-stranded (ds) as well as single-stranded (ss) DNA, representing high affinity anti-DNA antibodies, and some combinations, e.g., VH3H9 + Vκ8, specify antibody reactive only with ssDNA. VH3H9-only transgenic mice would express multiple light chains and should be capable of forming both anti-dsDNA and anti-ssDNA antibodies. Mice transgenic for both VH3H9 and Vκ8 should be able to form only anti-ssDNA.

It was found that 60–69 percent of B cells from VH3H9-Vκ8 transgenics bound ssDNA, but despite this predominance of transgene-expressing B cells, no elevation of anti-DNA antibody was found in the serum. Although the B cells did not show the downmodulation of sIgM found in the HEL–anti-HEL transgenic mice, the authors still believe they are dealing with B cell anergy, proposing that the B cells would have encountered DNA or DNA–protein complexes *in vivo*, conferring the negative signal. Evidence was also presented that mice singly

transgenic for VH3H9 did not produce B cells reactive with dsDNA, as opposed to ssDNA. Thus, the higher-affinity interaction between B cells using VH3H9 and an endogenous light-chain gene that creates an anti-dsDNA antibody might have led not to clonal anergy but to abortion/deletion. This paper refers to several interesting manuscripts in preparation and further exploration of the model is eagerly awaited. Oral presentations have produced evidence that the clonal anergy in VH3H9 +Vκ8 transgenics is broken when the transgenes are present in mice with background genes predisposing to autoimmunity, although to date no evidence of lupus has come forward. The studies to date suggest that antigens normally sequestered intracellularly but perhaps briefly rendered accessible after cell death can induce clonal anergy in B cells of low affinity for the antigen and clonal abortion in B cells with higher affinity. The former sort of tolerance may be negated by particular background genes through a dysregulation of the anergy induction process.

E. Antigen-Transgenic Models in Which B Cell Tolerance Is Absent or Only Partial

For each B cell, there must be some threshold below which the presence of antigen in the environment of the B cell does not produce a signal that the B cell can register. Three variables of clear importance are the affinity of the antigen–B cell receptor interaction; the concentration of the antigen; and the effective valency of the antigen which, if high, might override low affinity (Nemazee and Buerki, 1989a, b). It stands to reason that there must be some self antigens for which, by reason of concentration, accessibility, distribution, valency, or some combination thereof, this threshold is not reached. In such cases, self tolerance relies on T cell tolerance, which may be achieved at a lower threshold or on a lack of immunogenic efficacy because of sequestration or extremely low concentration. Transgenic models provide cases of both types. Whitely *et al.* (1990) investigated mice transgenic for human insulin, and found profound tolerance based entirely on T cell effects. Evidence for a peripheral rather than central thymic tolerance was produced. Arnold *et al.* (1988), in their transgenic soluble H-2 model, found neither B nor T cell tolerance. We have already dealt with the HEL transgenic model, in which lines yielding low endogenous HEL levels failed to give B cell tolerance despite the high affinity of the B cell receptor, yet complete T cell tolerance was seen. An interesting but complex tolerance model (Theopold and Köhler, 1990) involved mice rendered transgenic to β-galactosidase in a way that

made the antigen an integral membrane protein of B cells themselves. Such mice showed a near-normal frequency of anti-β-galactosidase B cells, but a complete absence of the capacity to form high-affinity anti-β-galactosidase antibody. Though the result is open to a number of interpretations, one (not favored by the authors) is that high-affinity anti-β-galactosidase B cells have been selectively anergized or deleted. Another fascinating model (Zinkernagel *et al.*, 1990) involves a transgenic viral antigen targeted to certain major viscera but not to lymphoid tissue. Here, T but not B cell tolerance supervenes and immunization with the viral antigen in adjuvant does not lead to antibody production; however, infection with the living virus triggers antibody production against the protein, perhaps because the "autoreactive" B cells themselves present nontolerated antigen fragments to T cells, permitting the latter to activate the nontolerant B cell. If this is the correct explanation, it shows just how dangerous "immunological ignorance" in B cells can be, and the experiment provides a good teleological rationale for the existence of B cell tolerance mechanisms.

Another viral transgenic experiment of great interest has recently been published by Oldstone *et al.*, (1991). A viral glycoprotein from the lymphocytic choriomeningitis virus (LCMV) was targeted to the insulin-producing β cells of the pancreatic islets of Langerhans via RIP. Such RIP-LCMV mice express only low levels of viral proteins, with minimal pathological consequences. When mice are challenged with live LCMV, a progressive insulitis develops, leading eventually to insulin-dependent diabetes. Evidently, the low level of viral gene product did *not* lead to irreversible tolerance in either the B or the T cell compartment; the viral infection triggered an antiviral immune response, including a cytotoxic T lymphocyte response; and islet cells expressed sufficient viral protein to make them into targets. The apparent "immunological ignorance" of the neo-self antigen again had drastic effects following an appropriate trigger.

Another approach to studying the effects of an important endogenous antigen was taken by Kenny *et al.* (1991). Mice were rendered transgenic for the rearranged M167 μ heavy-chain gene, the original μκ antibody having anti-phosphorylcholine (PC) reactivity of a characteristic M167$^+$ idiotype. Flow cytometric analysis showed an extraordinarily high incidence of M167 idiotype-positive (M167-id$^+$) B cells expressing the transgene and a single endogenous Vκ24Jκ5 light chain. In contrast, very few B cells of the T 15 idiotype were found; this idiotype would have required association with the Vκ22 light chain, and might have been expected to be equally frequent had there been no antigenic selection. The selective expansion of the M167-id$^+$ popu-

lation thus appears to be the result of an antigen-driven process. As M167-id$^+$ cells are PC specific, and M167VH-Vκ22 T15$^+$ B cells have little or no affinity for PC, the suspicion is that PC is the antigen responsible. If so, the antigenic drive must come from environmental (e.g., bacterial) antigens that include PC or, alternatively, autoantigens containing PC. Yet, there is no sugestion that this endogenous PC, wherever derived, has tolerized either B or T cells.

VII. B Cell Tolerance in the Secondary Repertoire

One of the most interesting aspects of B cell physiology relates to affinity maturation of the antibody response, where, on repeated or prolonged immunization, the median affinity of antibody progressively rises. Two aspects contribute to this phenomenon. The first is that V genes for immunoglobulins are capable of an extraordinarily high rate of mutation, approximating one mutation per division, following antigenic stimulation (Weigert *et al.*, 1970; Berek and Milstein, 1987; Kocks and Rajewsky, 1989). The second is that in the presence of increasing concentrations of antibody, and declining levels of antigen owing to neutralization and catabolism, only those B cells displaying receptors with higher affinity for antigen will compete effectively with previously formed antibody for access to the antigen required for further rounds of stimulation. Key events of B cell mutation and selection take place in germinal centers (reviewed in Nossal, 1992). One end result of the V gene hypermutation and antigenic selection processes is the production of a population of cells marked by multiple V gene mutations and certain functional and phenotypic characteristics (Linton *et al.*, 1989) that are termed memory B cells. Evidence has been presented that memory B cells are not the direct descendants of the virgin primary B cells that produce the primary antibody response, but represent a separate bone marrow-derived lineage (Linton *et al.*, 1989).

The fact that high-affinity antibody is derived chiefly from this mutation and selection pathway poses new questions for the student of B cell tolerance. Given that mutation is so frequent, and that there are so many self antigens, it must happen from time to time that a particular mutation occurring in a B cell proliferating quite appropriately to some foreign antigen fortuitously confers on that cell potential antiself reactivity. Are there any mechanisms that prevent this from happening? Linton *et al.* (1991) has provided an interesting answer. Murine splenocytes were injected intravenously in small numbers into lethally irradiated syngeneic, previously carrier-primed hosts together with an

optimal number of carrier -specific helper T cells. Attention was focused on a subset of J11D^{low} B cells typical of the secondary B cell lineage. Tiny fragments of spleen, containing no, one, or at most a very small number of hapten-specific B cells (according to the Poisson equation), were cultured and stimulated with hapten–carrier conjugates from culture initiation for 48 hours to yield a primary response. They were then washed and restimulated for 24 hours at day 7 to yield a secondary response. With few exceptions, J11D^{low} cells failed to form antibody on first stimulation but did form antibody when restimulated. Next, soluble hapten–protein tolerogen (i.e., the same hapten coupled to an irrelevant carrier) was added to cultures either *before* primary immunization or *between* primary and secondary immunization. As expected for mature B cells, tolerization before stimulation did almost nothing. The mature B cells of the secondary lineage were tolerance resistant as, in the same circumstances, are mature virgin primary B cells (Metcalf and Klinman, 1976); however, when the J11D^{low} cells were first sent into a primary round of division, geared to convert them into reactive memory B cells, and then exposed to a B cell tolerogen in the absence of T cell help, they were rendered tolerant and failed to react to the secondary stimulus. In other words, the recently activated secondary B cell passed through a "second window" of tolerance susceptibility, akin in its sensitivity to the first window experienced when primary B cells are immature. Linton *et al.* (1991) suggest that a B cell at risk of becoming an antiself B cell through a fortuitous mutation would encounter self antigen and be similarly tolerized. In this way the secondary or memory B cell repertoire would become purged of self-reactive B cells. The experimental protocol cannot differentiate whether anergy or deletion was involved.

Galelli and Charlot (1990) also reached the conclusion that tolerance could be a feature of the memory B cell population, but via a completely different experimental approach. They investigated the phenomenon of epitope-specific suppression (Herzenberg *et al.*, 1983) in which sequential immunization with carrier and then hapten–carrier leads to a specific downregulation of the antihapten IgG response. Hapten-specific B cells were isolated by the techniques we first introduced (Haas and Layton, 1975), and were found to be present in equal numbers in suppressed and control immunized mice; however, the memory B cells in the suppressed mice showed an intrinsic defect or anergy. When immunized T-dependently *in vitro*, they could proliferate but not differentiate into active IgG-secreting cells. This failure was not due to any defect in antigen-presenting capacity of the cells. Although not indicating the cellular or molecular mechanisms

whereby the carrier preinjection leads to anergy in memory B cells, the study enlarges the concept of anergic B cells now to encompass anergic cells also within the secondary repertoire.

Our group has addressed tolerance within the secondary repertoire in yet a different way (Nossal and Karvelas, 1990; Karvelas and Nossal, 1992). We used the capacity of IL-4 to cause an *in vitro* isotype switch, predominantly to IgG_1, to perform a repertoire analysis on B cells, scoring only antibody of high enough affinity to register as a bivalent IgG_1, rather than a decavalent IgM, in an enzyme-linked immunosorbent assay (ELISA). Cells from unimmunized mice were polyclonally activated in limiting dilution microcultures under carefully defined conditions where B cells were not too crowded but were supported by 3T3 filler cells; LPS acted as the polyclonal stimulus; and an optimal mixture of the three lymphokines IL-2, IL-4, and IL-5 was added (McHeyzer-Williams, 1989). Despite high cloning efficiency and excellent antibody formation of up to 20 ng per clone, repertoire analysis could find very few cells (~100 per spleen) the antibody product of which displayed sufficient affinity for a protein antigen, e.g., human serum albumin (HSA), to score in this high-affinity assay. Supernatants that were positive yielded a low optical density in the ELISA, i.e., bound poorly to antigen. Much the same was true of haptenic antigen when the hapten density on the protein conjugated to the ELISA plate as a capture layer was low. Next, mice were immunized with either protein or hapten–protein adsorbed onto alum together with *Bordetella pertussis* adjuvant. After a latent period of about 4 days, progressively more B cells with the requisite characteristics appeared and the antibody bound progressively better. A convenient time when maximal numbers of affinity-matured B cells had developed was 14 days after antigen.

Next, deaggregated antigen was administered to mice as a surrogate self antigen. This could drastically reduce (by a factor of 50–100) the genesis of affinity-matured B cells. In other words, tolerance had been achieved within the secondary B cell repertoire. Tolerogen worked if given before immunogenic challenge and even if given at any time up to 6 days after challenge; i.e., it could somehow halt the recruitment of high-affinity B cells. Was this an effect directly on the B cells or on the T cells needed to start T-dependent isotype switching and affinity maturation? Adoptive transfer experiments showed that the major effect of the tolerogen was on $CD4^+$ helper T cells. When B cells from normal mice and $CD4^+$ T cells from adult-tolerized mice were adoptively transferred and challenged, a greater than 90 percent reduction compared with nontolerized controls was achieved in the

numbers of clonable high-affinity B cells generated. In other words, the surrogate self antigen had caused clonal anergy or clonal deletion in the adult peripheral helper T cell population which indirectly led to a relative tolerance in the secondary B lymphocyte repertoire. In this same model, T cells from normal mice were adoptively transferred together with B cells from tolerized mice and a moderate but lesser degree of lowering of high-affinity B cell generation resulted. Thus, the tolerance lesion was actually within both the T and the B cell compartments; however, *in vitro* analysis has not yet revealed a special hypersusceptibility of B cells to tolerance induction shortly after their activation.

As well as adoptive transfer, another tool is available to determine which cellular compartment is involved in tolerance, namely, immunization with hapten A coupled to carrier X and tolerization either with hapten A coupled to carrier Y or with unconjugated protein X. The high-affinity anti-A B cell formation was studied (Nossal and Karvelas, 1992) in circumstances in which tolerogen was introduced 6 days after immunogenic challenge. Tolerogen AX virtually eliminated high-affinity B cell formation as expected, this being the appropriate negative control. If silencing of B cells had been the main mechanism of tolerance, tolerogen AY would have worked well. It, however, caused only a partial effect, but carrier X alone acted as a good tolerogen. This experiment also indicates that helper T cells are essential for affinity maturation even well after initial challenge, and that such T cells can be anergized or deleted up to 6 days after challenge immunization.

Taken together, the results suggest that the main bulwark against the hypermutation process driving B cells into high-affinity anti-self reactivity is T cell tolerance to self antigens providing a brake against the further development of such cells. Although high doses of tolerogen could have some direct effect on B cells, the studies (which are ongoing) have not yet provided evidence of a special window of tolerance susceptibility shortly after secondary B cells are activated. Using the NP hapten in C57/Bl mice, we are now in a position (McHeyzer-Williams *et al.*, 1991) to examine the V genes of single B cells for evidence of mutation. By 14 days (McHeyzer-Williams *et al.*, 1992) the majority of $sIgM^-$ $sIgG_1^+\lambda^+$ NP-binding B cells of NP-KLH immunized mice already display the critical tryptophan-to-leucine mutation at position 33 of CDR 1 of the VH gene known to confer higher affinity. It will now be a straightforward matter to determine whether adult tolerance represents simply a frustration of the mutation and selection mechanism in germinal centers that leads to this and other mutations.

VIII. Other Perspectives in Tolerance Including Antigen Presentation by B Cells

We have not yet dealt with another function of B lymphocytes, which is the capacity to process and present antigen to T cells. In the broadest sense, antigen presentation and processing are very important variables in tolerance induction. As a general rule, antigens that are aggregate free, circulate widely through serum and lymph, and manage to elude capture by macrophages and other accessory cells act as good tolerogens, whereas the same antigens may well induce immunity if given to the animal (e.g., in aggregated or particulate form) so as to be rapidly cleared by accessor cells. B cell processing of antigen represents only one facet of this still rather poorly understood phenomenon.

We have already considered the experiments of Theopold and Köhler (1990) dealing with antigen-transgenic mice. In their somewhat complex model, they actually ascribe the absence of high-affinity antibody production to a selective suppression by T cells of those B cells that are the most effective antigen presenters, namely, high-affinity B cells. A provocative recent paper is that of Eynon and Parker (1992). In their model, adult mice were injected with deaggregated Fab fragments of Rabbit anti-δ heavy-chain antibody. This antigen rapidly attached itself to essentially all mature B cells of the animal. It was found that when such mice were subsequently challenged with alum-precipitated *normal* rabbit Fab, they were profoundly and specifically tolerant. The degree of tolerance induced by normal rabbit Fab, deaggregated and intravenously injected, was very much lower. In other words, tolerance was largely dependent on antigen presentation by B cells. As in our model, both the helper T cell and the antigen-specific B cell compartments were found to be affected in the tolerant mice. The authors argue persuasively for a new and relevant mechanism of T cell tolerogenesis here. They believe the small resting B cell that has captured the tolerogen lacks the signaling machinery for delivery of the accessory signal (Lafferty *et al.*, 1983) required for immune activation. Macrophages, dendritic cells, and activated B cells would possess this capacity. As a consequence, the helper T cells for the antigen in question would be rendered anergic or deleted rather than activated. They argue that perhaps this is how ordinary freshly deaggregated antigens induce tolerance as well. Admittedly, then the antigen-capturing B cell would be a rare cell rather than every B cell, but at least they would have a major competitive advantage in capturing the antigen, and in low-molarity situations the "professional" antigen pre-

senting cells, lacking specific receptors for the low concentration antigen, may never capture enough to deliver any kind of signal. So the few B cells capturing the low-molarity tolerogen may have time to encounter the specific T cells one by one. This hypothesis also gets us over one of the biggest hurdles in understanding tolerization of T cells by deaggregated antigens. It is generally believed that T cell receptors interact not with soluble intact proteins but with peptides associated with MHC molecules. As deaggregated tolerogens elude accessory cells, it was previously believed that they must in some way interact directly with the T cell receptor. This formulation offers a possible way out. The B cell tolerance generated in the model can be explained on simple anergy induction grounds, particularly as it was shown that IgD *can* transmit negative signals.

It will be of interest to see whether further evidence in favor of this view accumulates. It would lend functional significance to anergic B cells and give an extra reason for their persistence in the body. It is also of interest to examine the opposite side of this coin. Autoreactive B cells, both those anergized as just described and perhaps some reactive with a self antigen that has caused T cell tolerance but not B cell tolerance, might become activated by some cross-reacting foreign antigen and appropriate T cell help. Having now acquired cosignaling capacity, such a B cell could present all T cell epitopes of the self antigen in question in an immunogenic mode to T cells, initiating an autoimmune cascade. A model suggesting such a process has recently been presented (Lin *et al.*, 1991).

IX. Resolution of Apparently Conflicting Models of B Cell Tolerance

It ought not to be imagined that all B cells with any degree of reactivity to self antigens exist in an anergized state. This all depends on affinity and strength of negative signal considerations. Indeed, antiself B cells capable of being readily activated by, for example, alloreactive T cells (Rolink *et al.*, 1987) form a significant proportion of the repertoire and, with this strong stimulation, can readily switch to downstream isotypes. Indeed, the spectrum of autoimmune syndromes that can be induced by initiating graft-versus-host disease forms a fascinating major research area in its own right (Gleichmann *et al.*, 1984; Rolink *et al.*, 1990). There has been somewhat of a tendency to create a "we don't believe in B cell tolerance" movement from these results; however, as we have repeatedly argued, by the very nature of the clonal selection hypothesis, B cell tolerance for self antigens can-

not be absolute. If it were, the only possible end result would be the purging of the total repertoire.

This is where the concept of clonal anergy exhibits such great force. It has been shown to be incomplete, with some limited B cell activation being possible; it can be reversed in some circumstances. This makes it very likely that, at the single cell level, there are various degrees of anergy. What much more subtle possibilities for regulation and fine tuning anergy offers than the killing of a cell! My close colleague, Dr. Ian Mackay, once said to me: "First it was clonal deletion, then clonal abortion during B cell formation, then clonal anergy during the pre-B to B transition, then clonal anergy induced in mature B cells, will it soon end with no tolerance at all?" My answer was, and is, all of the preceding are true, but to different degrees for different B cells against different self antigens.

Let us, then, survey the spectrum of regulatory mechanisms available to cover various autoimmune possibilities. At one extreme, self reactivity against self MHC really would be horrendous if one contemplates graft-versus-host disease or allograft rejection. Such ubiquitous self antigens situated at the cell surface invoke the strongest negative signaling mechanism. For the B cell, this is clonal abortion within the primary lymphoid organ, the bone marrow. Much the same may be true for the major blood group antigens, e.g., A, B, and D. I know of no spontaneous autoimmune process that targets such major antigens. As Nemazee has pointed out, for antigens of this sort, present in large numbers on the cell surface, affinity of binding may not be the main consideration in negative signaling because the avidity conferred through multiple receptor–ligand interactions creates very strong receptor crosslinking even for low-affinity receptors. Next in the negative signaling hierarchy come self antigens sparsely present at cell surfaces, or present in tissue fluids as monomers but with some oligomeric forms created through limited degrees of self-aggregation, binding to other serum proteins or to cell surfaces. Clonal anergy may be the B cell's most appropriate response to such antigens and, particularly for low-molarity antigens, may affect only the high-affinity end of the B cell repertoire. Low-affinity cells to such antigens persist in a competent state. They are a possible source of autoantibody formation should comcomitant T cell tolerance be bypassed through T cell activation by cross-reacting antigens or through T-independent (usually polyclonal) activation. Frequently, however, such autoantibodies do no harm either because the antigen in question is not accessible or because the affinity of the antibody is too low. Extremely strong helper

T cell activation might even activate anergic B cells, although the models in which this has been shown are hardly physiological and presumably this would pose a greater threat. Finally, some self antigens, because they are exclusively monomeric, or because they are not at sufficient concentration within extracellular fluid or too confined to particular anatomic locations, do not cause B cell tolerance at all, in which case the induction of autoantibodies becomes a simple matter in experimental circumstances.

This hierarchy of strength of negative signaling represents one dimension of the problem, but maturity of the B cell is an equally important second dimension. It has not escaped my notice that neither the Nemazee model nor the Goodnow–Basten model shows a significant effect of B cell immaturity on the respective tolerance events, namely, deletion and anergy. In both cases, special circumstances prevail. The MHC-based crosslinking signal is clearly so strong that it overrides the need for immaturity, as happens in our hands with highly multivalent hapten–protein conjugates. The HEL–anti-HEL doubly transgenic model features B cells of unusually high affinity that must effectively attract high local concentrations of antigen to their surface. The lack of a gap in HEL molarity required for tolerization of B cells growing up in the HEL environment versus mature B cells briefly parked in a HEL environment is difficult for me to explain. It would, however, be foolish to ignore the massive weight of evidence from research on normal B cells that speaks to the greater susceptibility of the immature cell.

What prevents a low-affinity antiself B cell left as a competent member of the primary B cell repertoire from hypermutating to a high-affinity one? Overwhelmingly the most important factor is lack of T cell help because of T cell tolerance. Without T cell help, a B cell cannot enter the germinal center V gene hypermutation process. Were the B cell to be triggered in this direction by cross-reactive help, a second factor might be the absence of the self antigen in question in the right relationship to follicular dendritic cells (FDCs). Antigen–FDC association depends on Fc binding, so there must be some preformed antibody, which is unlikely for a self antigen. Positive selection by FDC-bound antigen seems absolutely necessary for the survival and eventual export of the mutated germinal center B cell. What prevents anti-foreign B cells from mutating to high-affinity antiself? Negative selection of the B cell by soluble self antigen, as claimed for the "second window" of tolerance, certainly remains a possibility. Failure of positive selection (as above) must represent an alternative. Certainly T cell tolerance to the self antigen in question would be a major defense against the further proliferation of any antiself cells that

slipped through. The capacity for tolerogens to frustrate the mutation and selection process even after the germinal center reaction has begun and the fact that this postchallenge tolerance seems to be predominantly a T cell effect show just how important continued T cell help is to the whole sequence, and how readily it can be switched off by soluble antigen. There are some $CD4^+$ T cells in the germinal center, and perhaps these participate in some way in proliferation and even positive selection of germinal center B cells.

X. Summary and Conclusions

A paradox of immunology is that the immune system is distributed so widely in the body, as a large number of cells that discharge most of their effector functions as single cells; but, at the same time, the elements of the system are so very interdependent, not only via specialized cell clusters and microenvironments, but also by mobile feedback loops, cellular and molecular. The end result is that one cannot really understand one element of the system without understanding every other, at least to a degree. Certainly, tolerance cannot be isolated from immune activation, nor B cell from T cell tolerance, rendering the task of the reviewer somewhat thankless. This being said, the last few years have seen wonderful progress in our grasp of B cell tolerance, to which the transgenic revolution has contributed a great deal. The fact that B cell tolerance exists as an important component of self-tolerance has been firmly established, as have the limits of the process in terms of both the survival of low-affinity antiself clonotypes and the question of location and concentration of antigen required for tolerance induction.

Two processes have been identified as key alternatives: clonal abortion/maturation arrest/deletion and induction of clonal anergy. The latter requires a less strong Ig receptor crosslinking signal, may be partial, and is reversible. Recognition of these facts has prompted both experimentation and speculation on possible functions of the anergic cell. One unsatisfactory area, which we have not addressed because nothing like a consensus has been reached, is T cell-mediated suppression and its possible effects on tolerant states, including anergy induction in B cells. The phenomenology of suppression is too striking to sweep under the carpet, and suppressor T cell memory in particular (Adelstein *et al.*, 1990) requires much more investigation; however, suppression has not been shown to play a major role in any of the best-studied transgenic models. These can readily be explained on the basis of direct interactions between the B cell target for abortion or anergy and the self antigen in question.

The biochemical basis of discrimination between immunity and tolerance has also progressed, but not as fast. This is understandable, as so many signaling pathways have to come together for full immune induction, and as immaturity of the signal transduction pathway plays a profound role that must be studied in normal cells, with all the attendant difficulties of cell separation. The best paradigm for approaching the biochemistry of positive versus negative signaling remains the Bretscher–Cohn (1970) model. Two considerations give renewed hope. Transgenic mice will provide B cell populations of greater homogeneity, and the ancillary, Ig crosslinking-independent pathways are receiving their due attention. Two separate sets of these can be distinguished as being particularly important: those dependent on cytokines interacting with high-affinity receptors and those requiring cell contact and thus involving some membrane enzyme complex of as yet undetermined nature (Hodgkin *et al.*,1990) I therefore expect progress on our understanding of B cell signaling to accelerate.

Failure in B cell tolerance manifests itself in autoantibody formation. Just as it proved unrealistic to explain tolerance through one sweeping generalization, so it is unrealistic to expect a single mechanism for autoimmunity. Molecular mimicry or other forms of antigenic cross-reactivity; viral or toxic release of sequestered antigens; cytokine-induced MHC upregulation; genetically imposed hyperinducibility of B cells; Ir gene-dependent failures of T repertoire purging or pathogen elimination; unexpected antigen processing rendering self cellular constituents immunogenic; reversal of anergy; and many other factors will conspire in different situations. While we continue the search for general rules, we must also examine each particular autoimmune situation.

Despite our incomplete knowledge, the potential for immunointervention is creeping closer, although still dependent on a good deal of clinical empiricism as well. That ought not to deter us, because it is the same in virtually all cases of medical progress. We have done fairly well in immunology in keeping basic scientists and clinical scientists engaged in conversation. This becomes more difficult as the field grows wider. Tolerance is one area in which we must keep trying, as it clearly holds the key to transplantation, to autoimmunity, and perhaps to allergy and many other branches of immunopathology.

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Cell Surface Structures on Human Basophils and Mast Cells: Biochemical and Functional Characterization

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I. Introduction

Mast cells and basophils are specialized effector cells of the immune system. Both cells express high-affinity immunoglobulin E (IgE) binding sites and supposedly play an important role in host defense mechanisms and allergic reactions. Mast cells and basophils synthesize and store large amounts of pro-inflammatory mediator molecules, and on activation, these mediators may be released to the extracellular space.

Mast cells and basophils originate from uncommitted hemopoietic stem cells. Committed precursor cells giving rise to either basophils or mast cells, however, are nonidentical cells responsive to different growth factors. In contrast to blood basophils, mast cells are located primarily in extravascular areas. Basophils usually are intravascular, circulating cells but may emigrate into tissues on activation. A number of intrinsic, cellular, and external factors are involved in the control of growth, distribution, and function of basophil granulocytes and or mast cells.

Recent progress has led to the characterization of cell surface membrane structures expressed on human mast cells and human basophils. By the use of monoclonal antibodies (mAbs) to leukocyte differentiation antigens, the cell surface membrane antigen profile has been defined for both types of cells (deBoer and Roos, 1986; Bodger and Newton, 1987; Stain *et al.*, 1987; Bochner *et al.*, 1989b; Valent *et al.*, 1989b; Guo *et al.*, 1992). Mast cells and basophils clearly are different cells in terms of their immunological phenotype. Molecular and functional analyses of cell membrane antigens expressed on human blood basophils and human mast cells have also been performed. Thus, a number of cell surface receptors for basophil/mast cell growth factors and activating polypeptides, complement binding sites, receptors for immunoglobulins, recognition molecules, cell surface enzymes, and glycolipids have been identified. Many of these surface membrane antigens apparently play a very important role in inflammatory and allergic disease states involving blood basophils and tissue mast cells. This article gives an overview of cell surface membrane structures

expressed on human mast cells and human basophils, with special respect to similarities of and differences between these cells. Wherever possible, the respective situations in other species are discussed.

Before going into detail, we should make some (critical) comments on the material(s) and methods applied. For *in vitro* analysis of human mast cells or human basophils, primary cells or cell lines may be used. Primary cells may be obtained from the peripheral blood (basophils) or from dispersed tissues (mast cells), but the percentage of metachromatic cells in primary cell suspensions usually is very low. Subsequent purification techniques include gradient sedimentation/centrifugation and elution. Ultrapure preparations may be obtained by positive or negative selection techniques using mAbs. Several "technical problems" have to be taken into account when using such selection techniques. First, the cells may constitute a heterogeneous population in terms of cellular density (Leonard and Skeel, 1985; Morita *et al.*, 1989) or in terms of expression of cellular antigens. Thus, the possibility of loss of a subpopulation of cells exists. When basophils or mast cells are enriched by using a positive selection technique the effect of the ligand (often IgE) on (the function of) the respective cells has to be considered. Larger amounts of primary human basophils have been obtained from chronic myeloid leukemia (CML) patients (typically shortly before or during blastic transformation); however, these cells are clearly not normal, but are driven by the abl proto-oncogene. When analyzing tissue mast cells the purification procedure often includes exposure to enzymes (including proteases). The (possible) effect of such enzymes on mast cell surface (protein) structures should be considered. Mast cell heterogeneity (Irani *et al.*, 1986; Schwartz, 1989; Y. Kitamura, 1989; Galli, 1990) should also be taken into account.

Other problems may arise from limitations of the detection assays or reagents (mAbs) used. A negative result as assessed by mAbs and flow cytometry (done in most cases) does not exclude expression of very small numbers of the antigen, expression of alternative epitopes, or expression of masked antigen. Some of these difficulties can be resolved by the use of alternative (more sensitive) techniques and reagents and by the use of a panel of mAbs directed against a number of different epitopes. When the antigen (such as basophil CD15) is masked by sialic acid, sialidase may be used to "reexpose" this antigen (Stain *et al.*, 1987). Detection of specific mRNA in respective cells (in addition to surface labeling) as well as metabolic labeling experiments should provide useful information as to whether cells are capable of producing the antigen in question; however, such studies do not give

satisfactory answers as to whether the molecule is expressed on the cell surface in a functionally active form. *In situ* staining techniques may be useful for the confirmation of surface expression of antigens on tissue mast cells in vivo; however passive adsorption of the antigen as well as expression on neighboring cells must be considered.

Recently, human cell lines with characteristics of mast cells and basophils have been established. KU-812 cells were raised from a patient suffering from CML and exhibit many basophil properties (Kishi, 1985; Valent *et al.*, 1990a). In fact, the KU-812 progenitor represents a multipotent precursor cell and KU-812 cells express multilineage properties under a variety of conditions (Fukuda *et al.*, 1987). HL-60 cells (myeloid precursor, uncommitted) may express characteristics of basophils on appropriate induction with sodium butyrate following alkaline passage (Hutt-Taylor *et al.*, 1988; Denburg, 1992); however, a stable subclone of basophil-like HL-60 cells has not been established yet. The HMC-1 cell line was raised from a patient suffering from mast cell leukemia and clearly is associated with the mast cell lineage (Butterfield *et al.*, 1988; Valent *et al.*, 1990b). Yet, HMC-1 cells do not express all mast cell antigens; HMC-1 cells lack cell surface FcεRI α chain (FcεRI is the mast cell/basophil receptor for IgE; see Section VIII). The HBM-M cell strain has been grown from a patient suffering from diffuse cutaneous mastocytosis (Krilis *et al.*, 1991). HBM-M cells exhibit a number of mast cell antigens, but attempts to establish a stable subclone have been unsuccessful so far.

II. The CD Nomenclature: Cell Surface Typing with Monoclonal Antibodies—Immunophenotypic Characterization of Human Mast Cells and Basophils

The CD system (and nomenclature) of cellular antigens defined by groups of mAbs has widely been used for the immunological classification/characterization of human hemopoietic cells. A regular update of cluster (CD) formation has been achieved by leukocyte typing workshops and conferences (for a recent update, see Knapp *et al.*, 1989). Human basophils and human mast cells have also been analyzed by cell typing with mAbs. As defined by their CD phenotype, mast cells and basophils clearly are different cells. Table I gives an overview of CD antigens expressed on human blood basophils and on human tissue mast cells as determined by mAbs and immunostaining. Results obtained with immortal human mast cell (HMC-1) and basophil (KU-812) precursor cells are also given in Table I.

TABLE I
EXPRESSION OF CD ANTIGENS ON HUMAN BASOPHILS AND MAST CELLS AS DETERMINED BY MONOCLONAL ANTIBODIES AND INDIRECT IMMUNOFLUORESCENCE

CD	Structure	Basophils	KU-812	Mast cells	HMC-1
01a/b/c	gp49/45/43[a]	–	–	–	–
02	LFA-2	–	nk	–	+
03	TcR/T3	–	–	–	–
04	CL-II/HIV R	(–)	–	–	–
05	gp67/CD72 R	–	–	–	–
06	gp100	–	–	–	–
07	gp40	–	–	–	–
08	CL-I R	–	–	–	–
09	p24	+	+	+	+
10	Endopeptidase	–	nk	–	nk
11a	LFA-1	+	+	–	–
11b	Mac-1/C3bi R	+	+	–	–
11c	p150/95	+	+	(–)	–
13	Aminopeptidase N	+	+	–	(+)
14	LPS-R related	–	–	–	–
15	3-FAL	(–)	(–)	–	–
16	FcγRIII	–	–	–	–
w17	Lactoceramid	+	+	–	nk
18	β to CD11	+	+	(–)	–
19	p95	–	–	–	–
20	gp32/37	–	–	–	–
21	C3d R/EBV R	–	–	–	–
22	N-CAM related	–	–	–	–
23	FcεRII	–	nk	–	nk
24	gp42	–	nk	–	–
25	IL-2R/Tac	+	+	(–)	nk
26	Dipeptidase	+	nk	–	–
27	p55 dimer	nk	nk	nk	nk
28	gp44	–	–	–	–
29	VLA-β	+	+	+	+
30	gp120, Ki-1	–	nk	nk	–
31	gp140	+	nk	–	–
w32	FcγRII	+	+	(–)	nk
33	gp67	+	+	+	+
34	HPCA-1	–	nk	–	–
35	CR1	+	+	–	–
36	TSP R	–	nk	–	–
37	gp40-52	+	nk	–	nk
38	T10	+	nk	–	nk
39	gp80	+	nk	–	nk
40	NGF R homolog	+	+	–	nk
41	gpIIb/IIIa	–	nk	–	–
42a/b	gpIX/Ib	–	nk	–	nk
43	Leukosialin	+	+	+	+

(*continued*)

TABLE I *Continued*

CD	Structure	Basophils	KU-812	Mast cells	HMC-1
44	Pgp-1	+	+	+	+
45	LCA	+	+	+	+
45RA,RB	45 restricted	nk	nk	nk	nk
45RO	45 restricted	nk	nk	nk	nk
46	MCP	nk	nk	nk	nk
47	gp47-52	nk	nk	nk	nk
48	gp41	nk	nk	nk	nk
49b	VLA-2	nk	+	−	+/−
49d	VLA-4	+	+	+	+
49e	VLA-5	+	+	+	+
49f	VLA-6	−	−	−	−
50	gp180/108	nk	nk	nk	nk
51	VNR-α	nk	+	+	+
52	gp21-28	nk	nk	nk	nk
53	gp32-40	nk	nk	nk	nk
54	ICAM-1	+	+	+	+
55	DAF	nk	nk	nk	nk
56	NKH-1	−	nk	−	−
57	HNK-1	−	nk	−	−
58	LFA-3	nk	+	+	+
59	gp18-20	nk	nk	nk	nk
w60	NeuAc(2)-Gal	nk	nk	nk	nk
61	VNR-β, β3	nk	+	+	+
62	PADGEM, gp140	nk	nk	nk	nk
63	gp53	+	nk	nk	nk
64	FcγRI	−	nk	−	−
w65	Ceram-dodecasaccharide	(+)	nk	−	−
66	gp180–200	nk	nk	−	nk
67	p100	nk	nk	nk	−
68	gp110	−	−	(−)	(−)
69	gp32/28	nk	nk	nk	nk
w70	Ki24	nk	nk	nk	nk
71	Transferrin R	−	nk	−	nk
72	gp43/39	nk	nk	nk	nk
73	ecto 5-NT	nk	nk	nk	nk
74	CL-II invariant chain	nk	nk	nk	nk
w75	gp53	nk	nk	nk	nk
77	globo-ceramide	nk	nk	nk	nk
78	gp67	nk	nk	nk	nk

[a] gp, glycoprotein; R, receptor; CR, complement receptor; CL, MHC class (I, II); LFA, Leukocyte function-associated antigen; NGF, nerve growth factor; VNR, vitronectin receptor; TSP, thrombospondin; EBV, Epstein–Barr virus; VLA, very late antigen; ( ) expression seen in certain circumstances or in cytoplasm exclusively; nk, not known.

Mast cells and basophils express the "pan-leukocyte antigen" (CD45), also termed the leukocyte common antigen (LCA) (Reshef and MacGlashan, 1987; Stain *et al.*, 1987; Scheck *et al.*, 1987; Horny *et al.*, 1989; Bodger and Newton, 1987; Valent *et al.*, 1989b), a 180- to 220-kDa transmembrane glycoprotein with tyrosine phosphatase activity. Expression of LCA on human mast cells is consistent with the well-established concept that mast cells belong to the hemopoietic system (Y. Kitamura *et al.*, 1978, 1981; Y. Kitamura and Go, 1979). Human blood basophils express a number of myeloid cell surface (CD) antigens, likewise expressed in neutrophilic or eosinophilic blood granulocytes (Stain *et al.*, 1987). In contrast, human mast cells expose only a few of these molecules (Valent *et al.*, 1989b). Myeloid cell surface antigens detectable on human basophils but not on human mast cells include CD11b (LFA-1 α chain), CD13 (aminopeptidase-N), CD17 (lactosyl-ceramide), CD31 (gpIIa), and CD35 (CR1). Remarkably, basophilic and eosinophilic granulocytes share many immunophenotypic properties (Stain *et al.*, 1987); however, basophils differ from eosinophilic granulocytes in the constitutive expression of CD25 (IL-2 receptor α chain/Tac) and CD38 (T10 antigen). Strong expression of CD9 (p24) and CD17 is characteristic of basophils. Human mast cells also express CD9 but not CD38. Interestingly, human mast cells express some antigens otherwise expressed in macrophages including CD68 (cytoplasmic marker, not expressed on the mast cell surface) (Horny *et al.*, 1990) and as yet not clustered antigens defined by mAbs of the MAX series (Valent *et al.*, 1989b). Human mast cells and human basophils express CD43 (leukosialin), CD44 (Pgp-1), and CD54 (ICAM-1) (Valent *et al.*, 1990a,c; Bochner *et al.*, 1990). So far no major immunophenotypic differences among mast cells derived from different organs have been recognized (Valent *et al.*,1989b). Lymphoid membrane determinants (such as CD3, CD5, CD7, CD19, CD22) are not expressed on either human mast cells or human basophils.

Thus by using CD-specific mAbs, mast cells and basophils can clearly be distinguished from all other hemopoietic cells. Based on this approach mast cells and blood basophils could also be purified (from all contaminating cells) to homogeneity by using mAbs and complement (Stain *et al.*, 1987; Valent *et al.*, 1989b). CD antigens specific for either human mast cells or human basophils have not been defined so far. The YB5.B8 antigen (= c-kit = stem cell factor/SCF receptor) is associated with the mast cell lineage (Mayrhofer *et al.*, 1987; Valent *et al.*, 1989b) but is not exclusively expressed on mast cells (see later). Blood basophils do not express substantial amounts of YB5.B8 antigen. The Bsp-1 antigen (structure so far not defined) is almost exclusively

expressed on human basophils but is not expressed on human mast cells (Bodger *et al.*, 1987; Valent *et al.*, 1990c).

Basophil activation antigens have recently been identified. Activated basophils apparently express increased amounts of CD11b (Bochner *et al.*, 1989a, 1990) as well as of CD63 (Knol *et al.*, 1991) compared with resting cells. CD45 mAb may inhibit IgE-dependent release of histamine from human basophils (Hook *et al.*, 1991). Mast cell activation antigens have not been defined so far.

No consistent differences between normal human blood basophils and leukemic CML basophils in terms of their immunologic (CD) phenotype have been recognized (Stain *et al.*, 1987). CD antigens expressed on malignant cells derived from mast cell leukemia patients have not been studied extensively. In one case the malignant mast cells (in contrast to normal mast cells) were found to bind CD2 mAb (Dalton *et al.*, 1986). HMC-1 cells bind CD2 mAbs as well (Valent *et al.*, 1991), whereas HBM-M cells do not (Krilis *et al.*, 1991).

III. Receptors for Growth and Differentiation Factors

Growth and differentiation of hemopoietic cells is regulated by a number of polypeptides, mostly termed *cytokines*. Basophils and mast cells belong to the hemopoietic system and are derived from hemopoietic precursor cells giving rise to lineage-restricted progenitors. Multipotent hemopoietic precursor cells forming colonies in semisolid medium [colony-forming units (CFU)] and giving rise to metachromatic cells are located in the bone marrow, but also circulate in the peripheral bloodstream and can be found in extramedullary organs (Denburg *et al.*, 1983, 1992; Metcalfe *et al.*, 1981; Galli *et al.*, 1984; Marshall and Bienenstock, 1990; Galli, 1990; Schwartz and Huff, 1991). In the murine system the multipotent progenitor cell (CFU-MIX) has the capacity to generate larger amounts of erythrocytes, megakaryocytes, granulocytes (including basophils and eosinophils), as well as mast cells in response to appropriate growth factors (Y. Kitamura *et al.*, 1981). In humans, CFU-MIX also have the potential to generate erythrocytes and granulocytes (including basophils), but in normal circumstances, have only a limited capacity to generate mast cells (Yuen *et al.*, 1988; Kirschenbaum *et al.*, 1989, 1991; Valent *et al.*, 1989b). A substantial proportion of the mature precommitted, myeloid progenitor cells have the potential to give rise to basophilic and eosinophilic granulocytes (CFU eo/baso) (Leary and Ogawa, 1984; Denburg *et al.*, 1985b). The late-stage progenitors giving rise to either (human) mast cells or basophils clearly are distinct cells. The mast cell-committed progenitor cell

has recently been characterized in the murine system (Jarboe and Huff, 1989; Jarboe *et al.*, 1989; R. I. Ashman *et al.*, 1991) and more recently in the human system (Valent *et al.*, 1992). This cell appears to be a nongranulated, mononuclear cell sharing cellular properties with monocytic rather than lymphoid cells. Polypeptide cytokines controlling differentiation and function of basophilic myelocytes [interleukin-3 (IL-3), IL-5, granulocyte/macrophage colony-stimulating factor (GM-CSF)] or differentiation of mast cells [stem cell factor (SCF)] from their progenitor cells in vitro have recently been identified. At least in the human system a remarkable overlap exists between eosinophilic and basophilic granulocytes in their response to hemopoietic growth factors and expression of cytokine binding sites. In contrast, human basophils clearly differ from mast cells in this respect (i.e., response to growth factors). A number of observations suggest that basophil/eosinophil or mast cell progenitor cells and respective growth/differentiation factors are increasingly produced in inflammatory states and may accumulate in areas of local inflammation (Denburg *et al.*, 1985a, 1992; Otsuka *et al.*, 1987; Kay *et al.*, 1991). Recently, according to cDNA sequences, cytokine receptor supergene families have been defined. We refer to this novel classification in the next section(s).

A. Hemopoietic Growth Factor Receptor Superfamily: HRS

A group of hemopoietic growth factors (HGFs), including IL-3, IL-5, and GM-CSF, known to regulate differentiation and function of human basophils (and eosinophils, too) share some striking homologies in their molecular biology. These cytokines are encoded by genes located in a cluster on the long arm of human chromosome 5 (van Leuwen *et al.*, 1989). Moreover, these HGFs may be produced and released as a cluster of gene products during (antigen-induced) activation of (murine) mast cells (Plaut *et al.*, 1989; Wodnar-Filipowicz *et al.*, 1989; Burd *et al.*, 1989; Gordon *et al.*, 1990) or T cells (Niemeyer *et al.*, 1989; C. B. Thompson *et al.*, 1989) and they may appear *in vivo* as a cluster of cytokines, for example, in atopic skin during the late-phase cutaneous reaction (Kay *et al.*, 1991).

Receptors for HGFs (including basophil differentiation factors) belong to a novel receptor superfamily. This novel family, termed *hemopoietin receptor superfamily* (*HRS*), has been defined, on the basis of homologies in HGF binding domains (Bazan, 1989, 1990). The HRS includes binding sites for IL-3 (Itoh *et al.*, 1990; T. Kitamura *et al.*, 1991), IL-4 (Idzerda *et al.*, 1990) IL-5 (Tavernier *et al.*, 1991), GM-CSF (Gearing *et al.*, 1989), IL-2 (Hatakayama *et al.*, 1989), IL-6 (Yamasaky

et al., 1988), IL-7 (Goodwin *et al.*, 1990), erythropoietin (EPO) (D'Andrea *et al.*, 1989), G-CSF (Fukunaga *et al.*, 1990), prolactin (PRL) (Boutin *et al.*, 1988), and growth hormone (GRH) (Leung *et al.*, 1987).

The high-affinity HGF receptors usually are composed of (at least) two subunits, termed α and β *chains*, respectively (Tavernier *et al.*, 1991; T. Kitamura *et al.*, 1991; Nicola and Metcalf, 1991). When expressed alone, each subunit may also display binding capacity for the natural ligand; however, the affinity usually decreases compared with the multichain receptor complex. More recent studies have revealed that (at least) the human receptors for the basophil differentiation factors IL-3, IL-5, and GM-CSF share an identical β chain (Tavernier *et al.*, 1991; T. Kitamura *et al.*, 1991).

Human blood basophils express high-affinity receptors for IL-3 (Valent *et al.*, 1989c; Lopez *et al.*, 1990a), IL-4 (Valent *et al.*, 1990d), IL-5 (Lopez *et al.*, 1990a, 1992), and probably GM-CSF (Lopez *et al.*, 1990a) (Table II). Basophils also express low-affinity (but not high-

TABLE II
CYTOKINE RECEPTORS EXPRESSED ON HUMAN BASOPHILS, HUMAN (LUNG) MAST CELLS, KU-812 CELLS, AND HMC-1 CELLS

Cytokine	Receptor expression on			
	Basophils	KU-812	Lung mast cells	HMC-1
IL-1[a]	nk	nk	nk	nk
IL-2	+	+	(−)	nk
IL-3	+	+	−	−
IL-4	+	+	nk	+
IL-5	+	nk	nk	nk
IL-6	nk	nk	nk	nk
IL-7	nk	nk	nk	nk
IL-8	+	+	nk	+
IL-9	nk	nk	nk	nk
IL-10	nk	nk	nk	nk
GM-CSF	+/−	+	nk	nk
G-CSF	nk	nk	nk	nk
M-CSF/CSF-1	(−)	(−)	nk	(−)
SCF/MGF	(−)	(+/−)	(+)	(+)
NGF	(+)	nk	nk	nk
IFN-α	(+)	(+)	nk	nk
IFN-γ	+	+	nk	nk
TGF-β	(+)	(+)	nk	nk
TNF	nk	nk	nk	nk

[a] IL, interleukin; G, granulocyte; M, macrophage; CSF, colony-stimulating factor; SCF, stem cell factor; MGF, mast cell growth factor; NGF, nerve growth factor; IFN, interferon; TGF, transforming growth factor; TNF, tumor necrosis factor; nk, not known; ( ), no quantitative binding data available.

affinity) binding sites for IL-2 (Stockinger *et al.*, 1990). At present we do not know whether human blood basophils express receptors for IL-6 (a pleiotropic growth regulator), IL-7 (a stroma cell-derived growth factor), IL-9 (a putative growth factor for murine mast cells) (Moeller *et al.*, 1990), IL-10 [another growth factor for murine mast cells (Thompson-Snippers *et al.*, 1991)], or G-CSF (a neutrophil growth factor). The HGF receptor profile for human mast cells has not been established so far. A number of observations as well as the lack of response on agonist activation suggest that human mast cells lack many HGF receptors expressed on human basophils.

1. Interleukin-2 Receptor

Receptors for IL-2 are expressed on various leukocytes. The high-affinity receptor complex is composed of at least two subunits with molecular masses of 55–60 and 70–75 kDa, respectively (Sharon *et al.*, 1986; Dukovich *et al.*, 1987; Tsudo *et al.*, 1987). Each subunit may be expressed alone; however, the affinity for IL-2 is low (55- to 60-kDa subunit) or intermediate (70- to 75-kDa subunit) compared with the "complete" receptor. The 55- to 60-kDa subunit (also termed α chain) is identical to the Tac peptide and is recognized by CD25 mAbs. The 70- to 75-kDa subunit (also termed β chain) is a member of the hemopoietin receptor superfamily (Hatakayama *et al.*, 1989).

Human blood basophils were first described as binding CD25 mAbs by Stain *et al.* in 1987 (Table I). Biochemical characterization of the basophil receptor for IL-2 has been achieved by using highly enriched populations of human blood basophils (Stockinger *et al.*, 1990; Maggiano *et al.*, 1990). Immunoprecipitation experiments (using CD25 mAbs) suggest that human basophils express the 55- to 60-kDa subunit of the IL-2 receptor (= Tac peptide) (Stockinger *et al.*, 1990). Northern blot analyses on pure basophils showed two mRNA bands (3.5 and 1.5 kb) corresponding to Tac mRNA species expressed in T cells (Maggiano *et al.*, 1990; Stockinger *et al.*, 1990). As determined by metabolic labeling experiments, basophils actively synthesize the Tac peptide. Human (CML) basophils specifically bind ^{125}I-labeled recombinant IL-2 (Stockinger *et al.*, 1990). As determined by Scatchard transformation, CML basophils express a single class of 10,000–15,000 IL-2 binding sites per cell, with a calculated dissociation constant of 66×10^{-9} *M*. Human basophils lack detectable amounts of high-affinity IL-2 binding sites. In contrast to other granulocytes, blood basophils apparently express the Tac peptide in a constitutive manner (Stain *et al.*, 1987; Stockinger *et al.*, 1990). CML basophils also synthesize large amounts of CD25 antigen in culture and may even display

increased amounts of surface CD25 after several days in culture, compared with freshly isolated cells (Stockinger *et al.*, 1990).

Human tissue mast cells have also been tested for their ability to bind CD25 mAbs; however, controversial results have been obtained by utilizing different techniques and reagents. With immunohistochemical techniques using mAb 1HT4-4H3, human lung and intestinal mast cells (analyzed in tissue sections) were found to display Tac antigen on their cell surface (Maggiano *et al.*, 1990). In contrast, dispersed mast cells obtained from various tissues (lung, skin, intestine, ascites, uterus) by collagenase digestion were found to lack detectable amounts of CD25 antigen as determined by combined toluidine blue/immunofluorescence staining (Valent *et al.*, 1989b). These differences may arise (apart from the different techniques) because mast cells express extremely small numbers of IL-2 binding sites. Alternatively, CD25-reactive material was passively adsorbed rather than synthesized in tissue mast cells analyzed by immunohistochemistry. Another possibility would be that mast cells express IL-2 receptors under distinct conditions (or distinct receptor epitopes) exclusively.

IL-2 was first described as a T cell growth factor. Later, it was found that IL-2 in fact exerts effects on various immune cells. So far, however, no significant effect of IL-2 on either growth or function (histamine release, adherence, surface marker expression) of human basophils or mast cells has been observed (even when IL-2 was tested in very high concentrations, i.e., up to 10^{-5} *M*). This is probably due to the fact that (resting) basophils express (only) the Tac peptide (incapable of signaling) and not the multichain IL-2 receptor complex. The conditions under which human basophils would express high-affinity IL-2 binding sites and would respond to IL-2 constitute the subject of ongoing investigations.

2. *Interleukin-3 Receptor*

Interleukin-3 is produced by activated immune cells, in particular by T cells (Niemeyer *et al.*, 1989; Guba *et al.*, 1989; C. B. Thompson *et al.*, 1989), natural killer (NK) cells (Cuturi *et al.*, 1989), and (murine) mast cells (Plaut *et al.*, 1989; Wodnar-Filipowicz *et al.*, 1989; Burd *et al.*, 1989; Gordon *et al.*, 1990). IL-3 is a multipotent growth factor and exerts its effects via specific cell surface receptors.

Studies using radiolabeled IL-3 and mAbs suggest a multichain receptor complex and (at least) two classes of IL-3 binding sites on murine cells with higher and lower affinity for the natural ligand (Park *et al.*, 1986; Nicola and Peterson, 1986; Sugawara *et al.*, 1988; Sorenson *et al.*, 1989; Schreurs *et al.*, 1989, 1990; Yonehara *et al.*, 1990). A

complementary DNA (cDNA) for a subunit of the murine receptor for IL-3 was cloned by Itoh *et al.* 1990, and revealed a novel member of the HRS. More recently, a cDNA for the human receptor has been isolated and characterized by expression cloning using the β chain of the GM-CSF receptor (T. Kitamura *et al.*, 1991). The α chain is specific for IL-3. The β chain of the human receptor for IL-3 is identical to the β chain of the GM-CSF and IL-5 receptors and apparently is involved in signal transmission (T. Kitamura *et al.*, 1991; Tavernier *et al.*, 1991; Lopez *et al.*, 1992). Receptors for IL-3 are expressed on hemopoietic precursor cells (Park *et al.*, 1989; Budel *et al.*, 1989), blood monocytes (Elliot *et al.*, 1989; Park *et al.*, 1989), and eosinophil granulocytes but not on blood neutrophils (Lopez *et al.*, 1989).

Recently, human blood basophils have been tested for their ability to bind IL-3. In our own experiments basophils of two CML donors were purified to homogeneity (>95% purity) from mononuclear cell (MNC) samples by negative selection technique using mAbs and complement (Valent *et al.*, 1989c). Binding studies using ^{125}I-labeled recombinant human IL-3 (rhIL-3) revealed a single class of 500 and 2100 high-affinity IL-3 binding sites with calculated dissociation constants of 23 and 16 $\times$ 10^{-11} M, respectively. In a similar study conducted by Lopez *et al.* (1990a,b), one CML donor was tested. CML basophils were enriched by dextran sedimentation and subsequent centrifugation using metrizamide. Basophils expressed a single class of 840 IL-3 binding sites with a K_D of 2.6 $\times$ 10^{-11} M. Similar to primary CML blood basophils, KU-812 cells expressed a single class of high-affinity IL-3 binding sites (Valent *et al.*, 1990a,b) (Table II).

Binding of IL-3 to primary CML basophils is not inhibitable by addition of rhIL-5, rhGM-CSF, rhG-CSF, rhIL-1, or recombinant human tumor necrosis factor (rhTNF) (Lopez *et al.*, 1990a,b); however, the binding of both IL-5 and GM-CSF to human basophils is inhibitable by rhIL-3 (Lopez *et al.*, 1990a). The cross-competition between cytokines (IL-3, IL-5, and GM-CSF) on human basophils may occur because of the common (β) subunit of the receptors (see earlier text). The exact underlying mechanism of the affinity hierarchy remains unknown but may be the result of the different numbers of α chains expressed on human basophils (IL-3, n = 500–2000; IL-5, n = 800; GM-CSF, n = < 200 sites per cell). Interestingly, a similar hierarchy has been observed in the functional response of the basophils to the above cytokines (see later).

Accumulating evidence exists that IL-3 is a differentiation factor for human basophils. *In vitro* studies using short-term (14- to 21-day) suspension cultures have shown that rhIL-3 between 10^{-7} and

10^{-11} *M* induces differentiation of human basophils (and eosinophils) from their cord blood (Saito *et al.*, 1988) and bone marrow (Valent *et al.*, 1989a; Kirschenbaum *et al.*, 1989) progenitors. GM-CSF and IL-5, although far less potent compared with IL-3 (and not active in all donors), may also induce formation of basophils in these assays in certain circumstances, whereas other cytokines [IL-1, IL-2, IL-4, IL-6, IL-7, IL-8, G-CSF, M-CSF, interferons (IFNs), TNF] showed no effect. IL-3 also has the capacity to promote basophilic differentiation in human cell lines including KU-812 (Valent *et al.*, 1990a) and HL-60 (Denburg *et al.*, 1989). Interestingly, whereas IL-3 was found to induce formation of large numbers of basophils from bone marrow progenitor cells, IL-3 showed only a weak to moderate effect on peripheral blood basophil progenitors (Otsuka *et al.*, 1988; Denburg *et al.*, 1991). IL-3-dependent differentiation of myelocytes toward the basophil pathway is associated with expression of cellular histamine and synthesis and formation of FcεRI molecules (Saito *et al.*, 1988; Valent *et al.*, 1989a,d; H. L. Thompson *et al.*, 1990). *In vivo*, IL-3 may also induce differentiation of (i.e., elevations in) blood basophils. When administered to healthy rhesus monkeys, rhIL-3 induced a dose-dependent increase in the absolute and relative numbers of peripheral blood basophils (Donahue *et al.*, 1988; Mayer *et al.*, 1989; Dvorak *et al.*, 1989; Volc-Platzer *et al.*, 1990). Similarly, in cancer patients up to 10-fold elevations of blood basophils have been observed on administration of high (500 $\mu g/m^2$ per day) but not low doses of IL-3 (Merget *et al.*, 1990).

The signal transduction events in (murine) myeloid progenitor cells following stimulation with IL-3 have recently been reviewed in detail (Whetton and Dexter, 1988). The IL-3-induced differentiation of hemopoietic cells apparently is associated with activation of protein kinases such as c-raf (a cytosolic serine/threonine kinase) (Carroll *et al.*, 1990), protein kinase C (Whetton *et al.*, 1988; Chaikin *et al.*, 1990), and tyrosine kinases (Linnekin and Farrar, 1990). Specific signal transduction events involved in IL-3-induced progenitor cell differentiation toward the basophil (human) or mast cell (murine) pathway are poorly defined. IL-3 apparently induces *de novo* synthesis of histidine decarboxylase (and ornithine decarboxylase) in (murine) hemopoietic progenitor cells (Schneider *et al.*, 1987; Dy *et al.*, 1988).

IL-3 is a potent activator of mature human blood basophils. Between 10^{-9} and 10^{-12} *M*, IL-3 upregulates the capacity of human blood basophils to release histamine (as well as other mediators including sulfido-leukotrienes) on stimulation with complete agonists (substances requiring no second signal for inducing the release reaction)

such as anti-IgE, C5a, or calcium ionophore (Hirai *et al.*, 1988; Valent *et al.*, 1989c; Kurimoto *et al.*, 1989; Schleimer *et al.*, 1989). IL-3 (in the same concentration range) is also able to be a second (degranulation-inducing) signal for incomplete basophil agonists such as IL-8 (Dahinden *et al.*, 1989a,b), C3a (Bischoff *et al.*, 1990b) or platelet-activating factor (PAF) (Brunner *et al.*, 1991). Moreover, rhIL-3 has been described to counteract the glucocorticoid-, cyclosporin A-, and FK-506-induced downregulation of the releasability in human basophils (Schleimer *et al.*, 1989; dePaulis *et al.*, 1991a).

IL-3 per se is ineffective in inducing release of histamine from basophils in most normal donors; however, in a small and selective subset of allergic individuals [about 5–30% of atopics, apparently defined by IgE^+ (MacDonald *et al.*, 1989)], rhIL-3 has been reported to induce histamine release from human blood basophils (Haak-Frendscho *et al.*, 1988b; MacDonald *et al.*, 1989; Alam *et al.*, 1989). In most of the responsive atopics, the effect of IL-3 was found at higher concentrations of the agonist and the effect of rhIL-3 per se was weak compared with the effect of other mononuclear cell-derived histamine-releasing factors (HRFs) (Alam *et al.*, 1989). *In vivo* administration of rhIL-3 to tumor patients is associated with a dose-dependent increase in anti-IgE-induced release of histamine from the patients' blood basophils (Merget *et al.*, 1990). In contrast, no direct effect of IL-3 on basophil secretion content was observed in those patients, and even in the higher concentration range (up to 500 $\mu g/m^2$ per day) no (severe) allergic symptoms were noted (Merget *et al.*, 1990).

Direct release of histamine (from basophils obtained from atopics) induced by IL-3 is calcium dependent (Schleimer *et al.*, 1989), whereas the IL-3-dependent increase in basophil releasability does not require extracellular calcium (Kurimoto *et al.*, 1991). Moreover, C5a-induced release of histamine (usually being calcium dependent) becomes partially calcium independent after preactivation of the basophils with rhIL-3 (Kurimoto *et al.*, 1991).

The IL-3-dependent increase in basophil releasability lasts at least 24 hours when normal cells are kept in appropriate culture (MacDonald *et al.*, 1989; Schleimer *et al.*, 1989). Similar observations have been made with highly enriched CML basophils. On the other hand, pure CML basophils gradually lose their capacity to respond to IgE plus anti-IgE when cultured in control medium; however, when these unresponsive basophils (after 4 days in control culture) are (again) exposed to rhIL-3 they "reacquire" their capacity to respond to an allergen (after sensitization with specific IgE) within 30 minutes (see Fig. 1).

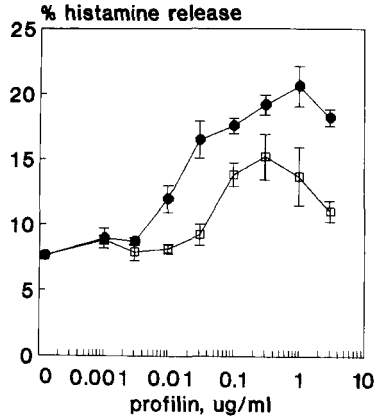

FIG. 1. Releasability in pure chronic myeloid leukemia (CML) basophils: dependence on interleukin-3. Basophils were isolated from a CML donor and purified to homogeneity (>95% purity) by monoclonal antibodies (mAbs) and complement (Stain *et al.*, 1987; Valent *et al.*, 1989b). Pure basophils were kept in culture in RPMI 1640 medium supplemented with 10% fetal calf serum, in a humidified atmosphere (5% CO_2, 37°C) for 4 days. Thereafter, cells were washed in buffer and exposed to allergen-specific immunoglobulin E (IgE) (obtained from an allergic patient) for 3 hours and to recombinant human IL-3 (rhIL-3) (100 U/ml) or control medium for an additional 30 minutes. Then, cells were again washed and exposed to various concentrations (as indicated) of allergen (i.e., recombinant profilin, □, and natural profilin, ●) at 37°C for 30 minutes. Cells were centrifuged and the amount of histamine released was measured in cell-free supernatants. Dose-dependent release on allergen challenge was observed after addition of IL-3. Control cells (not exposed to rhIL-3) could not be triggered for histamine release; that is, maximum release was below the baseline level of 8% (not shown). Recombinant profilin and natural profilin were kindly provided by Dr. Rudolf Valenta (Institute of General and Experimental Pathology, University of Vienna, Vienna, Austria).

Little is known so far about the signal transduction events involved in IL-3-dependent activation of human basophils. Studies on KU-812 cells suggest that IL-3 (between 10^{-9} and 10^{-12} *M*), but not IL-2, induces a rapid decrease in cellular cyclic AMP (cAMP) levels, with a maximum decline observed after 30 minutes and a slow phase of recovery to baseline levels during the following 24 hours (Virgolini *et al.*, 1992). Moreover, at higher concentrations, IL-3 but not IL-2 is capable of neutralizing the prostaglandin E_1 (PGE_1)-induced increase in cAMP in KU-812 cells.

A number of observations suggest that IL-3 is involved in the regulation of basophil migration and adherence to vascular endothelial cells. IL-3 (between 10^{-9} and 10^{-12} *M*) has been described as inducing the

in vitro chemotactic migration of highly enriched human (CML) blood basophils as well as the migratory response of the basophils to a second chemotaxin, such as IL-8 or C5a (Rot *et al.*, 1992). IL-3 also promotes *in vitro* adherence of human blood basophils to vascular endothelial cells via induction of Mac-1 (Bochner *et al.*, 1989a, 1990).

Previous studies have shown that (murine) IL-3 is a growth and differentiation factor for murine mast cells (Ihle *et al.*, 1983; Razin *et al.*, 1984). IL-3 by itself is capable of inducing growth of immature mast (progenitor) cells in short-term murine bone marrow cell cultures (Sredni *et al.*, 1983; Ihle *et al.*, 1983; Razin *et al.*, 1984); however, terminal maturation of (IL-3-induced) murine mast cells apparently requires the presence of additional factors such as stem cell factor (SCF) (Tsai *et al.*, 1991a,b) or nerve growth factor (NGF) (Matsuda *et al.*, 1991). Other studies suggest that IL-3 modulates the functional response (including chemotaxis and adhesion) of the murine mast cells (Matsuura and Zetter, 1989; H. L. Thompson *et al.*, 1989a,b). In contrast, although tested extensively (Saito *et al.*, 1988; Ishizaka *et al.*, 1989; Valent *et al.*, 1990b), no direct effect of rhIL-3 on growth or function (i.e., histamine release, releasability, proliferation, mRNA expression, surface marker expression) of human mast cells has so far been described.

A number of experiments were conducted to test the capacity of human mast cells to bind IL-3. Primary human mast cells enriched from lung tissue (of two donors) by the use of collagenase digestion and subsequent exposure to mAbs and complement failed to bind ^{125}I-labeled rhIL-3 in a specific manner (Valent *et al.*, 1990a,b). In a subsequent set of experiments, immortal human mast cells (HMC-1 cells) were analyzed and compared with immortal basophils (KU-812 cells). Although KU-812 cells bound IL-3 in a specific manner, HMC-1 cells failed to do so (Valent *et al.*, 1990a,b). These observations suggest that human lung mast cells and HMC-1 cells lack substantial amounts of high-affinity IL-3 receptors (Table II). The failure to detect IL-3 binding sites on primary human lung mast cells may be the result of a loss of receptors during the (mast cell) purification procedure. Another possibility could be that human mast cells express extremely small numbers of IL-3 binding sites or IL-3 receptors with very low affinity for the natural ligand [as has been shown for the α chain of the human receptor for IL-3 (Kitamura *et al.*, 1991)] which were not detected by the receptor assay used. At present, we also do not know whether, by using the same technique, human skin mast cells or intestinal mast cells would express or lack IL-3 binding sites.

Murine mast cell lines have been described as expressing IL-3 binding sites on their cell surface (Park *et al.*, 1986). Unfortunately, so far,

no comparative studies of IL-3 binding sites using human and murine mast cells in parallel have been performed. A provocative hypothesis aimed at explaining differences between human mast cells and murine mast cells with respect to their response (i.e., progenitor cell differentiation) to IL-3 would be that mast cell (high affinity) IL-3 binding sites have been lost during evolution (Valent *et al.*, 1990b).

3. *Interleukin-4 Receptor*

IL-4 is a multifunctional cytokine involved in the control of differentiation and function of lymphocytes and the production of IgE. IL-4 is also involved in the regulation of growth and differentiation of myeloid cells and mast cells (for a review, see Paul and Ohara, 1987). IL-4 is produced by activated T cells as well as by (murine) mast cells and (murine) basophils on crosslinkage of cell surface IgE (Paul and Ohara, 1987; Seder *et al.*, 1991).

High-affinity IL-4 binding sites have been detected on almost all hemopoietic cells and even on nonhemopoietic cells (Park *et al.*, 1987; Ohara and Paul, 1987). Recently, a receptor for IL-4 has been cloned and found to belong to the HRS (Idzerda *et al.*, 1990). Binding studies of murine and, more recently, of human cells suggest that basophils as well as mast cells express IL-4 binding sites (Brown *et al.*, 1987; Ohara and Paul, 1987; Valent *et al.*, 1990d, 1991) (Table II). CML basophils express a single class of approximately 200–1000 high-affinity IL-4 binding sites with a dissociation constant K_D of $7–10 \times 10^{-11}$ M (Valent *et al.*, 1990d). Similar data have been obtained with the basophil cell line KU-812. The mast cell leukemia cell line HMC-1 expressed a single class of 1000–3000 high-affinity IL-4 binding sites with a K_D of $4–20 \times 10^{-11}$ M (Valent *et al.*, 1991). Whether normal primary human mast cells and basophils express IL-4 binding sites remains to be elucidated.

In the murine system, IL-4 promotes (clonal) growth and differentiation of (IL-3-dependent) mast cell progenitor cells (Hamaguchi *et al.*, 1987). In addition, IL-4 has been shown to induce proliferation of immortal murine mast cells (Mosmann *et al.*, 1986). In the human system, IL-4 (alone or in combination with IL-3) has so far not been described as promoting growth or differentiation of mast cell progenitor cells. Rather, IL-4 inhibits uptake of thymidine by HMC-1 cells and inhibits SCF-dependent formation of human mast cells in long-term cultures (Valent *et al.*, 1992). Reasons for the species discrepancies are not known but they may result from the different growth requirements (IL-3 versus SCF). Interestingly, IL-4 downregulates expression of SCF receptors in HMC-1 cells and in other human myeloid progeni-

tors (Sillaber *et al.*, 1991) but not in murine mast cell progenitors (Welham and Schrader, 1991). IL-4 may also be involved in the regulation of production of proinflammatory cytokines (IL-1β) and adhesion molecules (ICAM-1 antigen) expressed in human mast cells; however, IL-4 (in contrast to SCF) failed to induce or promote histamine release from human mast cells (P. Valent and P. Bettelheim, unpublished observation).

The biological function of the basophil receptor for IL-4 remains unclear. In one study IL-4 was described as promoting IL-3-dependent growth and differentiation of basophil-like cells in cord blood cell cultures (Favre *et al.*, 1990); however, this observation has so far not been confirmed for normal bone marrow progenitor cells or other progenitors. So far no effects of rhIL-4 on the functional properties (histamine release, adherence, chemotaxis, surface marker expression) of primary mature human basophils have been described.

4. Interleukin-5 Receptor

Interleukin-5 (IL-5) is another multifunctional member of the HGF family (Takatsu *et al.*, 1988). IL-5 is produced primarily by activated T cells and may be detected together with IL-3 and GM-CSF (and IL-4) in local areas of ongoing inflammation. IL-5 may also be detected in patients suffering from the hypereosinophilic syndrome. Previous *in vitro* studies have shown that IL-5 promotes differentiation and function of human eosinophils (Lopez *et al.*, 1988; Saito *et al.*, 1988; Clutterbuck *et al.*, 1989; Rothenberg *et al.*, 1989). More recent studies suggest that IL-5, in addition, induces differentiation of human basophils from their (circulating) progenitor cells (Denburg *et al.*, 1991). IL-5 (between 10^{-12} and 10^{-9} *M*) also activates human blood basophils and increases their capacity to respond to degranulating stimuli (Hirai *et al.*, 1990; Bischoff *et al.*, 1990a). IL-5 acts on its target cells via cell surface receptors. Recently, cDNAs for the human receptor for IL-5 have been cloned (Tavernier *et al.*, 1991). The α chain of the receptor is specific for IL-5 (and belongs to the HRS), whereas the β chain is identical to the β chain of the GM-CSF and IL-3 receptors (Tavernier *et al.*, 1991). Studies of blood basophils purified from a patient suffering from (basophil) CML blast crisis using ^{125}I-labeled rhIL-5 revealed a single class of approximately 800 high-affinity IL-5 binding sites per blood basophil (Lopez *et al.*, 1992) (Table II). The binding of iodinated rhIL-5 to CML basophils is inhibitable by rhIL-3 and, to some degree, by rhGM-CSF (Lopez *et al.*, 1990a,b). On the other hand, rhIL-5 was found to compete with rhGM-CSF but not with rhIL-3 for binding to human basophils. Receptors for IL-5 have also been detected on human eosinophils (Chihara *et al.*, 1990; Lopez *et al.*, 1992). Other ma-

ture myeloid cells, however, do not express detectable amounts of IL-5 binding sites.

5. *Granulocyte–Macrophage Colony-Stimulating Factor Receptor*

GM-CSF is a multipotent regulator of myeloid cells. The target cell spectrum of GM-CSF is broad, and a marked (functional) overlap with IL-3 exists. High-affinity GM-CSF binding sites apparently are restricted to cells of hemopoietic origin. IL-3 and GM-CSF binding sites are members of the HRS and share an identical subunit, termed *β chain* (T. Kitamura *et al.*, 1991). Binding of GM-CSF to myeloid cells is inhibitable by preincubation with IL-3 (Taketazu *et al.*, 1991; Lopez *et al.*, 1990a). Initial studies of GM-CSF receptors on human basophils gave controversial results. Lopez *et al.* (1990a,b) analyzed the binding of iodinated rhGM-CSF to highly enriched (92% pure) CML basophils obtained from a CML patient. They were able to detect a single class of approximately 100 high-affinity GM-CSF binding sites with a calculated K_D of 4×10^{-11} *M*. In our own experiments, purified blood basophils (>90% pure) from two CML donors were analyzed, but no GM-CSF binding sites could be detected. This may be due to the fact that basophils (in contrast to neutrophils and eosinophils) express very small numbers of GM-CSF binding sites. As only a small number of patients have so far been tested, it could also be that expression of GM-CSF receptors is restricted to a subset of CML donors. Another possibility would be that human blood basophils express GM-CSF binding sites under certain conditions rather than in a constitutive manner. At present we favor the hypothesis that normal (mature) human blood basophils express minimal numbers of GM-CSF binding sites (Table II). In contrast to primary, mature CML basophils the basophil precursor cell line KU-812 was found to express substantial amounts (>1000 sites) of GM-CSF receptors (J. Besemer, unpublished observation). Whether human mast cells express GM-CSF binding sites remains unknown.

Like IL-3 or IL-5, GM-CSF (in nanomolar concentrations) promotes the releasability of human blood basophils. GM-CSF has also been shown to induce differentiation of basophils from their progenitor cells under certain conditions (Denburg, 1992), in particular in the presence of other growth regulators [such as NGF (Tsuda *et al.*, 1991) and transforming growth factor β (Sillaber *et al.*, 1992)].

B. RTK Family, c-kit, and Related Oncogenes

Cytokine receptors with tyrosine kinase activity (RTK) differ from other receptor families. Typically, members of the RTK family are products of cellular proto-oncogenes and contain a tyrosine kin-

ase domain in their cytoplasmic portion (for review, see Ullrich and Schlessinger, 1990). Recent data suggest that RTKs are involved in the control of growth and function of myeloid (precursor) cells and mast cells.

1. Stem Cell Factor Receptor: c-kit

The c-kit proto-oncogene is located on the long arm of human chromosome 4 (Yarden *et al.*, 1987; Qiu *et al.*, 1988). In mice, the c-kit proto-oncogene has been mapped to the dominant white spotting locus (W locus) on chromosome 5 (Geissler *et al.*, 1988; Chabot *et al.*, 1988). Cloning of a cDNA coding for (human) c-kit revealed a member of the Ig supergene family and predicted a 976-amino-acid transmembrane protein (Yarden *et al.*, 1987). The c-kit peptide is synthesized by translation of a single 5-kb mRNA (Yarden *et al.*, 1987). The mature protein on human cells exhibits a molecular weight of 140–150 kDa as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Yarden *et al.*, 1987; Lerner *et al.*, 1991). Recent data suggest that the c-kit product is identical to the receptor for mast cell growth factor (MGF), also termed stem cell factor, kit ligand (KL), or steel factor (SL) (Zsebo *et al.*, 1990; D. E. Williams *et al.*, 1990; Martin *et al.*, 1990).

Northern blot analyses have shown that c-kit mRNA is expressed in murine mast cell lines (Andre *et al.*, 1989; Nocka *et al.*, 1990; D. E. Williams *et al.*, 1990) as well as in the human mast cell line HMC-1 (Sillaber *et al.*, 1991). Circulating blood cells do not express substantial amounts of c-kit (Andre *et al.*, 1989; Lerner *et al.*, 1991); however, expression of c-kit is not restricted to mast cells. Transcripts for c-kit are also expressed in germ cells (Matsui *et al.*, 1990), brain (Yarden *et al.*, 1987), placenta (Yarden *et al.*, 1987), and melanoblasts, as well as in normal (human) bone marrow cells (Lerner *et al.*, 1991; L. K. Ashman *et al.*, 1991), leukemic myeloid cells (Sillaber *et al.*, 1991), and the human cell lines HEL (erythroid/myeloid leukemic) and A-172 (glioblastoma) (Lerner *et al.*, 1991).

The distribution of the MGF receptor within the human hemopoietic system has been investigated by using mAb YB5.B8 (L. K. Ashman *et al.*, 1991; Sillaber *et al.*, 1991). Sequential immunoprecipitation analyses and binding studies revealed that this reagent recognizes the MGF receptor and binds to or near the MGF binding domain on c-kit (Lerner *et al.*, 1991). Immunolabeling studies using mAb YB5.B8 suggest that the MGF receptor is constitutively expressed on the cell surface of human tissue mast cells (obtained from lung, skin, uterus, or gut) (Gadd and Ashman, 1985; L. K. Ashman *et al.*, 1987; Mayrhofer *et al.*, 1987; Valent *et al.*, 1989b; Guo *et al.*, 1992) as well as on the human

mast cell line HMC-1 (Valent *et al.*, 1990b) (Table II). The number of MGF binding sites on human mast cells and HMC-1 cells is supposed to be above 10,000 per cell (as an estimate from flow cytometric evaluations with mAb YB5.B8) and to be equal in, or even exceed the number of MGF receptors expressed on, normal myeloid precursor cells (P. Valent *et al.*, unpublished observations); however, so far no binding data using radiolabeled rhMGF on human mast cells have become available. Other mature hemopoietic cells do not express substantial amounts of YB5.B8 antigen (Gadd and Ashman, 1985; Valent *et al.*, 1989b; Lerner *et al.*, 1991); however, mAb YB5.B8 binds to immature bone marrow precursor cells (L. K. Ashman *et al.*, 1991; Sillaber *et al.*, 1991) and melanocytes, as well as to nerve cells (L. K. Ashman *et al.*, 1987). mAb YB5.B8 also binds to HEL cells and A-172 cells (Lerner *et al.*, 1991).

A number of studies have shown that c-kit and its ligand (MGF/SCF) are involved in growth and differentiation of mast cells. MGF is encoded by a gene on human chromosome 12 (between 12q14.3 and 12qter) (Geissler *et al.*, 1991). In mice the MGF gene has been mapped to the steel locus (SL) on chromosome 10 (Copeland *et al.*, 1990; Flanagan *et al.*, 1990). Recently, human MGF has been cloned (Martin *et al.*, 1990). MGF/SCF is produced primarily, although not exclusively, by stroma cells. MGF either may be released as a soluble (dimeric) growth factor or may be expressed on the cell surface of stroma cells (Anderson *et al.*, 1990). The membrane-bound form of SCF is determined by tissue-specific alternative splicing (Flanagan *et al.*, 1990) and may play a role in stroma-dependent growth and differentiation of hemopoietic cells expressing c-kit (Furitsu *et al.*, 1989; Valent *et al.*, 1992). Membrane-bound MGF may also play a role as a homing receptor for hemopoietic progenitor cells and mast cells (Flanagan *et al.*, 1990).

Recombinant human MGF (but not IL-3, IL-4, M-CSF, NGF, or IL-9) induces differentiation of human mast cells in long-term bone marrow or peripheral blood cell cultures (Valent *et al.*, 1992). Induction of differentiation of human mast cells by rhMGF in long-term cultures is inhibitable by preincubation of precursor cells with mAb YB5.B8. A similar response of mast cells to MGF was observed in the murine system (Anderson *et al.*, 1990; Tsai *et al.*, 1991a,b; Zsebo *et al.*, 1991). The biological significance of mast cell growth factor and c-kit has been best documented in animal models, however, in particular by the elegant transplantation experiments by Kitamura *et al.* on mast cell-deficient mice (Y. Kitamura *et al.*, 1978; Y. Kitamura and Go, 1979; Galli and Kitamura, 1987) with defects in MGF genes or MGF receptor

genes caused by mutations in the steel locus (SL) or dominant white spotting locus (W), respectively (Copeland *et al.*, 1990; Flanagan *et al.*, 1990). These studies suggest that *in vivo* differentiation of mast cells is dependent on expression of an active form of MGF in respective stroma cells and on expression of MGF receptors in the mast cell progenitors. Studies of mast cell-deficient rats suggest that the tyrosine kinase domain of c-kit plays a most crucial role in the signal transmission induced *in vivo* by MGF (Tsujimura *et al.*, 1991). Interestingly, a mutation in the tyrosine kinase domain of c-kit has been detected in human piebaldism, a dominant genetic disorder associated with white spotting of the skin (Giebel and Spritz, 1991).

Recent studies were conducted to analyze the potential role of MGF in activating mature mast cells (which express SCF binding sites). Recombinant MGF per se caused significant release of histamine from human mast cells in a subset of donors (Valent *et al.*, 1992). Moreover, rhMGF (0.01–100 ng/ml), but not IL-3 or IL-4, upregulates the capacity of human (lung and uterus) mast cells to release histamine on induction with anti-IgE (Bischoff and Dahinden, 1992; Valent *et al.*, 1992). Thus, SCF may be involved in allergic reactions involving mast cells and their products.

Recently, factors controlling expression of c-kit gene products *in vitro* have been identified. In the human system, IL-4 (but not IL-3, IL-5, IL-6, M-CSF, or GM-CSF) downregulates expression of c-kit mRNA and cell surface YB5.B8 antigen in HMC-1 cells (Sillaber *et al.*, 1991). Similar observations have been made with primary human (leukemic) myeloid cells, which, however, may also be regulated by IL-3 or GM-CSF. In the murine system, IL-3 and GM-CSF, but not IL-4, were found to downregulate expression of c-kit mRNA in hemopoietic mast cell lines (Welham and Schrader, 1991). The reason(s) for the species discrepancies with respect to IL-4 is not known.

2. *Other Receptors of the RTK Family*

Other RTK oncogenes and their products (supposed to regulate hemopoiesis) include c-fms (M-CSF-R), c-flk (ligand unknown), trk-A (NGF-R), erb (EGF-R), c-flg (aFGF-R), and c-bek (bFGF-R). The c-fms proto-oncogene encodes the receptor for M-CSF (Scherr *et al.*, 1985). The c-fms oncogene (as well as c-flk) is closely related to c-kit in its structural motifs (Ullrich and Schlessinger, 1990; Bazan, 1991). Furthermore, the c-fms gene complements the mast cell defect in W mice, suggesting common steps in signal transduction pathways transmitted by c-fms and c-kit (Dubreuil *et al.*, 1991); however, neither c-fms nor M-CSF receptors have been detected in human mast cells or basophils so far. M-CSF (unlike SCF) is also not capable of competing with mAb

YB5.B8 for binding to MGF receptors on human mast cells (Valent *et al.*, 1992) and no effects of rhM-CSF on growth or function of human mast cells have been described.

The trk oncogene encodes a receptor for nerve growth factor (NGF) (Klein *et al.*, 1991; Hempstead *et al.*, 1991). NGF, a neurotropic peptide produced by stroma cells, is well known to promote growth, differentiation, and function (i.e., histamine releasability) of (murine) mast cells (Aloe and Levi-Montalcini, 1977; Matsuda *et al.*, 1991; Bruni *et al.*, 1982; Sugiyama *et al.*, 1985; Tomioka *et al.*, 1888) and of (human) blood basophils (Matsuda *et al.*, 1988; Tsuda *et al.*, 1991; Bischoff and Dahinden, 1992). A direct effect of NGF on pure human basophils has been observed, suggesting the presence of functional cell surface receptors (Bischoff and Dahinden, personal communication). Other studies suggest that NGF-induced regulation of basophil/mast growth is dependent (at least in part) on accessory cells and their products (Tsuda *et al.*, 1991; Marshall *et al.*, 1991). So far we do not know whether human basophils (or mast cells) express c-trk or other cell surface NGF binding sites.

The c-erb proto-oncogene codes for a receptor for epidermal growth factor and transforming growth factor α. When transfected in murine stem cells, c-erb may complement the mast cell defect in W mice (similar to c-fms) and may induce mastocytosis (T. van Rüden, personal communication).

IV. Negative Regulators of Growth and Differentiation of Human Basophils and Human Mast Cells

Growth and differentiation of myeloid cells, including basophils and mast cells, is controlled by so-called "inhibitors of hemopoietic cell growth." So far, however, very little is known about the identity of these inhibitors.

A. Interferons

Interferon (IFN)-α and IFN-γ (in the nanomolar range) inhibit IL-3-dependent differentiation of human basophils from bone marrow precursor cells (Sillaber *et al.*, 1992) as well as IL-3-dependent (and spontaneous) growth of KU-812 cells. Similar observations have been made with IL-3-dependent growth/differentiation of murine mast cells (Takagi *et al.*, 1990) and SCF-dependent differentiation of human mast cells (P. Valent and Sillaber, unpublished observation). Moreover, IFN-α is capable of inducing a decrease in basophil counts in chronic myeloid leukemia patients and may successfully be used to treat aggressive systemic mastocytosis (Kluin-Nelemans *et al.*, 1992).

On the other hand, the IFNs have been shown to upregulate the releasability in human blood basophils (Ida *et al.*, 1977); however, in contrast to that induced by the HGFs GM-CSF, IL-5, and IL-3, the IFN-induced activation of human basophils for mediator release depends on a prolonged incubation period (at least 12 hours) and is supposed to require translation of new peptides (Hernandez-Asensio *et al.*, 1979).

To demonstrate a direct effect of IFNs on human basophils, we repeated some of the "earlier" experiments described by Ida *et al.* (1977) by using highly enriched human CML basophils. As shown in Fig. 2, both IFN-γ and IFN-α are capable of upregulating the capacity of the pure (>95%) basophils to release histamine (on incubation with IgE plus anti-IgE) after 24 hours. This experiment provides (further) evidence that human (CML) basophils express functional IFN binding sites. Preliminary binding data using ^{125}I-labeled IFN-γ exist as well and suggest that human basophils express high-affinity IFN binding sites.

B. Transforming Growth Factors

Transforming growth factor β (TGF-β) is another well-established negative regulator of hemopoietic cell growth. In mice TGF-β1 down-

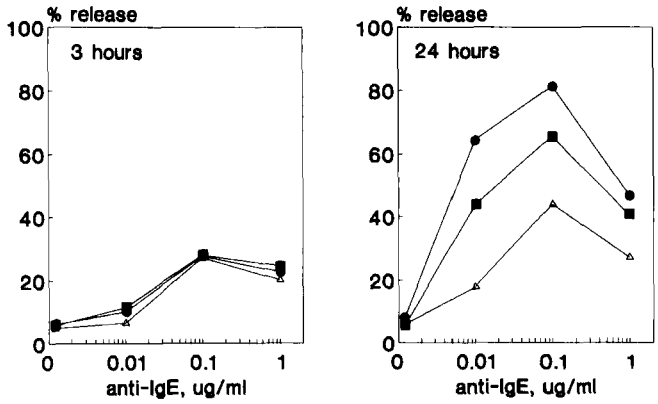

FIG. 2. Response of pure chronic myeloid leukemia (CML) basophils to recombinant interferons. Basophils were enriched to homogeneity as described by Stain *et al.* (1987). Pure (>95%) basophils were cultured in the presence of recombinant human interferon-α (rhIFN-α) (100 U/ml, ■) rhIFN-γ (100 U/ml, ●), or control medium (△) at 37°C/5% CO_2/10% fetal calf serum for 24 hours. After 3 and 24 hours, cells were exposed to human immunoglobulin E for 3 hours, washed, and then exposed to various concentrations of anti-IgE monoclonal antibody E124-2-8. Release is expressed as a percentage of total histamine.

regulates IL-3-dependent differentiation of mast cells (Broide *et al.*, 1989). Surprisingly, however, TGF-β1 (as well as TGF-β2) even upregulates IL-3-dependent differentiation (*in vitro*) of human basophils from their bone marrow progenitor cells, whereas in the same cultures TGF-β downregulates IL-3-dependent formation of eosinophilic granulocytes (Sillaber *et al.*, 1992). Moreover, in the presence of TGF-β, GM-CSF (which by itself has almost no basophil promoting activity in bone marrow cell cultures) becomes a strong basophilopoietin (Sillaber *et al.*, 1992). The differentiation-inducing effect of TGF-β on human basophils apparently is restricted to more immature stages of basophilic differentiation. In sharp contrast, TGF-β downregulates IL-3-dependent proliferation (i.e., uptake of thymidine) of highly enriched human CML basophils (Sillaber and Valent, unpublished observation). The latter effect of TGF-β1 on highly enriched (>90% pure) CML basophils suggests that these cells express functional TGF-β binding sites (Table II). Binding data on radiolabeled rhTGF-β did not become available during the preparation of this review. In contrast to the interferons tested, no effect of TGF-β (1–100 ng/ml) on the releasability of human blood basophils was observed over the time range (i.e., after 6, 8, 12, or 24 hours) of the tests (Sillaber *et al.*, 1992).

V. Adhesion Receptors and Recognition Molecules

Although both basophils and mast cells derive from circulating progenitor cells, basophils differ from mast cells in their distribution (Metcalfe *et al.*, 1981; Galli, 1990). Mature blood basophils typically are intravascular, circulating cells. Nevertheless, basophils have the potential to transmigrate through the endothelial cell layer to invade inflamed tissues. Mast cells, in contrast, are located in extravascular sites and usually do not appear in the peripheral bloodstream. Tissue distribution of mast cells, emigration of basophils, and migration of immune cells, in general, are controlled by cell surface recognition molecules (RMs) and adhesion receptors.

During the last few years a number of important RMs have been identified (Hynes, 1987; Kishimoto *et al.*, 1989; Hemler, 1990; Springer, 1990). Three major RM families have been recognized: the integrins, the selectins, and the (RM) members of the immunoglobulin (Ig) supergene family (Springer, 1990). RMs may interact with other RMs (intercellular recognition) or with extracellular matrix molecules (such as laminin, collagen, or fibronectin). Some of the RMs are involved in antigen recognition or presentation, and some are involved in binding to immunomodulating compounds or microbial (viral) prod-

ucts. Most RMs display a characteristic distribution within the mesenchymal cell system. Recently, RMs expressed on human blood basophils and tissue mast cells have been identified.

A. Integrins

Integrins are a family of structurally related RMs. They usually are expressed as heterodimers with a variable α chain and a common β chain. According to the β chain (and respective CDs), eight distinct integrin subfamilies have been identified, the most important being $\beta 1$ (CD29), $\beta 2$ (CD18), and $\beta 3$ (CD61) (Springer, 1990; Hynes, 1991). It is likely that many more integrin subfamilies exist.

1. β1 Integrins

$\beta 1$ (CD29) integrins, also termed very late antigens (VLAs) (Hemler, 1990) are involved primarily in the binding to extracellular matrix molecules. VLA-4 (CD49d/CD29), a receptor for fibronectin, is in addition a counterreceptor for VCAM-1 expressed on vascular endothelial cells (Elices *et al.*, 1990).

Human basophils express the common β chain of VLA integrins (CD29) as assessed by indirect immunofluorescence (Bochner and Sterbinsky, 1991; Sperr *et al.*, 1992) (Tables I and III). Studies with CD49d mAb HP2/1 suggest that human blood basophils express the α chain of VLA-4 (Bochner and Sterbinsky, 1991) (see also Table III). Preincubation of activated ECs with anti-VCAM-1 mAb 2G7 reduced their capacity to bind human basophils (and human eosinophils), but not their capacity to bind neutrophils (which lack VLA-4) (Bochner and Sterbinsky, 1991). Moreover, endothelial cells exposed to IL-4 exhibit increased capacity to express VCAM-1 antigen and to bind basophil (and eosinophil) granulocytes (Schleimer *et al.*, 1992).

Recently, expression of $\beta 1$ integrins on human mast cells has been evaluated. Mast cells obtained from the lung or the uterus by enzyme digestion were found to express CD29 (VLA-β), CD49d (VLA-4α) and CD49e (VLA-5α, involved in binding to fibrinogen) but not CD49b or CD49f (Guo *et al.*, 1992; Sperr *et al.*, 1992) (Table III). The mast cell leukemia cell line HMC-1 was found to express an almost identical pattern of $\beta 1$ integrins (Tables I and III). Treatment of endothelial cells with IL-1 results in increased expression of VCAM-1 and in increased binding to human mast cells (Guo *et al.*, 1992). Binding of mast cells to extracellular matrix molecules has recently been investigated in the murine system. Murine mast cells were found to express receptors for laminin and fibronectin under certain conditions (H. L. Thompson *et al.*, 1989a,b; Dastych *et al.*, 1991); however, the relationship of these binding sites to the $\beta 1$ integrins remains unknown.

TABLE III
EXPRESSION OF INTEGRINS ON HUMAN MAST CELLS AND BASOPHILS AS ASSESSED BY MONOCLONAL ANTIBODIES AND INDIRECT IMMUNOFLUORESCENCE

CD	Integrin	Natural Ligand	Surface expression on ba[a]	KU-812	l-mc	s-mc	u-mc	HMC-1
	β1 integrins							
CD29	β chain		+	+	+	+	+	+
CD49b/29	VLA-2	Lm, Co	nk	+	−	−	−	+/−
CD49d/29	VLA-4	Fn, VCAM-1	+	+	+	nk	+	+
CD49e/29	VLA-5	Fn	+	+	+	nk	+	+
CD49f/29	VLA-6	Lm	−	−	−	nk	−	−
	β2 integrins							
CD18	β chain		+	+	−	−	+/−	−
CD11a/18	LFA-1	ICAM-1/2	+	+	−	−	−	−
CD11b/18	Mac-1/CR3	C3bi,FX,Fb	+	+	−	−	−	−
CD11c/18	p150,95	C,?	+	+	−	−	+/−	−
	β3 integrins							
CD61	β chain		nk	+	+	+	+	+
CD41/61	gpIIb/IIIa	Fb,Fn,vWF	−	nk	−	−	nk	nk
CD51/61	VNR	Vn, Fb, Tsp	nk	+	+	+	+	+

[a] VLA, very late antigen; gp, glycoprotein; LFA, leukocyte function-associated antigen; ba, basophils; l-mc, lung mast cells; s-mc, skin mast cells; u-mc, uterine mast cells. FX, factor X; *v*WF, von Willebrand factor; Fn, fibronectin; Lm, laminin Fb, fibrinogen; Vn, vitronectin; VNR, Vn receptor; Tsp, thrombospondin; Co, collagen; VCAM-1, vascular cell adhesion molecule-1; nk, not known; +/−, small subset of cells positive.

2. *β2 Integrins*

β2 (CD18/CD11x) is restricted in its expression to leukocytes. Three distinct heterodimers have been identified: LFA-1 (CD18/CD11a), Mac-1 (CD18/CD11b), and p150,95 (CD18/CD11c). LFA-1 is the counterreceptor for ICAM-1/2. Mac-1 constitutes a multipotent binding site for various ligands including ICAM-1/2, fibrinogen, coagulation factor X, and the complement cleavage product C3bi. The ligand(s) for p150/95 (also termed *CR4*) has not been identified so far but CD11c may have the capacity to bind complement components.

Human blood basophils are recognized by mAbs specific for CD11a, CD11b, CD11c as well as mAbs specific for CD18. This has been demonstrated for normal blood basophils (deBoer and Roos, 1986; Stain *et al.*, 1987; Bodger and Newton, 1987; Bochner *et al.*, 1989a,b) and CML basophils (Stain *et al.*, 1987; Bodger and Newton, 1987; Valent *et al.*, 1990c), as well as for the human basophil precursor cell line KU-812 (Kishi, 1985; Fukuda *et al.*, 1987) (see also Tables I and III).

Basophils exhibit the potential to adhere to vascular endothelial cells (ECs). Adherence of basophils to ECs *in vitro* can be reduced by preincubation of basophils with mAbs to CD18 (Bochner *et al.*, 1988, 1990). Moreover, incubation with IL-1, TNF, or lipopolysaccharide (LPS) (promoting expression of ICAM-1 antigen) upregulates the capacity of the ECs to bind human blood basophils (Bochner *et al.*, 1988).

Recent studies have focused on the regulation of basophil β2 integrins. Indirect immunofluorescence staining experiments suggest that IL-3 (but not IL-2, IL-4, IL-5, IL-6, IL-8, or IFN) induces rapid upregulation of CD11b and CD18 on human basophils (Bochner *et al.*, 1990). In contrast, IL-3 failed to upregulate expression of CD11a on the same cells. The IL-3-dependent increase in expression of CD11b/CD18 on basophils is associated with an increase in their ability to adhere to vascular endothelial cells (Bochner *et al.*, 1990). Moreover, the induced increase in binding could be reduced by preincubation of the basophils with CD18 mAbs. An increase in expression of basophil β2 integrins was also found on crosslinkage of IgE binding sites (Bochner *et al.*, 1989a; Bochner and Sterbinsky, 1991). In addition, antigen-dependent crosslinkage induced upregulation of CD11b, CD11c, and CD18 in human basophils as determined by flow cytometry (Bochner and Sterbinsky, 1991). Interestingly, expression of CD11a antigen in the same cells essentially remained unchanged. Crosslinkage of FcεRI molecules is associated with an increased capacity of the basophils to adhere to ECs. Several lines of evidence suggest that the induced increase in β2 on human basophils is not directly linked to activation of the same cells for mediator release. For example, the induced increase in CD18 is rapid and precedes the release of mediator molecules from the basophils (Bochner *et al.*, 1989a). In addition, relatively low concentrations of antigen, ineffective in inducing basophil degranulation, may already induce an increase in expression of CD18 (Bochner *et al.*, 1989a). Altered expression of β2 integrins and of other RMs on human basophils and eosinophils may also be of functional significance in the recruitment to extravascular sites following immunological activation (Georas *et al.*, 1992).

Dispersed mast cells obtained from lung, skin, or gut by enzymatic digestion did not react with CD11a/b/c or CD18 mAbs by immunostaining (Valent *et al.*, 1989b). The human mast cell line HMC-1 also lacks detectable amounts of cell surface β2 integrins (Valent *et al.*, 1990b; Sperr *et al.*, 1992). Controversial data have been obtained for uterine mast cells. Guo *et al.* (1992) reported expression of CD11c as well as CD18 (but not CD11a or CD11b) on uterine mast cells by using mAbs SHCL-3 (CD11c) and H52 (CD18), whereas Sperr *et al.*, (1992)

were unable to confirm expression of CD11c or CD18 on uterine mast cells by using mAbs 3.9 (CD11c) and MHM23 (CD18). Reasons for these discrepancies are not known but they may be caused by the expression of selective epitopes or the expression of very small amounts of $\beta 2$ integrins on human mast cells.

3. β3 Integrins

So far very little is known about expression of $\beta 3$ integrins on human basophils or human mast cells. Preliminary evidence exists that basophils lack the IIb/IIIa complex likewise expressed on human platelets (P. Valent and P. Bettelheim, unpublished observation). Other studies suggest that human mast cells express CD51 and CD61, together forming the vitronectin receptor (Guo *et al.*, 1992; Sperr *et al.*, 1992) (Tables I and III). HMC-1 cells were found to react with CD61 and CD51 mAbs as well (Sperr *et al.*, 1992).

B. Recognition Molecules of the Immunoglobulin Supergene Family

1. LFA-2/CD2 and LFA-3/CD58

LFA-2 is expressed primarily on T cells and is supposed to play a crucial role in T cell activation and recognition. Normal human blood basophils or tissue mast cells have so far not been described as expressing CD2; however, in 1986, a case of mast cell leukemia was reported (Dalton *et al.*, 1986) in which the malignant cells were found to react with mAb OKT11 directed against CD2. Later, the human mast cell line HMC-1 was established (Butterfield *et al.*, 1988) and was found to be recognized by CD2 antibodies as well (Valent *et al.*, 1991). HBM-M cells (mast cell-like cells derived from a patient suffering from diffuse cutaneous mastocytosis) were found to lack CD2 (Krilis *et al.*, 1991). LFA-3 is the counterreceptor for LFA-2. Little is known about expression of LFA-3 on human basophils or mast cells. Binding of CD58 mAbs to primary lung and uterine mast cells as well as to HMC-1 cells has been observed (Valent *et al.*, 1991; Sperr *et al.*, 1992) (Tables I and IV).

2. ICAM-1/CD54

The ICAM-1 antigen (CD54) is broadly distributed on (activated) mesenchymal cells. ICAM-1 is a counterreceptor for LFA-1, Mac-1 (Springer, 1990), and CD43 and, in addition, a major receptor for rhinoviruses (Staunton *et al.*, 1990). Normal human blood basophils as

TABLE IV
EXPRESSION OF RECOGNITION MOLECULES BELONGING TO THE IMMUNOGLOBULIN SUPERFAMILY ON HUMAN MAST CELLS AND BASOPHILS AS DETERMINED BY MONOCLONAL ANTIBODIES AND INDIRECT IMMUNOFLUORESCENCE

CD	Name of RM	Natural Ligand	Surface expression on					
			ba[a]	KU-812	l-mc	s-mc	u-mc	HMC-1
CD2	LFA-2	CD58, LFA-3	−	nk	−	−	−	+
CD3	TcR complex	—	−	−	−	−	−	−
CD4	T4	Class II,HIV	(−)	−	−	−	−	−
CD8	T8	Class I	−	−	−	−	−	−
CD22	p130/14	CD45RO,CD75	−	−	−	−	nk	−
CD31	gp140	?	+	nk	−	−	nk	−
CD54	ICAM-1	LFA-1	+	+	+	+	+	+
CD58	LFA-3	LFA-2	nk	+	+	+	+	+
—	VCAM-1	VLA-4	−	nk	nk	nk	nk	nk
—	Class II	T4	(−)	nk	(−)	(−)	(−)	+/−
—	Class I	T8	+	nk	+	+	+	nk
—	SCF-R,c-kit	SCF	−	+/−	+	+	+	+

[a] ba, basophils; l-mc, lung mast cells; s-mc, skin mast cells; u-mc, uterine mast cells; SCF, stem cell factor; nk, not known; +, more than 90% of cells reactive; −, less than 10% of cells reactive with mAbs; ( ), expression seen under certain conditions.

well as CML basophils are recognized by mAbs specific for ICAM-1 antigen (Bochner *et al.*, 1989b; Valent *et al.*, 1990c). KU-812 cells expose ICAM-1 antigen as well. Dispersed human mast cells (derived from lung, skin, or gut) can be detected by using the CD54 mAbs 84H10 and My-13 and combined toluidine blue/immunofluorescence staining (Valent *et al.*, 1990c); however, the reactivity of these CD54 mAbs with human mast cells was low and three other CD54 mAbs tested (8F5, LB-2, and R.I.1.1.1.) gave almost no detectable signal. More recently, HMC-1 cells were found to be recognized by mAbs specific for ICAM-1 antigen (Valent *et al.*, 1991) (see Tables I and IV). In addition, HMC-1 cells were found to express ICAM-1 mRNA in a constitutive manner.

Resting mast cells apparently express relatively low levels of ICAM-1 antigen (see preceding text); however, IL-4 was found to promote expression of cell surface ICAM-1 antigen as well as expression of ICAM-1 mRNA in HMC-1 cells (Valent *et al.*, 1991). Other cytokines such as IL-2, IL-3, IL-8, and GM-CSF showed no effect on mast cell ICAM-1 antigen. Mast cells obtained from dispersed human lung were also found to react more intensively with CD54 mAbs in combined toluidine blue/immunofluorescence staining experiments after incu-

bation with IL-4, compared with control cells. IL-4 as well as IL-2, IL-3, and IL-8 failed to promote expression of cell surface ICAM-1 antigen in highly enriched CML basophils or the basophil leukemia cell line KU-812. Counterreceptors for ICAM-1 antigen are expressed on human basophils (CD11/CD18, CD43) as well as on human mast cells (CD43).

3. *VCAM-1*

VCAM-1 is the counterreceptor for VLA-4 (CD49d). Peripheral blood basophils apparently are not recognized by mAb 2G7 directed against VCAM-1 antigen (Bochner *et al.*, 1991).

4. *CD4 and CD8*

The T4 antigen (CD4) is the counterreceptor for MHC class II molecules and a major receptor for the human immunodeficiency virus (HIV). Primary resting (normal or CML) basophils do not express detectable amounts of CD4 as determined by indirect immunofluorescence; however, after short-term culture, purified CML basophils are reactive with mAbs clustered as CD4 (Stain *et al.*, 1987). Whether human blood basophils indeed express T4 antigen (or bind CD4 mAbs by an alternative mechanism) remains unknown. In patients suffering from the acquired immunodeficiency syndrome (AIDS), the histamine release induced by HIV peptide preparations is most probably due to type I allergic responses to a HIV antigen (Pedersen *et al.*, 1987). The T8 (CD8) antigen is not detectable on either human mast cells or human blood basophils (Stain *et al.*, 1987; Valent *et al.*, 1989b).

5. *Stem Cell Factor Receptor/c-kit*

Stem cell factor receptor (SCF-R), the product of the c-kit proto-oncogene, is expressed on human mast cells and on HMC-1 cells (Valent *et al.*, 1990a,b; Sillaber *et al.*, 1991). The c-kit receptor is a member of the RTK family (subclass III) and thus belongs to the Ig supergene family. SCF-R may play a crucial role as a recognition molecule because its ligand [SCF, also termed *kit ligand* (KL)] may be expressed in membrane-bound form on the cell surface of stroma cells (endothelial cells or fibroblasts) (Flanagan *et al.*, 1990; Anderson *et al.*, 1990; Adachi *et al.*, 1992). The membrane-bound form of SCF is determined by tissue-specific alternative splicing (Flanagen *et al.*, 1990). Membrane-bound SCF may play a role as a homing receptor for circulating mast cell progenitors (and other hemopoietic progenitors) expressing c-kit. The role of the SCF-R in mast cell growth and differentiation is described in Section III.

6. *MHC Class I and Class II Molecules*

MHC molecules are critical sites for antigen presentation and recognition. As assessed by different immunostaining techniques, basophils as well as tissue mast cells express class I antigen on their cell surface (deBoer and Roos, 1986; Reshef and MacGlashan, 1987; Bochner *et al.*, 1989b). In contrast, class II antigens could not be detected on the cell surface of primary normal human basophils (Stain *et al.*, 1987; Bochner *et al.*, 1989b; Valent *et al.*, 1990c). Primary CML basophils were also found to lack class II molecules; however, after culture for several days, a subset of (pure) CML basophils became reactive with anti-class II mAbs (Stain *et al.*, 1987). These molecules, however have so far not been defined in detail. Data on class II expression on human mast cells are divergent. In one study, dispersed human tissue mast cells were not found to express detectable amounts of cell surface class II molecules as determined by combined touluidine blue/immunofluorescence staining (Valent *et al.*, 1989b). In another study using an immunogold labeling technique, approximately 10–20% of the human lung mast cells analyzed were found to react with an anti-class II mAb (Reshef and MacGlashan, 1987). These differences at present cannot readily be explained. One possibility could be that activated mast cells express class II antigen, whereas resting mast cells do not. Interestingly, a small subset of HMC-1 cells express low levels of class II molecules under normal culture conditions (P. Valent, unpublished), and similar results were reported for HBM-M cells. The mast cell leukemia cell clone described by Dalton *et al.*, (1986), however, was found to lack cell surface class II molecules. Whether human mast cells are capable of presenting antigen remains to be determined.

7. *Other Recognition Molecules of the Ig Superfamily*

The glycoprotein CD31 is another putative adhesion receptor belonging to the Ig supergene family. Studies using CD31 mAbs suggest that this protein is expressed on human basophils but not on human mast cells (Valent *et al.*, 1990c) (see Tables I and IV). CD22, the B cell counterreceptor for CD45RO and CD75 (Stamenkovic *et al.*, 1991), could not be detected on either human blood basophils or human mast cells.

C. Selectins and Related Recognition Molecules

Selectins are involved in binding of (rolling) leukocytes to activated endothelial cells prior to emigration (into inflamed tissues) (Springer, 1990; Lawrence and Springer, 1991). Three major molecules have so

far been defined: LAM-1 (also termed *Mel-14*, *Leu-8*, and, more recently, *LECAM-1*); ELAM-1; and PADGEM (CD62). Human basophils express LAM-1 antigen as assessed by immunostaining (Bochner and Sterbinsky, 1991) (Table V), whereas mast cells were found to lack LAM-1 antigen (Guo *et al.*, 1992). Interestingly, a decrease in expression of Leu-8 was observed on induction of human basophils by degranulation-inducing compounds (Bochner and Sterbinsky, 1991). The functional significance of expression of LAM-1 on human basophils remains to be determined. Whether human blood basophils or mast cells express ELAM-1 or PADGEM is not known.

The CD15 antigen (3-FAL), also termed *x-hapten*, is expressed primarily on myeloid cells and their precursors. Recent data suggest that CD15 may constitute a counterreceptor for selectins. Blood basophils apparently express CD15; however, basophils are not recognized by CD15 mAbs unless treated with sialidases (Stain *et al.*, 1987) (Table I) suggesting the sialinated form of the x-hapten which is expressed on a variety of other myeloid and lymphoid cells, as well (Stockinger *et al.*, 1984). Human lung mast cells were not recognized by CD15 mAbs, even when exposed to sialidases (Valent *et al.*, 1989b). Human basophils also express sialyl-Lewis X, a putative counterreceptor for ELAM-1 antigen. So far a possible role for basophil CD15 or sialyl-Lewis X antigen remains unknown. Interestingly, desialidation of the basophil cell surface membrane is associated with an increase in their ability to respond to degranulating compounds (Jensen *et al.*, 1987).

TABLE V
EXPRESSION OF SELECTINS AND OTHER RECOGNITION MOLECULES ON MAST CELLS AND BASOPHILS AS DETERMINED BY MONOCLONAL ANTIBODIES AND INDIRECT IMMUNOFLUORESCENCE

CD	Name of RM	Natural Ligand	Surface expression on					
			ba[a]	KU-812	l-mc	s-mc	u-mc	HMC-1
—	LAM-1	nk	+	nk	nk	nk	−	nk
—	ELAM-1	nk	nk	nk	nk	nk	nk	nk
CD62	PADGEM	nk	nk	nk	nk	nk	nk	nk
CD9	p24	nk	+	+	+	+	+	+
CD15	3-FAL	Selectins?	(−)	(−)	−	−	−	−
CD36	Tsp-R	Tsp	−	nk	−	−	nk	−
CD43	Leukosialin	ICAM	+	+	+	+	+	+
CD44	Pgp-1	nk	+	+	+	+	+	+

[a] ba, basophils; l-mc, lung mast cells; s-mc, skin mast cells; u-mc, uterine mast cells; Tsp, thrombospondin; nk, not known; +, expression seen after treatment with neuraminidase.

D. Other Recognition Molecules

The Pgp-1 antigen (CD44), a putative homing receptor, is broadly distributed. mAb F10-44-2 directed against Pgp-1 has been described as binding to human blood basophils as well as to human mast cells (Valent *et al.*, 1990c). The CD36 antigen, a functional binding site for thrombospondin is expressed neither on human blood basophils nor on human mast cells as determined by immunostaining using mAb 5F1 (Valent *et al.*, 1990c). The CD9 antigen (p24) is another putative adhesion receptor and is expressed on both human basophils and human mast cells (Stain *et al.*, 1987; Valent *et al.*, 1989b) (Tables I and V).

VI. Receptors for Activating Peptides

A number of bioactive peptides have the capacity to regulate the functional properties of human blood basophils and/or the function of human mast cells. Such peptides may be classified according to their effects on the release reaction. Histamine-releasing factors (HRFs) directly induce release from the target cells. Histamine release-enhancing factors (HREFs) have no HRF activity per se, but may induce or potentiate the release reaction in the presence of a second agonist. HREF peptides act similarly on basophils as compared with the HGFs IL-3, IL-5, and GM-CSF. Histamine release-inhibitory factors (HRIFs) may reduce the magnitude of (or even abolish) the reaction induced by an (complete) agonist. Many peptide agonists also have the capacity to attract human basophils chemotactically.

Recently, the identities of a number of peptides regulating mast cells and human blood basophils have been established and evidence for specific binding sites on human basophils and human mast cells has emerged (Table VI). The seven-transmembrane-segment receptor superfamily (7-TMS-RS) is characterized by a transmembrane configuration of the receptor that spans the lipid bilayer of the cell surface membrane seven times (Dohlman *et al.*, 1991). 7-TMS receptors are also characterized by the coupling to G-proteins (Dohlman *et al.*, 1991). The 7-TMS-RS includes a number of peptide binding sites known to be involved in the regulation of various immune cells. Interestingly, the receptors for many (or most) of the defined basophil/mast cell-activating peptides appear to belong to the 7-TMS-RS. Although the molecular structures of the basophil/mast cell peptide binding sites have so far not been determined some observations suggest that indeed basophils and mast cells express such binding sites (Mousli *et*

TABLE VI
EXPRESSION OF FUNCTIONALLY ACTIVE[a] PEPTIDE RECEPTORS ON HUMAN BASOPHILS AND MAST CELLS

Peptide	Expression of receptors on		
	basophils	lung mast cells	skin mast cells
IL-8/NAP-1[b]	+	nk	nk
PBP	nk	nk	nk
CTAP-III	+	nk	nk
NAP-2	+	nk	nk
MGSA/NAP-3	nk	nk	nk
MCAF	+	nk	nk
fMet peptide	+	−	−
Substance P	−	−	+

[a] So far, quantitative binding data do exist for IL-8, but not for other compounds.

[b] IL, interleukin; NAP, neutrophil-activating protein; CTAP, connective tissue-activating protein; MGSA, melanoma growth-stimulating activity; fMet, formyl-methionine; nk, not known.

al., 1991; Fujimoto *et al.*, 1991; Hide *et al.*, 1991). In the following section we focus on member of the 7-TMS-RS and on their respective ligands.

A. Receptors for IL-8 and Related Intercrines

Intercrines (ICs) are a family of proinflammatory regulator peptides with molecular weights of 8–10 kDa (Oppenheim *et al.*, 1991). Two major types of ICs exist differing from each other by amino acid sequence and chromosomal location of the coding genes. Type α IC species are encoded by a gene cluster on human chromosome 4 (q11–21) (Modi *et al.*, 1990), whereas β ICs are encoded by a gene cluster located on the long arm of chromosome 17 (q11–21) (Irving *et al.*, 1990). α ICs include IL-8, platelet basic protein (PBP), connective tissue-activating protein III (CTAP-III), β-thromboglobulin (β-TG), neutrophil-activating peptide 2 (NAP-2), platelet factor 4, and melanoma growth-stimulating activity (Oppenheim *et al.*, 1991). α ICs have a preferential effect on granulocytes, whereas β-ICs include monocyte-activating peptides such as MCAF and RANTES (Oppenheim *et al.*, 1991). More recent data suggest that a number of basophil activating- or deactivating peptides including the lower molecular

weight forms of HRF (histamine releasing factor) or HRIF (histamine release inhibiting factor) may be members, or may even be identical to well recognized members, of the alpha IC family (Kaplan *et al.*, 1991).

IL-8 is the prototype of an α IC and has also been termed monocyte derived neutrophil chemotactic factor (MDNCF), neutrophil-activating factor (NAF), and neutrophil-activating peptide 1 (NAP-1) (reviewed by Oppenheim *et al.*, 1991). IL-8 is produced by (activated) hemopoietic and nonhemopoietic cells. The production of IL-8 can be induced by bacterial products, viruses, or endogenous pyrogens such as IL-1, TNF, and IFN. IL-8 was initially characterized as an activator and chemoattractant for neutrophils (Yoshimura *et al.*, 1987; Schroeder *et al.*, 1987; Walz *et al.*, 1987). It soon became clear, however, that IL-8 acts on many cells and activates and attracts human basophils and probably mast cells too. More recent studies have revealed that IL-8 binds to specific cell surface receptors on its target cells (Besemer *et al.*, 1989; Samanta *et al.*, 1989; Yoshimura and Leonard, 1990). The IL-8 receptor(s) belongs to the 7-TMS-RS (Holmes *et al.*, 1991; Murphy and Tiffany, 1991).

Human blood basophils enriched from CML blood (by the use of mAbs and complement) are capable of binding significant amounts of ^{125}I-labeled rhIL-8 in a specific manner (J. Besemer, P. Valent, and P. Bettelheim, unpublished observations, 1990). Scatchard plot analyses suggest that CML basophils express a single class of high-affinity IL-8 binding sites. The number of IL-8 binding sites on pure CML basophils varied between 500 and 10,000 sites per cell (range from three tested donors) with a calculated dissociation constant K_D of $4.4 \pm 3.4 \times 10^{-9}$ *M* (Valent, *et al.*, 1989d) corresponding to IL-8 receptors on neutrophils (Besemer *et al.*, 1989; Samanta *et al.*, 1989; Yoshimura and Leonard, 1990). KU-812 cells (basophil-like cell line) were found to express a single class of approximately 1000–2000 high-affinity IL-8 binding sites with a K_D of $0.5 \pm 0.11 \times 10^{-9}$ *M* (Valent, *et al.*, 1989d). So far little is known about IL-8 receptor expression on human mast cells. HMC-1 (mast cell-like) cells express a single class of high-affinity IL-8 binding sites (J. Besemer, unpublished observation, 1990).

IL-8 apparently is capable of modulating the secretory process in human blood basophils under certain conditions (Dahinden *et al.*, 1989a,b; White *et al.*, 1989). IL-8 by itself reportedly causes basophil histamine release *in vitro* between 10^{-4} and 10^{-6} *M*, but not between 10^{-7} and 10^{-9} *M* (White *et al.*, 1989). Between 10^{-4} and 10^{-6} *M*,

nonspecific activation of basophils may be operative. Other studies have suggested that IL-8 may induce histamine release *in vitro* from human basophils at lower (physiologic) concentrations (10^{-7} to 10^{-9} *M*), when cells are preincubated with IL-3, GM-CSF, or IL-5 (Dahinden *et al.*, 1989a,b); however, the effect of IL-8 on histamine release (even in the presence of IL-3) was not obtained with all donors tested and synergism between IL-8 and IL-3 could not be reproduced by all investigators (Kaplan *et al.*, 1991; P. Valent, unpublished data). This may be the result of the different experimental conditions and materials used. Of particular importance are the time and sequence of application of IL-8 when combined with other basophil agonists. Preincubation of basophils with IL-8 (10^{-7} to 10^{-9} *M*) may even inhibit histamine release from human basophils induced by HRF, CTAP-III/NAP-2 (Kuna *et al.*, 1991), and IL-3/IL-8 (Dahinden *et al.*, 1991; Bischoff *et al.*, 1991), but not by anti-IgE or formylmethionine. In this regard IL-8 seems functionally related to the 8-kD form of MNC-derived HRIF (Alam *et al.*, 1988, see also below), although the identities of the two molecules have so far not been established.

The chemotactic response of human basophils to rhIL-8 has recently been studied by several groups. Studies by Leonard *et al.* (1990) have shown that rhIL-8 (between 10^{-7} and 10^{-9} *M*) attracts normal human peripheral blood basophils (together with neutrophils) *in vitro*. This has more recently been confirmed by the use of highly enriched populations of CML blood pasophils by Rot *et al.* (1992). IL-8 by itself was found to be a rather weak agonist compared with C5a unless basophils had been preincubated with IL-3. On the other hand, IL-8 was found to enhance the chemotactic response of the basophils to a variety of basophil agonists including IL-3, formylated peptide, and C5a, which contrasts with the deactivating effect of (preactivation with) IL-8 obtained for basophil mediator release. Most surprisingly, however, IL-8 apparently even has the capacity to prime CML basophils for repetitive chemotactic activation with IL-8 (Rot *et al.*, 1992), which contrasts with the deactivating effect of IL-8 on neutrophil chemotaxis. These phenomena can at present not be explained and no evidence exists that similar mechanisms may be operative *in vivo*. If they are, the prolonged local production of IL-8 (and other cytokines) may lead to basophil accumulation, as has been observed in late-phase cutaneous reactions. Interestingly, IL-8 has no effect on basophil adhesiveness to endothelium (Bochner *et al.*, 1990).

Platelet basic protein (PBP) is stored in and released from (primarily

although not exclusively) platelet α granules. As compared with its derivatives, PBP by itself is biologically inactive. Proteolytic cleavage of amino acid residues from PBP results in the step-by-step formation of CTAP-III, β-TG, and NAP-2 (for review, see Oppenheim *et al.*, 1991). NAP-2 is less potent in stimulating neutrophils compared with NAP-1, whereas CTAP-III is ineffective. Recent data have shown, however, that both CTAP-III and NAP-2 alone cause basophil histamine release (Reddigari *et al.*, 1992). Histamine releasing factors have long been recognized in supernatants of stimulated MNCs (Thueson *et al.*, 1979; Kaplan *et al.*, 1985), lymphocytes (Sedgwick *et al.*, 1981; Haak-Frendscho *et al.*, 1988a), neutrophils (White and Kaliner, 1987), and monocytes and lung macrophages (Schulman *et al.*, 1985). Most significantly, however, human platelets have been described as producing and storing large amounts of HRFs (Orchard *et al.*, 1986). According to cell source, divergent molecular weight, and, more recently, dependence on IgE(+), the HRFs have been subclassified (Kaplan *et al.*, 1991; MacDonald *et al.*, 1987, 1991; Lichtenstein, 1988). Sequence analysis of an IgE-independent, low-molecular-weight (10–12 kDa) form of MNC-derived HRF revealed identity with CTAP-III/NAP-2 (Kaplan *et al.*, 1991).

Preliminary evidence exists that basophils express HRF binding sites (Ezeamuzie and Assem, 1987), although during the preparation of this review, no data on binding of iodinated NAP-2 to human basophils became available. Cross-competition analysis (performed on nonbasophil target cells as well as on basophils) suggests that NAP-2 (in high concentrations) may have the capacity to bind competitively to cell surface NAP-1 binding sites (Oppenheim *et al.*, 1991; Dahinden *et al.*, 1992). At low (more physiologic) concentrations of NAP-2, however, competition was incomplete (Leonard and Yoshimura, 1990; Oppenheim *et al.*, 1991). Whether the inhibitory effect of IL-8/NAP-1 on NAP-2-induced histamine release (see preceding text) reflects competitive binding to a basophil receptor or is due to nonspecific desensitization remains unknown.

Platelet factor 4 (PF-4), a potent heparin binding peptide, is another α IC. Like other ICs PF-4 is produced by and secreted from platelet α granules. PF-4 (as well as β-TG) exists as a tetramer under physiological conditions (i.e., at physiological ionic strength). The carboxy terminus of the peptide is highly acidic, whereas the amino terminus contains pairs of positively charged lysine residues separated by pairs of hydrophobic leucines or isoleucines. On a molar basis, PF-4 is far less potent than IL-8 in activating (or attracting) neutrophil granu-

locytes. Previous studies have shown that PF-4 (between 10^{-5} and 10^{-7} *M*) may induce histamine release from human basophils (Brindley *et al.*, 1983). Studies on structure–function relationships suggest that the carboxy terminus of PF-4 may be a critical site involved in the initiation of the release reaction. So far, however, no data on highly purified basophils have been reported, and whether human basophils or mast cells express receptors for PF-4 remains unknown. PF-4 apparently is unable to compete with IL-8 for IL-8 binding sites on myeloid target cells, whereas melanoma growth-stimulating activity (MGSA), also termed NAP-3, reportedly competes with IL-8 (NAP-1) in the binding to the IL-8 receptor.

Little is known so far about the effects of β ICs on human basophils or mast cells. Recent data suggest that MCAF (between 10^{-9} and 10^{6} *M*) induces release of histamine from human basophils (Kuna *et al.*, 1992). Enriched populations of human basophils were also found to respond to MCAF, suggesting the presence of a specific receptor. MCAF-induced release of histamine from human basophils is rapid (peak histamine release within 1 minute) and similar in magnitude to the release induced by anti-IgE.

B. Receptors for Formyl-methionine Peptides and Related Compounds

Formylated peptides are bacterial products modulating the functional properties of a variety of immune cells. Methionine-containing formyl tripeptides and dipeptides, between 10^{-7} and 10^{-4} *M*, induce release of histamine from human basophils (Hook *et al.*, 1976; Siraganian and Hook, 1977). Tripeptides usually are more effective than dipeptides (Hook *et al.*, 1976). Formylated peptides also induce chemotactic migration of human basophils together with neutrophils and other leukocytes. Cross-desensitization studies on human basophils induced with formylated peptides, inactive formyl peptides, C5a, and other secretagogues suggest that human basophils express a "specific" receptor (Siraganian and Hook, 1977; Hook *et al.*, 1976). Pharmacologic modulation of formyl-methionine (fMet) peptide-induced release from human blood basophils revealed a signal transduction pathway similar to that operative after addition of C5a. In contrast to the IgE-dependent reaction, the fMet-induced release of histamine from human basophils is rapid (several minutes), is optimal between 25 and 37°C, and is a single-step process.

Pepstatin A is a natural pentapeptide isolated from actinomycetes. Similar to the formylated peptides, pepstatin A has been shown to

induce release of histamine from human blood basophils (effective range 10^{-7} to 10^{-4} *M*) (Marone *et al.*, 1984). The kinetics of the release reaction on induction with pepstatin A and fMet peptides are very similar. Furthermore, pepstatin A-induced release from human basophils is competitively inhibitable by nonreleasing analogs of fMet peptides (dissociation constant 10^{-6} *M*) (Marone *et al.*, 1984). Thus, although the sequences of fMet peptides and pepstatin A are nonidentical, both agents apparently act via the same (or a closely related) binding site expressed on human basophils. Whether the basophil receptor for fMet peptides/pepstatin A is a member of the 7-TMS-RS remains to be elucidated. Microbial products and peptides acting on human basophils or mast cells may be involved in allergic reactions (or exacerbations) accompanying or following infectious disease states. Bacterial products such as fMet peptides may directly induce release of inflammatory mediators from basophils, whereas viral products (neuraminidases) usually upregulate releasability in respective target cells.

C. Receptors for Substance P and Other Neuropeptides

A number of observations support the concept of complex interactions between mast cells and the nerve system (Stead *et al.*, 1989; MacQueen *et al.*, 1989; Galli, 1990; Church *et al.*, 1991). Anatomical communications (appositions) between mast cells and nerve endings have previously been recognized by light microscopy (Stach, 1961; Newson *et al.*, 1983; Skofitsch *et al.*, 1985) and more recently by electron microscopy (Stead *et al.*, 1987, 1989; Stead and Bienenstock, 1989; Bienenstock *et al.*, 1987; Arizono *et al.*, 1990). Thus, histological examinations revealed mast cell apposition to (peptidergic) nerves in rat intestine, lung, skin, and heart, as well as in the human gastrointestinal mucosa. At present the reasons for the local association between mast cells and nerve endings is not fully understood. Some observations suggest that the local proximity is nonrandom and not (only) a function of chance (Bienenstock *et al.*, 1987); however, the intimate contact (often less then 100 nm) has also been observed between nerve processes and other hemopoietic cells in mucosal areas (Arizono *et al.*, 1990).

Functional studies in the murine system have shown that antidromic electrical stimulation of sensory nerves may induce degranulation of skin mast cells and may be associated with vasodilation, augmented vascular permeability, and increased granulocyte infiltration (Kiernan, 1972; Blandina *et al.*, 1984; Bani-Sacchi *et al.*, 1986). These antidromic reactions can in part be blocked by pretreatment of local areas with H-1

antagonists (Graham and Lioy, 1973; Powell and Brody, 1976; Lembeck and Holzer, 1979). Moreover, Pavlovian conditioning experiments on rats have suggested an asociation between the central nervous system and the mast cell system (MacQueen *et al.*, 1989). The peptidergic primary afferent neuron (as well as other neuronal cells) contains a number of neuropeptides known to induce or promote (pro)inflammatory reactions and vasodilation. These mediators include the tachikinins substance P, neurokinin A, and calcitonin gene-related peptide. The effects of substance P and the other aforementioned compounds are mediated via specific receptors.

Substance P (SP), an undecapeptide, apparently is one of the more important effector molecules involved in neurogenic inflammation. Injection of SP (10 p*M*) into human skin results in a dose-related wheal-and-flare reaction (Hägermark *et al.*, 1978; Foreman *et al.*, 1983). Injection of SP is associated with degranulation of mast cells in local (skin) areas. Antihistamines may in part attenuate the SP-induced skin response, in particular the flare reaction (Foremen, 1987). These observations together with more recent studies on mast cell-deficient (W) mice (Yano *et al.*, 1989) suggest that the SP-induced reactions in the skin are at least in part mast cell dependent.

A number of *in vitro* studies have shown that SP (between 10^{-4} and 10^{-7} *M*) induces release of inflammatory mediators from rat peritoneal mast cells and human skin mast cells in a dose-dependent manner (Johnson and Erdös, 1973; Foreman 1987; I. D. Lawrence *et al.*, 1987; Benyon *et al.*, 1987; Lowman *et al.*, 1988; Church *et al.*, 1991). The time course of SP-induced degranulation of human skin mast cells is rapid, with maximum release observed within 30 seconds. SP-induced release of histamine from human (skin) mast cells is accompanied by an increase in intracellular calcium but (in contrast to IgE-dependent release reactions) is independent of the presence of extracellular calcium (Benyon *et al.*, 1987; Church *et al.*, 1991). SP-induced release from mast cells also differs from IgE-dependent release by the failure to promote synthesis of larger amounts of prostaglandin D_2 (Benyon *et al.*, 1989). SP failed to induce histamine release from human lung or gut mast cells, supporting the concept of mast cell heterogeneity (Ali *et al.*, 1986; Lowman *et al.*, 1988; Church *et al.*, 1991). Human blood basophils were also found to be unresponsive to SP. The SP-induced release of histamine from (pure) human skin mast cells is accompanied by an elevation of cellular cAMP levels (Church *et al.*, 1991).

The response of pure populations of human skin mast cells to SP (Benyon *et al.*, 1987; Lawrence *et al.*, 1987; Church and Hiroi, 1987) has suggested the presence of functionally active binding sites. The

exact nature of the mast cell receptor for SP remains unknown. From functional analyses of skin mast cells using eledoisin and physalaemin (agonists at smooth muscle SP receptors that both failed to mimic the SP effect on skin mast cells) the investigators concluded that the mast cell SP receptor differs from the classical (P- and E-type or NK-1-, NK-2-, or NK-3-type) receptors for SP indentifiable on smooth muscle cells, in salivary glands, and in other tissues (Iversen, 1982; Lee *et al.*, 1986). Studies using synthetic SP antagonists (in particular SP_{4-11}[D-Pro^4, D-$Trp^{7,9,10}$] suggest that SP, vasoactive intestinal peptide, compound 48/80, morphine, and poly-L-lysine act via a common receptor on human skin mast cells (Lowman *et al.*, 1988; Church *et al.*, 1991). This receptor may be a receptor with low specificity for basic secretagogue compounds and may be (directly) associated with G-proteins. Whether this receptor type is associated with the 7-TMS-RS remains to be determined.

Studies of rat mast cells (Piotrowski *et al.*, 1984) and more recently of human skin mast cells (Lowman *et al.*, 1988; Church *et al.*, 1991) using SP fragments were conducted to investigate structure–function relationships. The octapeptide SP_{4-11} lacking the amino-terminal end Arg–Pro–Lys failed to induce release of histamine. A similar fragment, SP_{4-11}[D-Pro^4, D-$Trp^{7,9,10}$], however, inhibits SP-dependent release of histamine in a competitive way (Piotrowski *et al.*, 1984). These observations suggest that the amino terminus is in some way involved in the induction of release. The lipophilic carboxy terminus, in turn, appears to be involved in the binding to the mast cell receptor rather than being the critical site for initiating or promoting the release reaction. This hypothesis is supported by the fact that the stepwise removal of amino acids from the lipophilic carboxy terminus of SP is associated with a gradual loss of binding to mast cells and the capacity of the SP fragment(s) to induce release (Foreman, 1987; Lowman *et al.*, 1988). The tetrapeptide Arg–Pro–Lys–Pro, the amino-terminal sequence of SP, failed (at all) to induce release.

A number of other neuropeptides may be involved in mast cell activation and in neurogenic inflammatory responses. Calcitonin gene-related peptide (CGRP) and neurokinin A have been detected in primary afferent neurons together with substance P (J. Fischer *et al.*, 1985; Nawa *et al.*, 1983). CGRP induced release of histamine from rat mast cells but is less effective compared with SP (Foreman, 1987), whereas neurokinin A is ineffective. Somatostatin and vasoactive intestinal polypeptide (VIP) are two further candidates for neuronergic mast cell activators (see earlier text). Both peptides induce release from rat peritoneal mast cells and human skin mast cells and are at least equally potent (compared to SP) in this respect. Furthermore, both

peptides as well as CGRP induce a wheal-and-flare reaction in human skin (Piotrowski and Foreman, 1985, 1986).

VII. Complement Binding Sites

The complement (C) system is a group of plasma peptides termed C1, C2 . . . C9. In normal plasma the complement components are present in an inactive form. When activated during an immune response these components become potent effector molecules (reviewed by Gherbrehiwet, 1985). Activation of complement is usually initiated by antigen–antibody complexes involving IgG or IgM (classic pathway) or by alternative pathway(s). During complement activation the biologically active components of complement are formed (mostly by proteolytic cleavage) in a characteristic cascade, starting with C1 and C1q esterase and resulting in the formation of the membrane attack complex C5b,7,8,9.

The biological activities of the C components are pleiotropic and include induction and/or potentiation of chemotaxis, opsonization, cell recognition, cellular cytotoxicity, and anaphylactic responses. The specific interactions of the complement system with a variety of immune cells involve cellular receptors for the distinct components of complement (for review, see Schreiber, 1984).

Three structurally related complement components, C3a, C4a, and C5a, have collectively been termed *anaphylatoxins* because they were found to be able to elicit an anaphylactic response (with erythema and edema) *in vivo* (Hugli and Müller-Eberhard, 1978; Gorski *et al.*, 1979; Hugli, 1981). A number of previous studies have shown that anaphylatoxins also induce release of mediators from human basophils *in vitro* (Hook *et al.*, 1975; Siraganian and Hook, 1976; Hugli and Müller-Eberhard, 1978; Hugli, 1981; Dvorak *et al.*, 1981). C5a by itself (without a second stimulus) is a potent inducer of the release reaction (effective concentrations 10^{-10} to 10^{-6} *M*) (Siraganian and Hook, 1976; Hugli and Müller-Eberhard, 1978; Kurimoto *et al.*, 1989). C5a-induced release of histamine from human basophils requires extracellular calcium (in contrast to IgE-mediated relase), is optimal at 25°C, and occurs within several minutes of agonist stimulation (Siraganian *et al.*, 1975; Warner *et al.*, 1989; MacGlashan and Warner, 1991). C5a is unable to induce formation and release of LTC4 in human basophils; however, when primed with IL-3 (which also failed to induce LTC4 formation), basophils express and release substantial amounts of LTC4 (Kurimoto *et al.*, 1989). In addition, IL-3 upregulates C5a-induced release of histamine from human basophils. C5a also has been de-

scribed as inducing chemotactic migration of human blood basophils (Lett-Brown *et al.*, 1976). From the kinetics of the reactions (release and chemotaxis) as well as from studies using enriched target cell populations it might be concluded that human blood basophils express high-affinity C5a binding sites. So far, however, a cellular substrate has not been characterized. Interestingly, the recently cloned C5a receptor was found to belong to the 7-TMS-RS (Dohlman *et al.*, 1991). This may explain the similarity in the functional responses of the basophils to C5a, formylated peptides, and other activating peptides probably acting via 7-TMS receptors (see also Section VI).

In previous studies C3a (and C4a) has been reported to induce release of histamine from human basophils but the concentrations of C3a used to demonstrate an effect were high (micromolar) and, when ultrapure C3a was used, no significant effect was seen (Glovsky *et al.*, 1979; Hugli, 1981; Bischoff *et al.*, 1990a). Recently, however, Bischoff *et al.* (1990a) demonstrated that after preincubation with IL-3 or GM-CSF, human basophils release substantial amounts of histamine (and LTC4) in response to very low concentrations (nanomolar) of C3a. These observations raise the possibility that basophils possess (high-affinity) C3a receptors, which are inducible (or functionally upregulated/activated) in their expression by distinct cytokines. A cellular substrate for the C3a receptor expressed on basophils has not been defined so far.

For other components of the complement system no direct effect on human basophil release has been documented; however, serum-treated zymosan particles (zymosan activates the alternative pathway of complement) have been shown to enhance releasability in human blood basophils (Thomas and Lichtenstein, 1979). As C3b constitutes the "recognition unit" of the alternative pathway it was proposed that basophils express C3b binding sites. Evidence that human blood basophils indeed express a C3b binding site came from studies using mAbs to CR1 (CD35) (see later).

Human mast cells have also been tested for their response to C peptides. Highly enriched human lung mast cells do not release histamine on induction with C5a (Schulman *et al.*, 1988). Similar results have been obtained with adenoidal mast cells by Jürgensen *et al.* (1988). In contrast, human skin mast cells have been reported to respond to C5a, although the proportion of released mediator was rather low (below 10% of total histamine) and the possibility of another target cell type or indirect effect could not be excluded (Schulman *et al.*, 1988). Interestingly, C5a has been described as inducing release of histamine from rat mast cells (Johnson *et al.*, 1975).

The distribution and structure of a number of C receptors have recently been defined by molecular cloning of genes and the use of mAbs to leukocyte cell surface membrane structures. The C5a receptor is a member of the 7-TMS-RS as mentioned earlier. Complement receptor type 1 (CR1) as well as CR2, is a member of the C3b/C4b binding protein superfamily (Kristensen *et al.*, 1986). As determined by probing with CD35 mAbs, human basophils (but not human lung mast cells, intestinal mast cells, or human skin mast cells) express CR1 (deBoer and Roos, 1986; Stain *et al.*, 1987; Bochner *et al.*, 1989b; Valent *et al.*, 1989b). The CR2/CD21 binds C3d and constitutes the B cell binding site for the Epstein–Barr virus (reviewed by Cooper *et al.*, 1988). Neither human basophils nor human tissue mast cells bind CD21 mAbs (Stain *et al.*, 1987; Valent *et al.*, 1989b).

The C3bi receptor (CF3, CD11b) is a member of the CD11/CD18 (β2 integrin) family. In common with other myeloid cells human blood basophils bind CD11b and CD18 mAbs. Human mast cells enriched from lung, intestinal tissue, uterus, or skin (as well as HMC-1 cells) lack detectable amounts of CD11b as assessed by indirect immunofluorescence (Valent *et al.*, 1989b; Guo *et al.*, 1992) (see also Tables I and VII). The functional significance of the basophil C3bi receptor has so far not been defined. CR4 is also a member of the CD11 family (CD11c). Human basophils express CD11c as determined by indirect immunofluorescence. Human mast cells obtained from lung, skin, intestine, and ascites, as well as HMC-1 cells, were found to lack CD11c (Valent *et al.*, 1989b). Controversial results have been obtained for uterine mast cells (see Section V). Table VII summarizes the complement receptors expressed on human mast cells and human basophils.

TABLE VII
EXPRESSION OF COMPLEMENT RECEPTORS ON HUMAN BASOPHILS AND HUMAN MAST CELLS AS DETERMINED BY INDIRECT IMMUNOFLUORESCENCE

Complement receptor	CD	Expression of complement receptors on						
		ba[a]	KU-812	l-mc	s-mc	a-mc	u-mc	HMC-1
CR1	35	+	+	−	−	−	nk	−
CR2	21	−	−	−	−	−	nk	−
CR3	11b	+	+	−	−	−	−	−
CR4	11c	+	+	−	−	−	+/−	−

[a] ba, basophils; l-mc lung mast cells; s-mc, skin mast cells; a-mc, ascites mast cells; u-mc, uterine mast cells; nk, not known; +/−, small subset of cells positive.

VIII. Immunoglobulin Receptors

A. The High Affinity Receptor for IgE/FcεRI

1. Nomenclature

Immunoglobulins are highly specialized polypeptide molecules involved in antigen (Ag) recognition, Ag capture, and Ag processing. According to their different biochemical and functional properties, Ig subtypes have been defined. IgE was identified on the basis of its reaginic activity by K. Ishizaka *et al.* (1966a,b). Specific cell surface receptors for IgE are expressed on a variety of immune cells. Low-affinity IgE binding sites are widely distributed (Gordon *et al.*, 1989; Conrad, 1990). High-affinity binding sites for IgE, however, are expressed almost exclusively on mast cells and basophils (Metzger *et al.*, 1986; Kinet, 1990; Ravetch and Kinet, 1991). In common with other receptors for Igs, high-affinity IgE binding sites bind the Fc portion of Fcε. The mast cell/basophil receptor for IgE has been termed FcεRI (Metzger *et al.*, 1986; Kinet, 1990) to distinguish it from the low-affinity FcεRII (CD23), expressed on lymphocytes, macrophages, eosinophils, and platelets (Ravetch and Kinet, 1991). The mast cell/basophil receptor for IgE apparently is involved in the immediate type response to an antigen/allergen (Lichtenstein and Osler, 1964; K. Ishizaka *et al.*, 1966a, 1970a,b; Osler, 1966; Sampson and Archer, 1967; Sullivan *et al.*, 1971; Lichtenstein, 1971; T. Ishizaka *et al.*, 1972; Becker *et al.*, 1973; T. Ishizaka and K. Ishizaka, 1975, 1978).

Primary cells as well as cell lines expressing FcεRI molecules have been used to characterize the receptor in detail. Earlier studies in the human system were performed with primary cells exclusively, as human cell lines expressing FcεRI molecules were not available. More recent studies were performed with the human basophil leukemia cell line KU-812. Other species analyzed in detail are mouse and rat. The rat basophilic leukemia cell line, RBL (a mucosal mast cell-like cell line) (Kulczycki *et al.*, 1974; Conrad *et al.*, 1976), has been widely used to analyze rat FcεRI molecules.

2. Topology of the FcεRI Multichain Complex and Biochemical Characterization of Its Subunits

The high-affinity receptor for IgE represents a tetrameric complex (Metzger *et al.*, 1983, 1986, 1989; Kinet, 1990). It consists of three distinct peptides: the α chain (Kanellopoulos *et al.*, 1980), the β chain (Holowka *et al.*, 1980; Holowka and Metzger, 1982), and a disulfide-linked homodimer formed by two identical γ chains (Perez-Montfort *et al.*, 1983). The tetrameric model holds true for the three species analyzed (rat, mouse, and human).

a. α Chain

The α chain of the mast cell receptor for IgE is a heavily glycosylated protein (Kulczycki *et al.*, 1976). Approximately 30% of its molecular mass represents carbohydrate (Goetze *et al.*, 1981; Hempstead *et al.*, 1979a; Kanellopoulos *et al.*, 1980). Depending on the species and cell types analyzed, the molecular mass of the α chain (as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis) varies from 45 to 65 kDa (Conrad and Froese, 1976; Kulczycki *et al.*, 1976; Hempstead *et al.*, 1979a,b; Conrad *et al.*, 1983). Differences in the molecular mass of α chains are most probably due to different grades of glycosylation. The protein backbone of α has been the subject of extensive biochemical analysis (Kulczycki *et al.*, 1976; Hempstead *et al.*, 1979a,b; Goetze *et al.*, 1981; Kumar and Metzger, 1982; Perez-Montfort *et al.*, 1982; Basciano *et al.*, 1985).

The recently cloned cDNA for the (human) α chain predicted a protein trunc of 260 amino acids with molecular weight of 26.4 kDa for the unprocessed peptide (Shimizu *et al.*, 1988; Kochan *et al.*, 1988). The deduced amino acid sequence for rat (245 residues) and mouse (250 amino acid residues) α chains revealed a similar size (Kinet *et al.*, 1988; Ra *et al.*, 1989b; Liu *et al.*, 1988; Tepler *et al.*, 1989). The slight differences between the species analyzed are due to the varying length of the cytoplasmic portion of α. This might be the result of transcriptional modification(s). Sequence analysis and hydropathicity plots of (human, mouse, and rat) α revealed a single transmembrane domain (Shimizu *et al.*, 1988). The extracellular tail contains the amino-terminus bearing a characteristic signal peptide. The hydrophobic carboxy terminus apparently is embedded within the cytoplasm (Shimizu *et al.*, 1988). The extracellular domain is composed of about 180 amino acid residues. It contains two immunoglobulin-like domains of 40 and 42 residues, respectively. These domains each expose two cysteines supposed to form two disulfide bonds (by analogy to the tertiary structure of Fcγ receptors, see Ravetch and Kinet, 1991). The extracellular domains of α contain six (human and mouse) or seven (rodent) amino-linked glycosylation sites (Shimizu *et al.*, 1988). The glycosylation sites on α apparently are not directly involved in binding to IgE (Kulczycki and Vallina, 1981).

b. β Chain

The nonglycosylated β chain represents a highly hydrophobic polypeptide with a molecular mass of 30–35 kDa (Holowka *et al.*, 1980; Holowka and Metzger, 1982). Two different fragments have been obtained by proteolytic digestion: The β1 fragment (23 kDa) is associated with the plasma cell membrane. β2 is a phosphorylated pep-

tide of 9–10 kDa and apparently represents the cytoplasmic fraction of β.

Complementary DNAs for mouse and rat β chains have been isolated (Ra *et al.*, 1989b; Kinet *et al.*, 1988). A cDNA for the human β chain has recently been obtained as well, but has not been characterized in detail so far. Preliminary results suggest a rather high (about 70%) identity between rodent and human β chains (see Kuster *et al.*, 1990). The amino acid sequences deduced for mouse and rat β chain(s) predicts proteins of 235 and 243 residues, respectively, with corresponding molecular masses of 25.9 kDa and 27 kDa for the processed peptide(s). Unlike the α chain, the β chain contains no hydrophilic leader sequence at the amino terminus. Sequence analyses and hydropathicity plots of the β chain sequence have shown that it contains four transmembrane segments. Both the amino and carboxy termini are located within the cytoplasm. Thus, the most likely topology for the β chain is a fourfold membrane-spanning peptide with intramembranous and cytoplasmic domains and minimal external regions located close to the transmembrane sites. This model is also consistent with earlier studies and labeling experiments on FcεRI molecules (Holowka *et al.*, 1980; Lee and Conrad, 1985; Rivnay *et al.*, 1982, 1984) as well as with studies using monoclonal antibodies to β (Kinet *et al.*, 1988).

c. γ Dimer

The γ subunit is a disulfide-linked homodimer (Perez-Montfort *et al.*, 1983). Each monomer exhibits a molecular mass of approximately 7–9 kDa as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Perez-Montfort *et al.*, 1983; Alcaraz *et al.*, 1987). A corresponding molecular mass (7.8 kDa) was predicted for the processed peptide from sequencing of cDNA clones isolated from human (Kuster *et al.*, 1990), mouse (Ra *et al.*, 1989b), and rodent (Blank *et al.*, 1989) libraries. Hydropathicity plots have suggested a single transmembrane peptide with a typical leader sequence. The mature peptide contains two cysteine but no tryptophan, histidine, or methionine residues. A predicted histidine residue at the carboxy terminus was identified within the precursor protein but not within the processed peptide (Blank *et al.*, 1989). The disulfide link is located within the transmembrane region close to the amino-terminus at Cys 7. Studies using mutated γ subunits suggest that the second, carboxy-terminal cysteine residue (Cys 26) is not involved in γ chain dimerization (Varin-Blank and Metzger, 1990). The γ chain is phosphorylated on four threonine residues (Perez-Montfort *et al.*, 1983). The amino-

terminal portion contains two tyrosine residues on which the mature protein can be iodinated on inverted vesicles but not on intact cells (Holowka *et al.*, 1985), suggesting that the amino terminus is embedded within the cytoplasm. A small external domain with five residues at the amino terminus was predicted from sequence analyses.

Thus, each of the subunits of FcεRI contains external and internal domains and the external domains are linked to the cell interior via distinct transmembrane regions. The IgE receptor complex appears to contain seven transmembrane domains, four of which are part of the β chain. The exact tertiary structure of the FcεRI complex has so far not been delineated.

3. *Assembly*

A number of recent observations and, in particular, gene transfer analyses suggest that interactions between FcεRI subunits play a crucial role in receptor assembly and display. The critical functional region of FcεRI is the α chain, as it contains the IgE binding site. Appropriate expression of α apparently depends on the presence of other components of the receptor complex. Thus, expression of α chains alone in transfected cells is not accompanied by significant exposure of functional IgE binding sites on the cell surface (Kinet *et al.*, 1988; Shimizu *et al.*, 1988; Ra *et al.*, 1989a,b); however, cotransfection of human α and γ chains results in appropriate expression of functional α subunits (Ra *et al.*, 1989a,b; Blank *et al.*, 1989; Kuster *et al.*, 1990; Miller *et al.*, 1989). The β subunit of the human receptor was not required in such transfer experiments. Interestingly, in rats expression of both β and γ is required for surface expression of the α subunit in transfected cells.

Studies on detergent-induced disruption of the receptor (Metzger *et al.*, 1986) as well as more recent studies on mutated receptors (Varin-Blank and Metzger, 1990) suggest that the transmembrane regions of the FcεRI subunits are the most critical loci for receptor assembly and display. This is probably because selective interaction(s) between receptor subunits takes place within the cell surface membrane. Recent studies have focused on the mode of interaction of FcεRI subunits. Several observations support the notion of lipid-dependent interactions. The FcεRI complex dissociates in mild (Kinet *et al.*, 1985a,b; Rivnay *et al.*, 1982) but not in submicellar detergent (Kinet *et al.*, 1985a,b; Alcaraz *et al.*, 1984). Moreover, disruption of FcεRI complexes in mild detergent is inhibitable by addition of phospholipid (Kinet *et al.*, 1985a,b; Rivnay *et al.*, 1982). Studies on detergent-induced disruption of receptors as well as studies using antibodies to β

suggest that β and γ chains always dissociate from α in unison (Kinet *et al.*, 1985a,b; Rivera *et al.*, 1988). Whether the interactions between β and γ involve disulfide bridging remains to be elucidated. If so, Cys 26 of γ would be the candidate because it is topologically in plane with Cys 80 of β and because the second cysteine of γ (Cys 7) apparently is involved in γ dimer formation and is not in the vicinity of the cysteine on the β chain (Varin-Blank and Metzger, 1990). The disulfide bond of the γ chain (Cys 7–Cys 7) is located within the transmembrane region. Interestingly, Cys 7 apparently is nonessential for surface expression of functional FcεRI molecules as assessed by site-directed mutagenesis of γ (Varin-Blank and Metzger, 1990).

4. Chromosomal Location, Genomic Organization, and mRNA Species

The subunits of the high-affinity receptor for IgE are encoded by distinct genes. The gene encoding for the α chain is located on the long arm of human chromosome 1, in a region (1q23) containing the genes for several Fcγ receptors (Ravetch and Kinet, 1991). The mouse α gene also maps to chromosome 1 (Huppi *et al.*, 1989). The (rat) gene encoding for the α chain of FcεRI is composed of five exons and spans about 6.6 kb (Tepler *et al.*, 1989). Exons 1 and 2 code for the leader peptide, exons 3 and 4 for the immunoglobulin-like domains, and exon 5 for the transmembrane and cytoplasmic domains (Tepler *et al.*, 1989). Transcripts specific for the α chain have been detected in mast cells and basophils of human (1.1 kb) and rodent (1.3 kb) origin. Expression of additional mRNA species in RBL cells (Tepler *et al.*, 1989; Liu *et al.*, 1988) most probably is due to alternative splicing. In one case, 163 base pairs at the 3′ end of the fourth exon were removed, supporting the possibility of a secreted form of α; however, a secreted form of α has so far not been identified by mAbs in extracellular fluids.

The gene for the β chain of the FcεRI complex is located on mouse chromosome 19, in the vicinity of a related gene encoding for CD20 (Huppi *et al.*, 1989; see also later). The chromosomal location of the human β chain is not available at present. The rat and mouse sequences revealed lengths of 729 and 705 bp, respectively. Two major species of β mRNA (2.7 and 1.75 kb) have been detected in rodent cells. The existence of these two major mRNA species apparently is the result of alternative polyadenylation.

The γ chain of FcεRI is located on human chromosome 1 (1q23) in the vicinity of the α chain (see preceding text). The mouse γ chain gene is also located on chromosome 1. The human γ subunit contains 5 exons (Kuster *et al.*, 1990). The first exon contains the leader peptide sequence, exon 2 encodes for the extracellular and transmembrane

domains, and exons 3, 4, and 5 code for most of the cytoplasmic tail. A single mRNA species (0.85 kb) has been detected in human, rats, and mice. In contrast to α and β subunit mRNAs, transcripts for γ have been detected in various hemopoietic cells (Ravetch and Kinet, 1991) consistent with the observation that γ is not restricted to Ig receptors on mast cells or basophils (see later).

5. *Sequence and Structural Homologies*

a. Conservation during Evolution

Cloning of rat, mouse, and human subunits of the high-affinity receptor for IgE revealed significant interspecies homology (Kinet, 1990). The level of conservation differs among the different subunits as well as between different sites in a given subunit. The γ chain apparently has best been conserved with a consensus sequence for the three species (rat mouse, and human) containing 86% identical amino acid residues (Kinet, 1990). The α chain has less perfectly been conserved (38 percent identical residues in consensus sequence). An extremely high conservation was observed for the transmembrane domains (Kinet, 1990). Maximal conservation was found for residues in transmembrane regions predicted to be buried by analysis of hydrophobic moments (Varin-Blank and Metzger, 1990).

b. Molecules Related to FcεRI α Chain

The FcεRI α chain revealed sequence homology to Fcγ receptor subunits and, in common with these receptors, belongs to the immunoglobulin supergene family. Moreover, the genes coding for these receptors are clustered on chromosome 1 and are likely to be derived from a common ancestral precursor (Ravetch and Kinet, 1991). The most significant homology (95% sequence identity in both intron and exon sequences) was found between FcεRI α and FcγIII-A α molecules expressed on macrophages and natural killer cells. Expectedly, structural similarities at the DNA and protein level between FcεRI and FcγRIII-A molecules also exist. No significant sequence homology exists between IgERI α chain and other known IgE binding factors, in particular the low-affinity receptor for IgE (CD23) expressed on lymphocytes, macrophages, and eosinophils. No differences in the sequence or structure of FcεRI molecules expressed on basophil granulocytes versus tissue mast cells have ever been suspected.

c. Molecules Related to FcεRI β Chain

The β subunit of the FcεRI complex revealed substantial homology to CD20. Both CD20 and FcεRI β chain contain four transmembrane domains and are encoded by genes on mouse chromosome 19. Prelimi-

nary evidence exists that CD20 and related peptides are associated with transmembrane ion gates.

d. Molecules Related to FcεRI γ Chain

The FcεRI and the FcγRIII-A complex (CD16) appear to share an identical γ subunit (Ra *et al.*, 1989a,b; Hibbs *et al.*, 1989). Surprisingly, the γ chain also revealed significant sequence homology (up to 50% in the respective exon structures) to the CD3 ζ subunit of the T cell receptor (TcR)/CD3 complex (Kuster *et al.*, 1990). Like the FcεRI γ chain, the TcR ζ subunit is a disulfide-linked homodimer involved in receptor assembly. More recently, transfection experiments have shown that (human) CD3 zeta may even substitute for rat FcεRI γ chain in Fcε receptor assembling (Howard *et al.*, 1990).

6. Control of Synthesis and Expression of FcεRI Molecules

a. Synthesis during Differentiation of Basophils/Mast Cells from Their Hemopoietic Precursor Cells

Basophils and mast cells are derived from immature hemopoietic progenitor cells lacking FcεRI molecules. Under a variety of conditions, growth and differentiation of metachromatic cells as well as expression of IgE binding sites can be induced by T cell (IL-3, IL-5, GM-CSF)- or stroma cell (SCF, GM-CSF)-derived factors (Razin *et al.*, 1981; Ogawa *et al.*, 1983; Tadokoro *et al.*, 1983; Leary and Ogawa, 1984; T. Ishizaka *et al.*, 1985a; Denburg *et al.*, 1985b; Seldin *et al.*, 1986). IL-3 induced growth of metachromatic cells (i.e., basophils) and expression of IgE binding sites in human bone marrow cell cultures (Saito *et al.*, 1988; Valent *et al.*, 1989a; Kirschenbaum *et al.*, 1989). A similar response of murine mast cell precursors to T cell factors (including IL-3) was observed in the murine system (Ihle *et al.*, 1983; Razin *et al.*, 1984). IgE receptors expressed on cultured basophils/mast cells have similar (although not identical) biochemical and functional characteristics compared with primary cells (Ogawa *et al.*, 1983; T. Ishizaka *et al.*, 1985a). Moreover, as determined by Northern blot analyses, cultured basophils/mast cells express FcεRI α mRNA, likewise expressed in normal mast cells (H. L. Thompson *et al.*, 1990). Interestingly, synthesis of FcεRI α chain mRNA precedes the formation of metachromatic granules in culture (H. L.Thompson *et al.*, 1990). Therefore, induction of synthesis of FcεRI α chain mRNA appears to be an early step in IL-3-dependent differentiation of progenitor cells toward the basophil/mast cell pathway. IL-3 was also found to upregulate binding of IgE to cell surface receptors on the human basophil cell line KU-812 (Valent *et al.*, 1990a) and to promote expres-

sion of IgE binding sites on primary (pre)mature CML basophils (Valent *et al.*, 1989c). Whether IL-3 is capable of inducing FcεRI molecules on fully mature, normal basophil granulocytes remains to be determined. Other cytokines inducing expression of human FcεRI molecules under certain conditions include GM-CSF, IL-5, TGF-β, NGF, and SCF. Induction of expression of FcεRI on mast cell progenitors by human SCF is restricted to long-term culture conditions (Valent *et al.*, 1992).

b. Synthesis during the Cell Cycle

Expression of IgE binding sites during the cell cycle has been studied in RBL cells using radiolabeled IgE (Isersky *et al.*, 1975). During mitosis the density of the receptor declines, whereas the total number of IgE binding sites per culture remains unchanged. This is most likely because no further receptor is acquired by the dividing cells. An accumulation of receptor was found in cells arrested in G_1, but not in S or G_2.

As known for other surface proteins, an inverse relationship exists between the growth rate and expression of molecules. In the presence of a differentiation-inducing factor the situation may be different and the receptor density may even increase. Receptor labeling studies on RBL cells suggest coordinate and parallel synthesis, assembly, and degradation of all subunits of the FcεRI complex (Quarto *et al.*, 1985).

c. Dependence on IgE

Studies on binding of iodinated IgE to circulating blood basophils suggest a positive correlation between the number of IgE binding sites per basophil and the amount of circulating IgE. The observed relationship appeared to be significant in one study (Malveaux *et al.*, 1978) but not in another (T. Ishizaka *et al.*, 1973). To explain the possible relationship between IgE level and number of FcεRI molecules per cell, a number of hypotheses have been raised. The most likely comes from *in vitro* experiments with RBL cells suggesting that IgE, once bound to IgE receptors, prevents the disappearance of these (now occupied) IgE binding sites from the cell surface membrane (Furuichi *et al.*, 1985a,b; Quarto *et al.*, 1985; see also later).

d. Half-Life, Internalization, Degradation, and Reexpression

Studies on RBL cells suggest that unliganded FcεRI molecules are constantly replaced by novel receptors (Furuichi *et al.*, 1985a,b). The half-life of unliganded FcεRI molecules on RBL cells appears to

be 8–12 hours. The half-life of the receptor liganded with (monomeric)IgE, however, appears to be much longer (more than 30 hours) (Isersky *et al.*, 1979; Furuichi *et al.*, 1985a,b). Thus, liganded cells expose large amounts of FcεRI molecules for a long period (Isersky *et al.*, 1979). The exact mechanisms by which IgE upregulates expression of FcεRI molecules at present remain unknown. One speculation is that IgE simply prevents receptor internalization. Another possibility could be that IgE prevents molecular degradation of the (recycling) receptor.

Antigen-induced bridging of IgE binding sites on FcεRI-bearing cells results in formation of aggregates, patches, and caps, with subsequent internalization of IgE–IgE receptor complexes (see later). Internalization of aggregated FcεRI molecules has extensively been analyzed by using oligomeric IgE and RBL cells (Isersky *et al.*, 1983; Furuichi *et al.*, 1984, 1986). In contrast to the unliganded receptor (or the receptor liganded with monomeric IgE), the half-life of the receptor liganded with oligomeric IgE or multivalent antigen on RBL cells is quite short (several minutes) because of rapid internalization of the IgE–IgE receptor complexes (Isersky *et al.*, 1983). Once internalized with oligomeric ligand or antigen, the IgE receptor seems not to be reusable or to recycle to the cell surface within a short time (Isersky *et al.*, 1983; Furuichi *et al.*, 1986). Recycling IgE and FcεRI may be found in a degraded form (in cell supernatants) after endocytosis (Furuichi *et al.*, 1986). Interestingly, empty receptors might be co-internalized with oligomer-liganded receptors. How FcεRI molecules are internalized and degraded remains unknown. The interaction of FcεRI molecules with cytoskeletal elements following aggregation may be of importance (see later). Unlike the release process, neither the process of cluster formation nor the process of receptor internalization requires external calcium.

7. *Numbers and Binding Constants on Primary Cells*

Basophils and mast cells constitutively express a single class of high-affinity IgE binding sites on their cell surface. The number of IgE receptor complexes on human basophil granulocytes ranges from 10,000 to 500,000 (average 170,000) sites per cell (K. Ishizaka *et al.*, 1970a,b; T. Ishizaka *et al.*, 1973; Conroy *et al.*, 1977; Malveaux *et al.*, 1978; Pruzansky and Patterson, 1986). The density of FcεRI molecules on purified human lung mast cells exhibits a similar range (MacGlashan *et al.*, 1983b). No significant differences were found for atopic individuals compared with nonatopic controls. A positive correlation exists between the total number of FcεRI molecules expressed on

blood basophils, the number of IgE molecules per basophil, and the serum IgE level (Malveaux *et al.*, 1978). The affinity of the FcεRI complex to its ligand is high. Usually 70–90% of FcεRI molecules on primary cells are occupied by the natural ligand. Binding studies suggest a dissociation constant K_D of 10^{-9} to 10^{-11} M depending on the cell types analyzed and the methods applied (K. Ishizaka *et al.*, 1970a,b; T. Ishizaka *et al.*, 1973; Kulczycki *et al.*, 1974; Malveaux *et al.*, 1978). *In vitro* as well as *in vivo* studies suggest a nonlinear mode of dissociation of IgE from FcεRI-bearing target cells. Dissociation of iodinated IgE from RBL cells could be resolved into two phases with fast ($t_{1/2}$ = 7–12 hours) and slow ($t_{1/2}$ = 6–11 days) half-times (Isersky *et al.*, 1979). The slow half-time was also observed in experiments using solubilized FcεRI molecules (Rossi *et al.*, 1977). In an *in vivo* study by K. Ishizaka and T. Ishizaka (1971), iodinated IgE disappeared from monkey skin with biphasic half-times of 3–4 and 8.5–14 days, respectively. The slower component of dissociation of IgE from FcεRI complexes may provide a selective protection (of IgE) against degradation and catabolism and explain the long half-time of reaginic activity (up to several weeks) found in the early studies by Prausnitz and Küstner (1921) as well as in later studies (Augustin, 1967; Cass and Anderson, 1968; K. Ishizaka and Ishizaka, 1971; T. Ishizaka and Ishizaka 1975). The extremely long half-time of IgE–IgE receptor complexes *in vivo* might in part be due to the fact that dissociated IgE (in contrast to internalized, recycling IgE) is functionally active and capable of rebinding to neighboring target cells. Changes in the pH may alter the binding of IgE to the basophil/mast cell receptor (T. Ishizaka and K. Ishizaka, 1974).

8. *Functional Characterization of the Receptor*

a. Binding to IgE

The extracellular portion of the α chain contains the IgE binding site. The specificity for IgE is almost perfect. Other immunoglobulins are not able to compete with IgE in binding to α, with the exception of very high concentrations of IgG. The binding domain for IgE on α is supposed to be located within the immunoglobulin-like domains. The exact location of the binding domain has so far not been characterized. Labeling studies suggest that the binding domain might be closely associated with tyrosine residues on α (Metzger *et al.*, 1986). The carbohydrate moieties on α are not required for binding to IgE. Studies using antibodies to α domains (Basciano *et al.*, 1981, 1986; Baniyash *et al.*, 1987; Miroli and Spitz, 1987) should be helpful in identifying IgE binding epitopes. Usually one IgE molecule is bound to one α mole-

cule (i.e., molar ratio 1 : 1). Free, soluble IgE differs from receptor-bound IgE in its molecular conformation as assessed by energy transfer experiments (Holowka *et al.*, 1985). IgE binds to FcεRI molecules in an asymmetric manner and in bent conformation and may lose some of its flexibility (Baird *et al.*, 1989). The binding domain for FcεRI α on human IgE represents a sequence of 76 amino acids (Gln 301–Arg 376) located in a cleft between the $C_{\epsilon 2}$ and $C_{\epsilon 3}$ of Fcε (Helm *et al.*, 1988). This site is distinct from the B cell binding site on the $C_{\epsilon 3}$ domain (Vercelli *et al.*, 1989). Studies using antibodies to β suggest that the stoichiometry of the receptor is not affected by binding to IgE. Moreover, binding of IgE to α in itself is not associated with changes in receptor mobility (McCloskey *et al.*, 1984; Pecht *et al.*, 1991) or significant signs of signal transduction. Nevertheless, differences exist between liganded and unliganded FcεRI molecules (see also earlier text). Thus, unliganded (but not liganded) FcεRI molecules can be oxidatively iodinated on intact RBL cells (Conrad and Froese, 1976; Conrad *et al.*, 1976) and have a shorter half-life on the mast cell surface compared to unliganded receptors.

b. Signal Transduction Events

Usually FcεRI molecules are highly mobile. They are randomly and independently distributed in the lipid bilayer of the plasma cell membrane (Metzger *et al.*, 1986; Pecht *et al.*, 1989). The critical event in IgE-dependent activation of basophils/mast cells is bridging of FcεRI complexes to aggregates by multivalent antigen (or experimental compound) (K. Ishizaka *et al.*, 1970a,b; Siraganian *et al.*, 1975; Isersky *et al.*, 1978; MacGlashan and Lichtenstein, 1983; MacGlashan *et al.*, 1983b, 1986). A very small amount of cell-bound IgE or IgE–IgE receptor clusters per cell may be sufficient to mediate/initiate a release reaction (Pruzansky *et al.*, 1980; Pruzansky and Patterson, 1988). Dimeric crosslinking of the receptor by anti-FcεRI antibodies is also sufficient for specific activation of the cell (Segal *et al.*, 1977; Kane *et al.*, 1988); however, multimer stimulation usually is more effective compared to dimer activation (Fewtrell and Metzger, 1980; MacGlashan *et al.*, 1983b; Menon *et al.*, 1984, 1986).

Aggregation of the FcεRI on RBL cells is associated with a decrease in its lateral mobility as assessed by photobleaching techniques (Schlessinger *et al.*, 1976; McCloskey *et al.*, 1984). The decrease in FcE receptor mobility has been confirmed for its rotational dynamics by analyzing phosphorescence emission kinetics (Pecht *et al.*, 1991). In addition to mobility and rigidity, configuration and orientation of the receptor (subunits) within aggregates may be of importance for early events in signal transduction (Ortega *et al.*, 1988a,b). Crosslink-

ing of IgE receptors on RBL cells is also associated with detergent insolubility (Robertson *et al.*, 1986; Apgar, 1990; Seagrave and Oliver, 1990). Both insolubility and immobilization of the receptor are most probably due to interaction(s) with the surrounding (detergent-insoluble) membrane skeleton (Apgar, 1990, 1991). At least the α chain is capable of interacting with membrane skeletal elements. Transfection studies using mutated α subunits (including the truncated ectodomain of α) suggest that the membrane skeleton may extend close to the cell surface (Mao *et al.*, 1992). Components of the (activated) membrane skeleton may interact with (and affect) the cytoskeleton and thereby transmit signals; however, the exact regulatory interactions involving the cell skeleton during IgE-dependent events remain to be determined. Pfeiffer *et al.* (1985) analyzed the cytoskeletal changes in RBL cells on immunological activation by the use of F-actin-specific probes. They observed a rapid (10- to 30-second) depletion of F-actin in the isolated cell matrices followed by actin recovery within 1 minute. A subsequent increase in F-actin is associated with a transformation of the cell membrane (from a finely microvillous to an extensively folded topography) and cell spreading (Pfeiffer *et al.*, 1985). These changes may result in part from actin polymerization and redistribution. Interestingly, profilin, a major regulator of actin polymerization, appears to be a potent allergen present in plants and to be an autoallergen active in sensitized individuals (Valenta *et al.*, 1991, 1992). Studies using D_2O, colchicine, and cytochalasin B suggest that microfilaments and microtubules may be involved in the activation of basophils/mast cells for mediator release (Gillespie and Lichtenstein, 1972a,b).

FcεRI bridging is followed by a cascade of specific biochemical events. This cascade involves the phosphorylation of the receptor subunits, activation of signal transduction enzymes (such as phosphatases and kinases), and transmembrane flux of calcium ions, and results in the formation and release of mediator molecules. A number of observations suggest that the phosphorylation of the receptor represents an early (or the earliest) step in signal transduction (Perez-Montfort *et al.*, 1983; Hempstead *et al.*, 1983; Quarto and Metzger, 1986). Recent studies on RBL cells suggest that FcεRI engagement is associated with a rapid (few seconds) increase in serine phosphorylation of β and threonine phosphorylation of γ, as well as tyrosine phosphorylation of both β and γ (Paolini *et al.*, 1991). Substantial evidence exists that members of the src family (i.e., lyn and yes) are involved in the activation of mast cells via FcεRI molecules (Eiseman and Bolen, 1992). Tyrosine phosphorylation of FcεRI clearly precedes the other (well-known) biochemical events following receptor aggregation and

is reversible on disengagement of receptors by addition of monovalent hapten (probably via an undefined phosphatase) (Paolini *et al.*, 1991). Studies using mutated receptor subunits suggest that the carboxy terminus of the β chain may play a role in the early events of signal transduction, transmitted by activated FcεRI molecules (Alber *et al.*, 1991).

Subsequent events in the signal transduction pathway are mechanistically separable from the components of the FcεRI (Lichtenstein, 1971; Lichtenstein and DeBernardo, 1971; MacGlashan and Lichtenstein, 1983; T. Ishizaka *et al.*, 1983). These events include activation of proteases (Austen and Brocklehurst, 1960; T. Ishizaka *et al.*, 1985b), activation of methyltransferases (Hirata *et al.*, 1979; Hirata and Axelrod, 1980; T. Ishizaka *et al.*, 1981, 1983; Morita and Siraganian, 1981; Morita *et al.*, 1981), activation of the adenylate cyclase/cAMP system (Lichtenstein and DeBernardo, 1971; Lichtenstein *et al.*, 1978; T. Ishizaka and Ishizaka, 1984), breakdown of phosphatidylinositides (PIs) (Metzger *et al.*, 1986; Siraganian 1988, 1989), changes in protein kinase C (PKC) (Warner *et al.*, 1989; Warner and MacGlashan, 1990), activation of calmodulin (Marone *et al.*, 1983, 1986a), increase in intracellular calcium (T. Ishizaka *et al.*, 1980, 1983; Crews *et al.*, 1981; MacGlashan, 1989), organization of the cytoskeleton (Gillespie and Lichtenstein, 1972a,b), and depolarization of the plasma cell membrane (Sagi-Eisenberg and Pecht, 1984; Sagi-Eisenberg *et al.*, 1984; Kanner and Metzger, 1983). The pattern of signal transduction events may (slightly) differ from cell type to cell type (Metzger *et al.*, 1986; MacGlashan and Guo, 1991), and the physiological significance of each of the steps of the signal cascade remains to be determined.

Interestingly, the release reaction (in human basophils) may also be associated with changes in cell surface expression of membrane antigens. Most significantly, an increase in expression of CD11b was observed (Bochner *et al.*, 1989a). This increase even precedes the release reaction in human basophils. Human basophils have also been reported to display large amounts of CD63 on their cell surface membrane when activated for mediator release (Knol *et al.*, 1991).

A very small number of FcεRI bridges (fewer than 20 per cell) can be sufficient to provide an activating signal in basophils/mast cells (Pruzansky and Patterson, 1988). A positive correlation exists between the amount of membrane-bound IgE and the capacity of basophils/mast cells to respond to (optimal concentration of) releasing reagent. Usually, only a certain percentage (but not all) of basophils (in a given cell population) respond to releasing stimuli. At present, however, it remains uncertain whether for individual cells the release reaction rep-

resents an all-or-nothing or an overall graded process. A graded process has been proposed for the IgE-mediated changes in cytosolic free calcium (MacGlashan, 1989).

c. *Effector Mechanisms and Functional Changes of Mast Cells/Basophils following Activation through IgE Binding Sites*

Crosslinking of FcεRI molecules on basophils or mast cells is followed by formation and/or release of a number of mediator molecules and by a change in the functional "shape" of the cell. Mediators released from activated mast cells/basophils include vasoactive/coaguloactive molecules such as histamine, heparin-like proteoglycans (Schwartz *et al.*, 1981; Reilly *et al.*, 1988), proteases (such as tryptase), prostaglandins (such as mast cell PGD2; Lewis *et al.*, 1980), and leukotrienes (LTC4; Kaliner *et al.*, 1972; Lewis and Austin, 1984; MacGlashan *et al.*, 1986); growth regulators such as interleukins and colony-stimulating factors (CSFs) (Plaut *et al.*, 1989; Burd *et al.*, 1989; Wodnar-Fillipowicz *et al.*, 1989; Gordon *et al.*, 1990; Seder *et al.*, 1991); and effector molecules involved in defense reactions such as tumor necrosis factor (Young *et al.*, 1987; Gordon *et al.*, 1990), chemotactic factors, and many more. The physiological and biochemical events following mast cell/basophil activation and mediator secretion are manifold and have been reviewed extensively (Lewis and Austen, 1981; Austen, 1982; T. Ishizaka and K. Ishizaka, 1978, 1984; Serafin and Austen, 1987; Johnston and Holgate, 1990; Schwartz and Huff, 1991).

IgE-mediated activation of basophils/mast cells may also be associated with changes in the (physiological) functions of the cells. Activated mast cells/basophils may exhibit increased cell surface adhesion molecules (Bochner *et al.*, 1989a,b; H. L. Thompson *et al.*, 1989a,b) and increased chemotactic properties (H. L. Thompson *et al.*, 1989a,b) and cytotoxicity (Shiver and Henkart, 1991; Benyon *et al.*, 1991). In contrast to blood basophils mast cells are supposed to recover and regranulate after activation and degranulation (Dvorak *et al.*, 1988). For mast cells, IgE-mediated crosslinking of FcεRI may even provide a growth-promoting signal (Takagi *et al.*, 1989).

9. *Cell Surface Structures Functionally Associated with FcεRI Molecules*

The FcεRI apparently is the critical site involved in the initiation of signal transmission, culminating in the secretion of mediators from basophils/mast cells; however, a number of observations and in particular gene transfer experiments suggest that additional cell membrane

components may be required for the initiation of signal transduction (Kinet, 1989). A number of previous as well as more recent studies have focused on putative ion channels and on other cell surface molecules probably associated with FcεRI in functional terms.

The influx of calcium from the extracellular space into the cell is closely associated with (antigen-induced) release of mediators from human basophils and mast cells, although an absolute requirement for extracellular calcium does not exist. Great efforts have been undertaken during recent years to characterize the (IgE receptor-associated) calcium gate expressed on the cell surface of mast cells. The FcεRI complex in itself contains no calcium gate.

a. Mast Cell Cromolyn Binding Site

Cromolyn [1,3-bis)2-carboxychromon-5-yloxy)-2-hydroxypropane], is a widely used antiasthmatic drug (Cox, 1967). Cromolyn has been shown to interfere with signal transmission processes involved in stimulus–secretion coupling in murine mast cells. A receptor for cromolyn, also termed cromolyn binding protein (CBP), was first detected on RBL-2H3 cells (Mazurek *et al.*, 1980). Biochemical characterization of a cromolyn-associated cell surface glycoprotein has recently been achieved by using cromolyn derivatives (Hemmerich and Pecht, 1988). As assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis this protein (CBP) exhibits molecular masses of 110 kDa under nonreducing and 50 kDa under reducing conditions (Hemmerich and Pecht, 1988). Biochemical analyses suggest a glycoprotein dimer (glycosylation grade 25%). Scatchard analyses revealed a single class of 4000–8000 sites per (RBL) cell with a dissociation constant K_D of $3.8 \pm 0.2 \times 10^{-8}$ *M* (Hemmerich and Pecht, 1988). Apparently, CBP is no member of the FcεRI complex. A number of observations suggest that cromolyn binding site(s) are in some way associated with calcium flux and mediator secretion (Mazurek *et al.*, 1983a,b; Corcia *et al.*, 1986, 1988; Hemmerich and Pecht, 1988). RBL-2H3 variants with impaired capacity to bind cromolyn do not respond to an immunological stimulus, usually leading to calcium influx and degranulation in normal RBL-2H3 cells (Mazurek *et al.*, 1983a,b). In addition, the impaired capacity of these RBL-2H3 variants to respond to the calcium gating signal can be restored by implantation of the CBP (Mazurek *et al.*, 1983a,b). Although cromolyn inhibits mediator release in serosal mast cells, it does not inhibit the release reaction in RBL-2H3 cells or in mucosal mast cells. More recent data suggest that cromolyn may also interact with an intracellular acceptor, probably being a nucleoside 5′-diphosphate kinase (Hemmerich *et al.*, 1991).

Interestingly, in contrast to the native drug (CG), the cell-permeant form of cromolyn (CG/AM) is able to reduce the capacity of RBL-2H3 cells to release mediator molecules on immunological activation (Hemmerich *et al.*, 1991).

b. ME491 (CD63)

The ME491 antigen (CD63) is a 40- to 60-kDa cell surface membrane glycoprotein with four putative transmembrane domains. Cloning of the respective cDNA predicted a 237-amino-acid backbone (Kitani *et al.*, 1991). The human gene is located on chromosome 12. At least the amino terminus has been conserved during evolution (Kitani *et al.*, 1991). ME491 (CD63) is expressed in melanoma cells and in a number of secretory cells including endocrine glands. Recent data suggest that, in addition, human basophils (Knol *et al.*, 1991) (Table I) and (murine) mast cells (Sikora *et al.*, 1987; Vennegoor *et al.*, 1985; Atkinson *et al.*, 1985; Kitani *et al.*, 1991) express CD63. Studies using anti-IgE receptor mAbs and anti-ME491 mAb AD1 have shown that CD63 and the FcεRI are closely located and associated on the cell surface membrane of mast cells and basophils. In high concentrations AD1 inhibits IgE-mediated release from RBL cells. Moreover, binding of (some) anti-FcεRIm Abs to mast cells may be inhibited by mAb AD1, most probably as a result of steric hindrance (Kitani *et al.*, 1991); however, clearly CD63 is not part of the FcεRI complex. The putative structure of CD63 suggests the possibility of an ion gate-associated molecule. Interestingly, IgE-mediated activation of basophils is associated with an increase in expression of CD63 (Knol *et al.*, 1991).

c. Cell Surface Glycolipids

A number of previous observations suggest that cell surface (glyco) lipids, in particular gangliosides, control the (IgE-mediated) release reaction. Desialylation of the basophil plasma membrane is associated with an increase in their releasability (Jensen *et al.*, 1986). The reason for the functional shift remains unknown. One hypothesis is that depletion of lipid reduces the mobility of the FcεRI complex. Desialyation of the basophil membrane may also be associated with exposure of (masked) functional epitopes (such as 3-FAL/CD15 and other adhesion receptors). A number of glycolipids or lipids expressed on human basophils and/or mast cells have been defined by mAbs. Human basophils express lactosylceramide (CD17), leukosialin (CD43), and VIM-2-reactive sialofuco-oligosaccharide-containing gangliosides (Stain *et al.*, 1987) (see Table I). Human mast cells express CD43 but not CD17 or VIM-2 antigen (Valent *et al.*, 1989b) (Table I). The func-

tional significance of these surface glycolipids on human basophils/mast cells remains at present unknown.

Monoclonal antibody AA4 binds to a unique ganglioside (an α galactosyl derivative of GD_{1b}) expressed close to the FcεRI complex on RBL cells (Guo *et al.*, 1989). AA4 inhibits binding of IgE to RBL cells and IgE-dependent release from the cells. On the other hand, IgE was not able to inhibit binding of mAb AA4 to mast cell surface membranes (Guo *et al.*, 1989). Another mAb directed against GD_{1b} (associated components), B17, revealed similar effects on mast cells (Ortega *et al.*, 1990). From studies using radiolabeled ligand, RBL cells express approximately $1–2 \times 10^5$ GD_{1b} sites per cell. A number of functional studies and recent observations suggest that gangliosides are involved in the regulation of activation and mediator secretion in murine as well as human basophils and mast cells. Whether human mast cells (or basophils) express GD_{1b} remains to be determined.

d. G63

Recently the G63 protein, another putative mast cell activation antigen, has been characterized (Ortega *et al.*, 1988a,b). mAb G63 inhibits FcεRI-mediated secretion from RBL cells and the rise in cytosolic free calcium but not calcium ionophore-induced release of mediators from RBL cells, suggesting interference at an early phase of cellular activation. G63 also has no effect on binding of IgE to FcεRI molecules. RBL cells express $1–2 \times 10^4$ G63 molecules per cell as determined by ^{125}I-labeled ligand (Ortega *et al.*, 1988a,b). Immunoprecipitation experiments revealed a molecular mass of 58–70 kDa. G63 is expressed on primary (serosal and mucosal) as well as on malignant (RBL) murine mast cells. Whether a human correlate exists remains unknown. Apparently G63 antigen is distinct from FcεRI molecules; however, colocalization of immobilized, crosslinked FcεRI molecules and G63 has been observed (Ortega *et al.*, 1991). The exact physiological role and the molecular identity of G63 remains to be determined.

B. Fcγ Receptors

Fcγ receptors (FcγR) are widely distributed on human leukocytes. Subtypes of FcγR can be distinguished by their affinity to a particular IgG, by their molecular weight, and by specific binding to (CD) antibodies.

In 1970, Parish demonstrated the presence of anaphylactic IgG in patients sensitized to β-lactoglobulin by passive transfer experiments. Later, a number of observations suggested the possibility of IgG being a reaginic compound (Grant and Lichtenstein, 1971; Parish, 1981; Fagan *et al.*, 1982).

Human basophils were first described as binding IgG on their cell surface in a specific manner by T. Ishizaka *et al.* (1979) in autoradiographic analyses of blood basophils incubated with heat-aggregated human IgG (HGG) and ^{125}I-labeled anti-IgG. Binding of IgE to FcεRI or capping of FcεRI molecules on human basophils has no influence on the binding of IgG to its binding site. Binding of IgG, in turn, does not influence binding of IgE to human basophils or IgE-dependent release of histamine (T. Ishizaka *et al.*, 1979).

A number of investigations have attempted to identify IgG subclass-specific binding sites on human basophils. These studies have focused on type 4 IgG based on *in vivo* data suggesting an increase in IgG_4 levels in atopic patients (Dewey and Panzani, 1975; Bruynzeel and Berrens, 1979; Gwynn *et al.*, 1982; Nakagawa and de Weck, 1983; Merret *et al.*, 1984). So far, studies aimed at demonstrating IgG_4 molecules on the basophil surface membrane have been inconclusive; however, under certain conditions, antibodies to IgG_4 have the capacity to induce release of histamine from human basophils, whereas anti-IgG_1, anti-IgG_2, anti-IgG_3, anti-IgA_1, and anti-IgM antisera are ineffective (Fagan *et al.*, 1982; Poulsen *et al.*, 1988; Beauvais *et al.*, 1990). In the presence of D_2O, anti-IgG_4 is able to elicit histamine release from human basophils in approximately 20–30% of allergic subjects and in some normal donors (Fagan *et al.*, 1982; Beauvais *et al.*,1990). The response (i.e., percentage histamine release) to anti-IgG_4 is far less pronounced compared with the response found to anti-IgE, and the amount of anti-IgG_4 required to induce the release usually is very high (micromolar); however, even at concentrations of 1–100 pg/ml, anti-IgG_4 may be capable of inducing release of histamine in human basophils under certain conditions, in particular in the presence of blood eosinophils (Beauvais *et al.*, 1990). Eosinophil cationic proteins (ECPs) may represent the accessory molecules responsible for basophil activation in this process. In the absence of eosinophils or their products, basophils cannot be induced to release histamine on induction with picogram concentrations of anti-IgG_4 (Poulsen *et al.*, 1988; Beauvais *et al.*, 1990).

More recent studies were performed to characterize IgG binding sites on human basophils by the use of receptor-specific mAbs. As assessed by combined toluidine blue/immunofluorescence staining peripheral blood basophils bind CDw32 mAbs directed against the 40-kDa low-affinity Fcγ receptor (FcγRII) (Stain *et al.*, 1987). This observation has been confirmed by flow cytometric analyses of highly enriched CGL basophils (Stain *et al.*, 1987), KU-812 cells (Valent *et al.*, 1990a), and normal human blood basophils (Bochner *et al.*, 1989b). In contrast, human basophils did not react with CD64 mAbs directed

TABLE VIII
DISTRIBUTION OF IMMUNOGLOBULIN RECEPTORS EXPRESSED ON HUMAN BASOPHILS AND MAST CELLS AS DETERMINED BY INDIRECT IMMUNOFLUORESCENCE

		Expression of Ig receptors on						
Receptor	CD	ba[a]	KU-812	l-mc	s-mc	u-mc	a-mc	HMC-1
FcεRI	—	+	+	+	+	+	+	–
FcεRII	23	–	nk	–	–	–	–	nk
FcγRIII	16	–	–	–	–	–	–	–
FcγRII	w32	+	+	–	–	(+)	–	–
FcγRI	64	–	nk	–	–	–	–	–

[a] ba, basophils; l-mc, lung mast cells; s-mc, skin mast cells; u-mc, uterine mast cells; a-mc, ascites mast cells; nk, not known.

against the 75-kDa Fcγ receptor (FcγRI) expressed on monocytic cells (Stain *et al.*, 1987; Bochner *et al.*, 1989b; Valent *et al.*, 1990c) and basophils also did not react with CD16 mAbs directed against the 50- to 70-kDa Fcγ receptor (FcγRIII) expressed on neutrophilic granulocytes and natural killer cells (see also Tables I and VIII).

Human mast cells obtained from various sites of the body by collagenase digestion (i.e., from lung, skin, intestinal tract, or ascites) and analyzed with mAbs were found to lack CD16, CDw32, and CD64 (Valent *et al.*, 1989b) (Table VIII). Recently, however, human mast cells obtained from the uterus have been described as binding CDw32 mAbs (Guo *et al.*, 1992). Whether these differences are due to mast cell heterogeneity or to the different techniques and reagents applied remains unknown. So far no effect of IgG or anti-IgG on growth or function of human mast cells has been described. Table VIII summarizes immunoglobulin receptors expressed on human basophils and human mast cells.

IX. Receptors for Low-Molecular-Weight Regulators and Pharmacological Compounds

The ability of human basophils and mast cells to respond to degranulating stimuli (releasability) is controlled by a number of deactivating factors. In this section we focus on receptors for low-molecular-weight and pharmacological regulators of basophil/mast cell function.

A. Receptors for Adenosine

Adenosine is a natural nucleoside consisting of the purine base adenine and the primary sugar ribose. Adenosine is a purinergic regu-

lator of many biological processes (Fredholm and Sollevi, 1980; Snyder, 1985; Marone, 1987). Adenosine receptors are widely distributed on leukocytes, including inflammatory effector cells (Daly, 1985; Marone *et al.*, 1985a,b). At least two types of cell surface receptors for adenosine can be distinguished by biochemical and pharmacological criteria: A_1/Ri and A_2/Ra (Londos and Wolff, 1977; Van Calker *et al.*, 1979; Londos *et al.*, 1980). In addition, a separate, intracellular adenosine receptor, the so-called p-site, exists (Londos and Wolff, 1977).

A_1/Ri differs from A_2/Ra in its functional interaction with the cAMP system and in its binding (properties) to adenosine (and a number of chemically modified adenosine analogs). Activation through the A_1/Ri site is usually associated with a decrease in cAMP levels in the effector cells, whereas activation via A_2/Ra is linked to an increase in cellular cAMP. The A_1/Ri receptor binds adenosine with high and low affinity, whereas the A_2/Ra receptor binds adenosine with low affinity only. *N*-Ethylcarboxyamido-adenosine (NECA), an adenosine derivative, is a more potent agonist at the A_2/Ra binding site compared with phenylisopropyl-adenosine (PIA), whereas at the A_1/Ri site, PIA usually is more effective than NECA. The p site requires an intact purine ring and differs from the external A/R sites in several functional aspects (Londos and Wolff, 1977). Most significantly, unlike A/R, p sites are not affected by xanthines.

By ligand binding techniques (Bruns *et al.*, 1980; Williams and Risley, 1980; Marone *et al.*, 1987) as well as by photoaffinity labeling (Klotz *et al.*, 1985) and more recently by the use of monoclonal antibodies directed against adenosine derivatives (Braas *et al.*, 1986), A/R sites have been detected in various tissues.

Basophils and mast cells differ in their response to adenosine. A number of previous studies have shown that preincubation with adenosine in micromolar (but not submicromolar) concentrations inhibits anti-IgE- and antigen-induced release of histamine and formation of LTC4 in human blood basophils (Marone *et al.*, 1979a,b, 1985a,b; Church *et al.*, 1983). This effect of adenosine could be mimicked more effectively by NECA than by PIA and could be antagonized by methylxanthines (Hughes *et al.*, 1987; Peachell *et al.*, 1989). These results, suggesting an A_2/Ra-like receptor, have more recently been confirmed by the use of highly enriched (>90% pure) populations of human blood basophils (Peachell *et al.*, 1991). As expected, adenosine was found to upregulate cellular levels of cAMP in pure basophils. Preincubation of pure human basophils with low (submicromolar) concentrations of adenosine apparently has no significant effect on the release reaction induced by antigen or anti-IgE; however, when basophils are incubated first with anti-IgE and thereafter with adeno-

sine (0.01–1μM), potentiation of the release reaction occurs (Church *et al.*, 1983). Again NECA was found to mimic this effect of adenosine more sufficiently compared with PIA. So far no indication for the existence of an A_1/Ri-like receptor on human blood basophils exists. Surprisingly, the releasability-reducing effect of adenosine on (pure) human basophils could be inhibited by dipyridamole, a purinergic transport inhibitor; however, dipyridamole only reduced the capacity of the basophils to release histamine, not their capacity to form LTC4 on induction with anti-IgE. These observations raise the possibility that human blood basophils possess intracellular p sites, and this has more recently been confirmed by studies using adenosine analogs acting on p sites (Hughes and Church, 1986).

When human lung mast cells are preincubated with low concentrations (nanomolar) of adenosine, their capacity to respond to release-inducing compounds such as antigen or anti-IgE increases, whereas when mast cells are pretreated with high (micromolar) concentrations of adenosine, their capacity to release histamine and form LTC4 declines (Hughes *et al.*, 1984; Kagey-Sobotka *et al.*, 1985; Peachell *et al.*, 1986, 1991; Marone *et al.*, 1986b). The observation that highly enriched mast cells respond to adenosine (Peachell *et al.*, 1991) suggests a receptor-mediated mechanism. Adenosine analogs showed a similar effect on mast cell releasability compared with adenosine. NECA was more potent compared with PIA for both the inhibitory and potentiating activities (Peachell *et al.*, 1991). These observations suggest that human lung mast cells express an (functionally active) A_2/Ra-like receptor. The releasability-potentiating effect of low concentrations of adenosine may also involve a separate, as yet undefined binding site. In micromolar (but not nanomolar) concentrations, adenosine also induced an increase in cAMP levels in purified lung mast cells (Peachell *et al.*, 1991).

B. Receptors for Arachidonic Acid Metabolites

Arachidonic acid is a ubiquitous fatty acid fixed to (phospho)lipids present in the cell (surface) membranes. Enzymatic liberation and modification of arachidonic acid results in the formation of a cascade of bioactive molecules. Two major pathways, the cyclooxygenase/prostaglandin (PG) pathway and the lipoxygenase/leukotriene (LT) pathway, have been delineated. Details have been reviewed extensively (Vane, 1971; Samuelsson *et al.*, 1975; Goetzl, 1980; Kuehl and Egan, 1980; Lewis and Austen, 1981; Weissmann, 1983; Peters, 1985). The amount and type(s) of arachidonic acid derivatives formed and released from the cell depend on the cell type, the expression of

specific (arachidonic acid-active) enzymes, and the functional shape (resting or activated) of the cell. Activated basophils and mast cells are a rich source of arachidonic acid products. The biological effects of the PGs and LTs suggest a rather important role for these substances in a number of inflammatory and allergic reactions (Samuelsson *et al.*, 1975; Lewis and Austen, 1981; Peters, 1985; Weissmann, 1983; Serafin and Austen, 1987). Many of the arachidonic acid derivatives are potent regulators of the (micro)vascular system, the smooth muscle system, and the immune system. Moreover, PGs and LTs have been shown to modulate directly the function of the effector cells (i.e., basophils and mast cells) of allergic reactions. PGs are supposed to downregulate rather than upregulate basophil releasability, whereas LTs may increase the ability of the basophils to release mediators of allergy. More recent data suggest that PGs and LTs act on human basophils and mast cells via specific cell surface binding sites.

1. Prostaglandin Binding Sites

Prostaglandins are multifunctional modulator molecules involved in a variety of biological processes (Samuelsson *et al.*, 1975; Peters, 1985). PGs are produced by many different cells including activated immune cells such as mast cells (PGD_2). The PGs exert its biological activities on its target cells via specific cell surface receptors. Although specific receptors for each of the PGs have been postulated, cross-competition between the PGs in binding to the target cell receptor is a well-recognized phenomenon. A number of studies have shown that PGs modulate the functional properties of blood basophils and mast cells (Lichtenstein and Henney, 1972; MacGlashan and Lichtenstein, 1983; MacGlashan *et al.*, 1983a,b; Tauber *et al.*, 1973; Sullivan and Parker, 1976; Borne *et al.*, 1972; Peters *et al.*, 1983). PGs of the E series (i.e., PGE_1 and PGE_2) usually downregulate the capacity of human blood basophils to respond to histamine release-inducing agonists. Most investigators have observed an effect of PGs on basophil releasability between 10^{-9} and 10^{-6} *M*. A rank order of potency for the PGs was also observed with $PGE_1 > PGE_2 > PGA_1 > PGA_2 > PGB_2 > PGF_{1a}$ (Lichtenstein and Henney, 1972). In the late 1980s, highly enriched populations of human blood basophils (>90% pure) have been shown to respond to PGs, suggesting the presence of specific binding sites.

More recently, the PG binding sites expressed on highly enriched populations (>95% pure) of human basophils (obtained from CML patients) were characterized by means of a radioreceptor assay using ^3H-labeled PGE_1, PGE_2, PGI_2, iloprost (a stable analog of PGI_2),

PGD_2, and $PGF_{2\alpha}$ (Virgolini *et al.*, 1992). Two CML donors were tested extensively. Basophils from these donors as well as the human basophil cell line KU-812 (raised from a CML patient) were found to bind PGs in a specific manner. Scatchard plots revealed two classes, a high-affinity low-capacity class and a low-affinity high-capacity class, of PGE_1 and PGI_2/iloprost binding sites. In the first CML donor, basophils were found to express 1029 high-affinity PGE_1 binding sites per cell with a calculated dissociation constant K_D of 0.4×10^{-9} *M* and 13,195 low-affinity PGE_1 binding sites with a K_D of 33×10^{-9} *M*. In the second patient pure basophils were found to express 1583 high-affinity PGE_1 binding sites with a K_D of 0.7×10^{-9} *M*) and 16,446 low-affinity PGE_1 binding sites with a K_D of 61×10^{-9} *M*.

In cross-competition analyses the PGs were found to bind to the basophil PGE_1 receptor in affinity hierarchy: $PGE_1 > PGI_2 > PGD_2 > PGE_2 > PGF_{2\alpha}$. CML basophils were also found to express two classes of iloprost/PGI_2 binding sites in both donors. KU-812 cells and primary basophils revealed similar numbers and similar affinity constants of PG receptors. Specific receptors for $PGF_{2\alpha}$ could not be detected on human basophils or KU-812 cells. Unexpectedly, a specific receptor for PGE_2 could also not be detected on either primary or immortalized (KU-812) CML basophils, although a functional response of the basophils to PGE_2 was observed. This might be due to the fact that PGE_2 acts via another PG receptor such as the PGE_1 binding site which also binds PGE_2. Another possibility would be that PGE_2 receptors could not be detected because of extremely small receptor numbers or selective expression of PGE_2 receptors during cell activation (rather than in the resting basophils).

CML basophils also express PGD_2 binding sites (Virgolini *et al.*, 1992); however, the basophil PGD_2 receptor differs from the other basophil PG binding sites in several aspects. Unlike the PGE_1/I_2 receptor, the basophil PGD_2 receptor lacks a low-affinity high-capacity binding component. The basophil receptor exhibited only a single class of low-capacity PGD_2 binding sites. Compared with the PGE_1/I_2 binding sites, the affinity of the PGD_2 receptor for its natural ligand is intermediate. In the two CML donors tested, 1860 and 2697 PGD_2 binding sites per cell with K_D of 16 and 11×10^{-9} *M*, respectively, could be detected. The PGD_2 receptor (unlike the PGE_1/I_2 receptor) is incapable of transmitting a releasability-reducing signal in the basophils but in certain circumstances may even upregulate the capacity of the basophils to release proinflammatory mediators (MacGlashan *et al.*, 1983b; Virgolini *et al.*, 1992). PGD_2 (unlike PGE_1 or PGI_2) also failed to counteract the IL-3-induced downregulation of cAMP levels

in KU-812 cells (Virgolini *et al.*, 1992). The reason for the functional differences found between basophil PG receptors remains unknown. An attractive hypothesis would be that the low-affinity high-capacity PG binding sites play a more critical role in basophil deactivation for mediator release. This would explain the observation that optimal releasability-reducing effects are transient and are seen at relatively high concentrations of PGs and the inability of the PGD_2 receptor (lacking a low-affinity PG binding class) to provide a releasability-reducing signal.

So far, little is known about the half-life, metabolism, internalization, or degradation of the basophil PG receptors. The basophil receptors for PGE_1, PGI_2, and PGD_2 apparently are expressed in a constitutive manner. When basophils or KU-812 cells are exposed to rhIL-3 for 6 or 12 hours, a significant (down to 50% of control) decrease in the number of PGE_1/PGI_2 binding sites (without any change in the affinity constants) can be observed (Virgolini *et al.*, 1992). In parallel, IL-3 induced a decrease in cellular cAMP levels in these (pure) basophils with maximum downregulation occurring after 30 minutes. To which extent the IL-3 induced modulation of the PG receptor/cAMP system is associated with the IL-3-induced upregulation of basophil releasability remains to be determined. Human mast cells are also supposed to express specific PG binding sites (Peters *et al.*, 1982a,b) although no quantitative binding studies have been presented so far.

2. *Other Arachidonic Acid Derivatives*

The effects of arachidonic acid and arachidonic acid products of the lipoxygenase pathway on human basophils have also been analyzed. Blood basophils preincubated with high concentrations of arachidonic acid (between 10^{-7} and 10^{-5} *M*) exhibited an (slightly) increased ability to release mediators of allergy, whereas blood basophils pretreated with the arachidonic acid analog eicosa-3,8,11,14-tetraynoic acid (ETYA), a synthetic blocker of arachidonic acid metabolism (in both the cyclooxygenase and lipoxygenase pathways), revealed decreased releasability (Marone *et al.*, 1979a,b). The mechanism of action of arachidonic acid on human basophils remains unknown. Selective blockage of the cyclooxygenase (but not lipoxygenase) pathway or activation by products of the lipoxygenase pathway enhanced basophil releasability (Marone *et al.*, 1979a,b; Peters *et al.*, 1979, 1982a,b, 1983). Of the lipoxygenase products, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE) are the most potent compounds, with biological activity found between 10^{-9} and 10^{-8} *M* (Marone *et al.*, 1979a,b). Other lipoxygenase products (leuko-

triences C, D, and B, 11-HPETE, 12-HPETE, 15-HPETE) showed no effect on human basophils except when toxic (micromolar) doses were used (Marone *et al.*, 1979a,b). 5-HPETE and 5-HETE also were found to counteract the PGE-induced inhibition of basophil releasability (Peters *et al.*, 1982a,b).

C. Receptors for Histamine

Histamine is produced and stored primarily in mast cell and basophil precursor cells and is released from granules of mature basophils and mast cells on appropriate activation. Histamine is a potent regulator of the vascular and immune systems. Histamine acts on a variety of target cells through specific cell surface receptors. At least three types of histamine binding sites exist: H-1, H-2, and H-3 receptors (Ash and Schild, 1966; Black *et al.*, 1972; Arrang *et al.*, 1987). Histamine receptors are widely distributed on human leukocytes (Clark *et al.*, 1977; Wescott and Kaliner, 1983; Casale *et al.*, 1985; Cameron *et al.*, 1986). On the basis of functional analyses a basophil H-2 receptor has previously been postulated. In particular, histamine as well as dimaprit, a selective H-2 agonist, decreased the releasability of human blood basophils (Lichtenstein and Gillespie, 1973), whereas neither histamine nor dimaprit is able to modulate the releasability in human lung mast cells. Moreover, H-2 antagonists have been shown to upregulate releasability in human blood basophils (Tung *et al.*, 1982). Whether human basophils or mast cells express H-1 or H-3 receptors remains unknown. So far, H-1 and H-3 agonists have failed to mimic the effect of H-2 agonists on basophil histamine release. The observation that histamine deactivates the basophil leukocyte (i.e., reduces releasability and the chemotactic response) suggests a negative feedback mechanism controlling reactions to agonists in human basophils (Lichtenstein and Gillespie, 1973; Lett-Brown and Leonard, 1977).

D. Receptors for Antiinflammatory Drugs

A number of antiinflammatory drugs (AIDs) are used to treat anaphylactic reactions and some drugs are supposed to have a direct effect on human basophils and human mast cells. We distinguish three groups of AIDs: the steroids, the nonsteroidal AIDs (NSAIDs), and cyclosporin A and related compounds.

Steroids are the most widely used AIDs. They are supposed to interact with a variety of immune cells through specific receptors. Steroids have also been described as regulating the functional properties of human basophils and probably mast cells, too. Thus, steroids are perhaps the most effective antiallergy drugs available. Steroids are

very effective in the immediate response to an allergen, but most effective in late-phase reactions (Lichtenstein and Bochner, 1991). A number of observations suggest that steroids inhibit IgE-dependent release of mediators from human basophils but not release from human mast cells (Schleimer *et al.*, 1981, 1982, 1983; Pipkorn *et al.*, 1987). Corticosteroids also inhibit the accumulation of basophils in tissues and the number of circulating basophils (as well as eosinophils); however, steroids do not inhibit the adhesiveness of human blood basophils to (resting or activated) endothelial cells (Bochner *et al.*, 1988). The exact mechanisms of action of steroids (and respective cell surface receptors) on human basophils remain to be characterized.

A number of NSAIDs influence the functional properties of human basophils and mast cells. Many of these drugs have a direct effect on the adenylcyclase/cAMP system and may directly interact with and deactivate human basophils/mast cells. These drugs include β-adrenergic agonists such as isoproterenol, fenoterol, and salbutamol (Lichtenstein and Margolis, 1968; Assem and Schild, 1969; Lichtenstein, 1975; Butchers *et al.*, 1979; Peters *et al.*, 1982b; Schulman *et al.*, 1982; Church and Hiroi, 1987; Morita and Miyamoto, 1987; Undem *et al.*, 1988; Chazan *et al.*, 1991). Thus, human basophils and human mast cells apparently express adrenergic receptors. The mechanisms of action and the exact types of cell surface receptors for adrenergic compounds expressed on human basophils and human mast cells, however, remain to be determined.

Cyclosporin A (CsA) is a potent and widely used immunosuppressive drug. CsA deactivates a number of immune cells including (activated) T cells and mast cells as well as blood basophils. Recent studies suggest that CsA inhibits release of histamine and formation and release of PGD_2 from human lung mast cells (Marone *et al.*, 1988; Triggiani *et al.*, 1989). CsA also inhibits release of mediators from human basophils (Cirillo *et al.*, 1990; Ezeamuzie and Assem, 1990).

FK-506, a macrolide isolated from *Streptomyces tsukobaensis*, has also been shown to inhibit mediator release in human basophils (dePaulis *et al.*, 1991a,b). FK-506 is active between 1 and 300×10^{-9} *M* and inhibits antigen-, anti-IgE-, and calcium ionophore A23187-induced release of histamine from human basophils as well as formation of LTC4 on induction with anti-IgE (dePaulis *et al.*, 1991a,b). In contrast, FK-506 failed to inhibit fMet peptide-induced release from human basophils. FK-506 is more effective than CsA in deactivating human basophils. Interestingly, IL-3, a potent activator of human basophil releasability, reversed the inhibitory action of FK-506. More recent studies suggest that FK-506 deactivates human mast cells (dePaulis *et al.*, 1991a,b; Stellato *et al.*, 1992).

Rapamycin (RAP), a novel macrolide product of *Streptomyces hygroscopicus*, is related to FK-506. RAP ($30–1000 \times 10^{-9} M$) was found to inhibit IgE-mediated release of histamine from human basophils. In contrast to FK-506 (causing complete inhibition), however, RAP reduced only the IgE-dependent releasability of the basophils and showed no effect on A23187-induced release. As expected from studies using T cells RAP competitively inhibits FK-506-induced inhibition of A23187-mediated release (dePaulis *et al.*, 1991a,b).

Recently, the mechanisms of action of CsA, FK-506, and RAP have been investigated. Corresponding cytosolic binding sites, so-called immunophilins (identical to peptidyl-prolyl isomerase), have been identified (Siekierka *et al.*, 1989a,b; Harding *et al.*, 1989; Takahashi *et al.*, 1989; Fischer *et al.*, 1989; Dumont *et al.*, 1990). Cyclophilin binds CsA, and FK-506 binding protein (FKBP) is the receptor for FK-506. Both corresponding complexes (cyclophilin–CsA and FKBP–FK-506), but not FKBP–RAP, are supposed to bind to and modulate calcineurin, a calcium/calmodulin-dependent phosphatase (Liu *et al.*, 1991). From functional analyses on enriched cells, human basophils and human mast cells are supposed to express cytosolic cyclophilins (Cirrillo *et al.*, 1991). More recent data suggest that specific cell surface receptors for cyclosporin A and FK-506 also exist; however, a cellular substrate on human basophils or mast cells has not been characterized so far.

X. Concluding Remarks

Mast cells and basophils share many phenotypical and functional properties. Despite this, striking differences exist. The cell surface phenotype documents that both cells are different and respond to different signals. In fact, human mast cells differ from blood basophils in their binding and response to differentiation-inducing (growth) factors, activating (poly)peptides, pharmacological compounds, and adhesion molecules. The concept that mast cells and basophils are such different cells and the concept of mast cell heterogeneity raise a number of important questions and should be of major interest to those involved in the analysis of allergy and in the regulation and biology of human mast cells and basophils in general.

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Animal Models for Acquired Immunodeficiency Syndrome

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I. Introduction

The current epidemic of acquired immunodeficiency syndrome (AIDS) has had profound worldwide consequences. The number of deaths attributable to this disease has reached over 100,000 in the United States and as many as 1,000,000 in the world (Blattner, 1991). The number of persons currently infected with human immunodeficiency virus-1 (HIV-1), the causative agent, may be as high as 2 million in the United States and 8 million in the world as this virus continues to spread in the industrialized as well as in developing nations. There is an indisputable need for effective vaccines to stop viral spread and for pharmaceutical agents to arrest disease progress in infected persons; however, a formidable barrier to facile development of weapons to fight AIDS has been the lack of readily available animal systems to test candidate vaccines and drugs.

A number of animal models have been proposed for AIDS research and these models have been used to gain valuable insight into the effects of HIV-1 or similar viral infection on the intact organism (Desrosiers and Letvin, 1987; Gardner and Luciw, 1989; Letvin, 1990). Before discussing specific models it may be useful to first consider the features of an ideal system for the study of AIDS (McCune, 1991). The animal should be small, abundant, inexpensive to purchase and house, and easy to maintain under biosafety conditions required for working with HIV-1. Ideally, the animal would respond to HIV-1 infection with an immunodeficiency syndrome involving depletion of helper T cells similar to that seen in HIV-infected humans. It should be possible to monitor progress of infection by simple tests and within a relatively short time frame. Infection should be transmitted by routes implicated for human HIV-1 infection. Techniques for culturing and infecting various cell types should be available to facilitate basic studies of virus attachment and replication. Further assets to the model would include a wide range of reagents to study molecules of importance in the immune system, including lymphokines and lymphoid cell surface

markers, as well as a backlog of information about the immune system of the animal.

With these rigid and admittedly unrealistic criteria in mind, potential models may be explored. As Table I indicates, the proposed models vary considerably in choice of animal species and in the viruses used for infection. The proposals range from use of an endangered species, the chimpanzee, which supports infection with HIV-1 without apparent disease, to infection of the abundant and easily maintained laboratory mouse with murine leukemia viruses, which are unrelated to HIV but cause a form of immunodeficiency. To date the most promising animal models, at least for vaccine development studies, have been those involving the use of simian immunodeficiency viruses (SIVs) to infect some of the more abundant primate species. SIVs are retroviruses related to HIV-1, and macaques infected with these viruses display signs of AIDS-like disease, including immunodeficiency accompanied by depletion of helper T cells. Models involving HIV-1 infection of immunodeficient (SCID) mice reconstituted with human immunocompetent cells appear to have good potential for evaluating therapeutic agents. The feline viral systems and the infection of rabbits with HIV-1 have promise for AIDS research, but these models require further study and development to explore their practical uses.

The present discussion covers the major animal models used for the study of AIDS. Prior to discussion of specific models, relationships among the different viruses used are briefly reviewed. In addition to a description of each model, an attempt is made to evaluate its utility for development of vaccines and antiviral therapeutic agents and for studies of the basic pathogenesis of HIV-1 infection.

II. Relationships among the Viruses Used as Animal Models for AIDS

It is obvious that the models listed in Table I cover a broad range of animal species with different evolutionary relationships to humans. An equally important consideration in model choice is the virus to be used and its relationship to HIV-1. A useful means of comparing these viruses is according to their genomic organization relative to HIV-1 (Fig. 1). Whereas the overall structures are quite similar and all the retroviruses share certain genes, there is considerable difference in the regulatory elements produced (see Fig. 1).

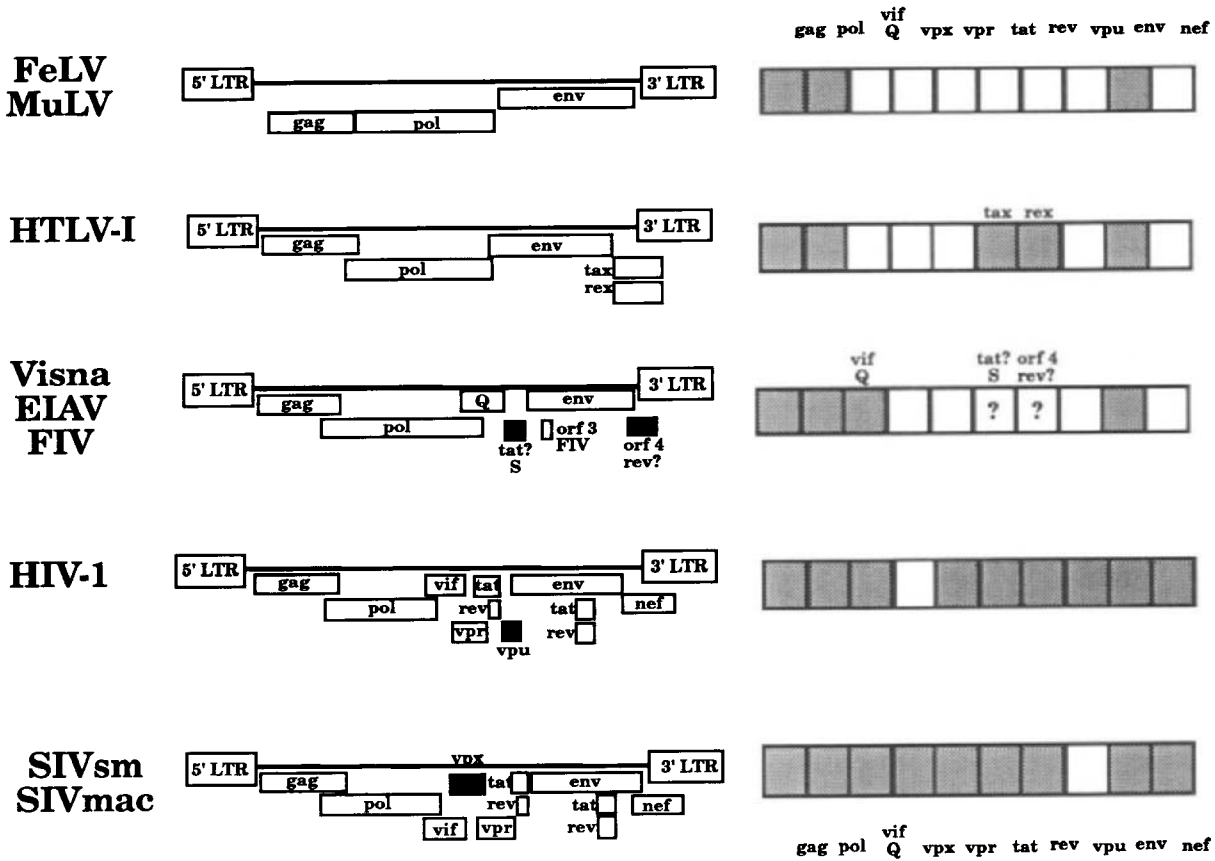
A general classification of retroviruses is by virion morphology (A, B, C, and D); most viruses described in the present review are type C retroviruses, which may be further classified on the basis of disease association on oncornaviruses, lentiviruses, and spumaviruses (foamy

TABLE I

POTENTIAL ANIMAL MODELS FOR HUMAN AIDS

Virus classification		Animal model	Disease parameters and *in vitro* correlates			
			Immune dysfunction	Central nervous system disease	*In vitro* Tropism	
					CD4 lymph	Macrophage
Distantly Related to HIV-1						
Oncornaviruses	FeLV[a]	Cats	Yes	No	Yes	Yes
	MuLV	Mice	Yes	No	—	—
	HTLV-1	Rabbits	No	No	—	—
Lentiviruses	Visna Virus	Sheep	No	Yes	No	Yes
	EIAV	Horses	No	No	No	Yes
	BIV	Cattle	—	No	—	Yes
	FIV	Cats	Yes	Yes	Yes	Yes
Closely related to HIV	SIVsm/mac	Macaques	Yes	Yes	Yes	Yes
	HIV-2	Macaques	No	No	Yes	Yes
Models Using HIV-1	HIV-1	Chimpanzee	No	No	Yes	Yes
		SCID mice	No	No	n.a.	n.a.
		Rabbits	No	No	—	—

[a] HIV, human immunodeficiency virus; FeLV, feline leukemia virus; MuLV, murine leukemia virus; HTLV-1, human T lymphotropic virus type 1; EIAV, equine infectious anemia virus; BIV, bovine immunodeficiency virus; FIV, feline immunodeficiency virus; SIVsm/mac, simian immunodeficiency virus of macaques or sooty mangabeys; HIV-2, human immunodeficiency virus, type 2; HIV-1, human immunodeficiency virus, type 1; n.a., not applicable. A dash indicates that this factor is unknown.


FeLV
MuLV
5' LTR
3' LTR
env
gag
pol
gag pol vif Q vpx vpr tat rev vpu env nef
HTLV-I
tax
rex
tax rex
Visna
EIAV
FIV
Q
orf 3
FIV
tat?
S
orf 4
rev?
vif
Q
tat? orf 4
S rev?
HIV-1
vif
tat
rev
vpr
vpu
nef
SIVsm
SIVmac
vpx

viruses). Infection with this last group appears to be asymptomatic and therefore spumaviruses have not been used as a model for immunodeficiency; they are not further discussed. Oncornaviruses as their name implies are best known for their association with oncogenesis, particularly leukemias, and this is frequently reflected in their names (i.e., feline leukemia virus). However, oncornavirus infections are frequently associated with immunosuppression; for example, more FeLV-infected cats die as a consequence of immunosuppression than of neoplastic disorders. As shown in Fig. 1, the classical oncornaviruses have a simple, basic genome structure expressing only the structural proteins gag and envelope (env) and the polymerase (pol), without additional regulatory proteins. In contrast, human T lymphotropic virus type 1 (HTLV-1), also classified as an oncornavirus, has an atypical genome organization, expressing, in addition to gag, pol, and env, two transactivator proteins (tax and rex) encoded by a short open reading frame in the 3′ portion of the genome.

HIV-1 falls into the classification of lentivirus, a term that implies the slow disease course that characterizes infection with this group of viruses. Lentiviruses have a more complex genome organization. The nonprimate lentiviruses including visna virus of sheep (termed an ungulate lentivirus) and feline immunodeficiency virus of cats have a number of short open reading frames (orfs) that have been variably designated Q, S, or more simply orf-1 to orf-4. The function of the proteins encoded from these open reading frames is still poorly understood (this is implied by the question marks in Fig. 1). Many may be analogous to regulatory proteins of HIV-1, such as Q of visna virus and vif of HIV-1. Finally, the primate lentiviruses including HIV-1 have the most complex genome organization, encoding six additional regulatory proteins. Although SIV from sooty mangabey (SIVsm) and SIV

FIG. 1. Genome organization of viruses used as animal models for AIDS are shown schematically on the left, with open reading frames represented by open boxes. The sequences span about 10 kb; the lengths of boxes are not accurate representations of gene size. Genes that appear to be unique to a particular virus are shown as black boxes. Some genes such as orf S of the visna virus group have unknown function. On the right is a schematic representation of the genes found in each representative virus. Shaded boxes indicate the presence of and open boxes represent the absence of a gene. Genes considered equivalent are listed in corresponding positions; for example, the *tax* and *rex* genes of HTLV-1 are analogs of *tat* and *rev* of HIV-1. FeLV, feline leukemia virus; MuLV, murine leukemia virus; HTLV-1, human T lymphotropic virus type I; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; HIV-1, human immunodeficiency virus-1; SIVsm, simian immunodeficiency virus from sooty mangabey; SIVmac, simian immunodeficiency virus from macaque.

from macaque (SIVmac) are similar in structure to HIV-1, some subtle differences exist. Thus, SIVsm lacks the *vpu* gene found in HIV-1 and HIV-1 lacks the *vpx* gene seen in SIVsm. Differences in genome organization clearly have implications to the applicability of a particular virus as a model for AIDS; SIV is the most similar to HIV-1 and, as such, is most likely to share pathogenic mechanisms with HIV-1. As discussed next, this prediction is borne out by experimental studies.

III. Models Using Viruses Distantly Related to HIV-1

The ideal human disease models are those in which the administration of the etiological agent reproduces the disease conditions in well-characterized laboratory animals. In the case of AIDS, the etiological agent, HIV-1, does not cause reproducible disease in any laboratory animals tested so far (Desrosiers and Letvin, 1987; Gardner and Luciw, 1989; Letvin, 1990). Even in those species capable of supporting persistent HIV infection, the animals do not develop AIDS-like disease. Accordingly, investigators are searching for alternative models by studying related viruses that may cause the AIDS-like symptoms in laboratory animal species. It is hoped that such models can be used as drug testing or vaccine testing tools and will provide insights and strategies to combat HIV infection.

Some AIDS model candidates are chosen for their abilities to induce immunodeficiency or other symptoms reminiscent of AIDS in a natural host. These models employ distantly related viruses that include members of the oncovirinae family such as murine leukemia virus (MuLV) and feline leukemia virus (FeLV), as well as members of lentivirinae family such as feline immunodeficiency virus (FIV) and many other ungulate viruses. Some of the more appealing viruses such as FIV, FeLV, and MuLV can cause an immunosuppressive state; however, with the possible exception of FIV, their pathological mechanisms appear to be different from that of HIV. Other viruses such as equine infectious anemia virus and caprine arthritis–encephalitis virus do not cause immunosuppression but involve a long-term infection. Overall, these distantly related viruses may have value for testing antiretroviral drugs and new vaccines. More importantly, their study may uncover novel HIV pathological mechanisms and reveal how some animals combat long-term retroviral infection.

A. Ungulate Lentiviruses

Numerous retroviruses belonging to both lentivirinae and oncovirinae exist naturally in several ungulate species. Domestic livestock such as sheep, goat, horse, and cow all have diseases associated with

retroviral infection. These retroviruses are distantly related to HIV-1 and do not cause immunodeficiency. Even though the ungulate retroviral diseases have long been recognized clinically, very little is known about the viruses and their immunological effects on the hosts. Contributing to these apparent problems is the fact that very little is known about the ungulate immune system. Perhaps for these reasons, most ungulate viruses are overlooked as potential AIDS animal models. There are certain characteristics of these viruses that may be of interest in AIDS research and these warrant mention here.

Maedi-visna virus (MVV) and caprine arthritis–encephalitis virus (CAEV) are closely related retroviruses of sheep and goat, respectively (Crawford *et al.*, 1980). Among the ungulate viruses, MVV and CAEV are most closely related to HIV-1 (Gonda *et al.*, 1987). Both lentiviruses infect macrophages and cause slow progressive disease involving the central nervous system (CNS), lungs, and joints (Specter *et al.*, 1989). Typically, these viruses can be transmitted horizontally via respiratory secretions and vertically via milk (Evermann, 1990). After a long incubation period, MVV-infected animals experience interstitial pneumonia and severe demyelinating encephalomyelitis. As for CAEV, chronically infected goats develop immune-mediated arthritis and encephalitis (Fenner, 1987). Interestingly, similar to HIV these viruses persist in the infected host despite the presence of neutralizing antibodies. Furthermore, similar to HIV-1, these viruses undergo frequent envelope mutation resulting in antigenic drift. Although there is no evidence of immunosuppression in these animals, two AIDS-like clinical features, wasting and leukoencephalopathy, are observed. These similarities give these viral systems potential for the study mechanisms of lentivirus mutation and its role in avoidance of immune surveillance.

Equine infectious anemia virus (EIAV) is the best studied ungulate system for viral antigenic drift. EIAV infection is characterized by episodal clinical signs such as fever, weight loss, anemia, edema, and leukopenia (Montelaro *et al.*, 1990). The anemia appears to result from immune-mediated hemolysis. These clincal profiles are precipitated by the emergence and rapid replication of new antigenic variants of EIAV. In as little as 2 weeks, virus populations differing by one or two amino acids can emerge and cause another round of viremia leading ultimately to chronic, debilitating diseases. EIAV infects monocytes (Salinovich *et al.*, 1986); however, this virus does not appear to be tropic for T lymphocytes and therefore does not result in notable immune dysfunction. Nonetheless, it is worth considering the EIAV infection model as a potential model for studying long-term interactions between lentivirus and the host.

Cattle harbor two retroviruses, an oncornavirus, bovine leukemia virus (BLV), and a lentivirus, bovine immunodeficiency-like virus (BIV), that cause lymphocytosis and lymphadenopathy in infected cattle. In addition, BIV infection can cause CNS lesions, weakness, and emaciation. The BIV provirus has been molecularly cloned and shown to be biologically active and capable of inducing syncytium formation *in vitro* (Braun *et al.*, 1988). Although some abnormal humoral and cellular immune responses have been reported (Trainin *et al.*, 1976; Thorne *et al.*, 1981), severe immunosuppression has not been linked to either of these viruses. In fact, BIV is named "immunodeficiency-like virus" for its antigenic and genetic similarities to HIV-1 rather than for its clinical manifestation. Interestingly, both viruses can productively infect rabbits (Burney *et al.*, 1985; Gonda *et al.*, 1990), inducing seroconversion, persistent infection, and viremia. Further development of the rabbit BIV model may prove to be beneficial for testing antiretroviral agents in the future.

B. Murine Leukemia Virus

Several strains of MuLV cause immunosuppression following experimental inoculation of laboratory mice (Salamon and Wedderbrun, 1966; Bendinelli *et al.*, 1985). By far, Friend and Rauscher are the best characterized immunosuppressive MuLV strains. The mixture of replication-defective and replication-competent helper virus found in these strains infects cells of lymphoid and myeloid lineage, causing immune dysfunction in susceptible animals. Such infection alters the normal T cell, B cell, and macrophage interactions and induces severe immune dysfunction. Numerous studies have identified host susceptibility factors linked to genes such as *Fv-1*, *Fv-2*, *Rfv-1*, *Rfv-2*, and *Rfv-3* (Lilly and Pincus, 1973; Chesebro and Wehrly, 1976). Despite their immunosuppressive properties, the Friend and Rauscher MuLV strains are not used as animal models for AIDS.

More recently, Mosier and co-workers described an immunosuppressive syndrome similar to early phases of AIDS in C57Bl/6 mice infected with the LP-BM5 strain of MuLV. The LP-BM5 MuLV strain is a derivative of radiation-induced Duplan–Laterjet leukemia virus. The observed abnormalities included humoral and cellular immunodeficiencies, susceptibility to mousepox infection (Buller *et al.*, 1987), hypergammaglobulinemia (Mosier *et al.*, 1985), polyclonal B cell activation (Mosier *et al.*, 1985), and an occasional occurrence of aggressive B cell lymphoma (Klinken *et al.*, 1988). This syndrome is now referred to as the murine acquired immunodeficiency syndrome (MAIDS) (Klinken *et al.*, 1988).

The LP-BM5 strain is a mixture of ecotropic subgroup B, mink cell focus-inducing MuLV, and a defective MuLV genome (BM5d). This defective virus is only 4.8 kb because of deletion of the pol and env genes; the remaining gag gene contains a large open reading frame that encodes the $Pr65^{gag}$ protein. This protein appears to be the pathological determinant of MAIDS, perhaps acting as an oncoprotein (Jolicoeur, 1991). Recently, two independent laboratories (Aziz *et al.*, 1989; Chattopadhyay *et al.*, 1989) convincingly showed the BM5d defective viral component as the etiological agent in the LP-BM5 mixture. The defective genome is cytopathic for fibroblasts if rescued with a nonpathogenic helper virus. Furthermore, the helper-free defective viral stock can cause MAIDS in the absence of viral replication (Huang *et al.*, 1989).

Despite similarities in clinical expression of immune-mediated dysfunctions, the immunosuppressive mechanism in infected mice differs significantly from the pathogenesis of human AIDS, limiting the direct utility of this model. MuLV-induced mouse immunodeficiency appears to be a paraneoplastic syndrome characterized by abnormal lymphoproliferation rather than the immunocytopathic syndrome seen in AIDS. Also, unlike human AIDS, which specifically infects and depletes the $CD4^+$ subset of T lymphocytes, the development of MAIDS requires the presence of both T and B lymphocytes, suggesting that both cell types are involved in pathogenesis. In addition, abnormal levels of cytokines including interleukin-1 (Cheung *et al.*, 1991), interleukin-2 (Morse *et al.*, 1989), tumor necrosis factor (Cheung *et al.*, 1991), and interferon (Pitha *et al.*, 1988) are detected in mice with MAIDS, suggesting a perturbation of T and B cell regulation.

Certain features of MAIDS may shed light on events in AIDS. The association of immunodeficiency with a defective viral genome such as in MAIDS [and feline AIDS (FAIDS); see next section] leads to renewed interest in the potential role that a defective viral genome may have in the pathogenesis of AIDS. In addition, these studies encouraged AIDS researchers to rely less heavily on tissue culture-derived viruses because of the selection against defective (or non-culture-adapted) viral genomes under these conditions; however, although studies of AIDS patients have demonstrated genomes with multiple in-frame stop codons (Meyerhans *et al.*, 1989), the significance of such viruses in the pathogenesis of AIDS is still unclear. Furthermore, the mechanisms used by these two viruses to generate defective genomes, point mutations in HIV-1 versus the extensive deletions in the MuLV genome, are quite different.

The MAIDS model may have potential for testing of antiviral drugs.

For example, 3′-azido-3′-deoxythymidine (AZT) (Jolicoeur, 1991) and 9-(2-phosphonylmetoxyethyl)adenine (PMEA) (Gangeni *et al.*, 1989) can inhibit development of MAIDS, if administered immediately after the virus inoculation. As the MAIDS syndrome is a lymphoproliferative disorder, immunosuppressive drugs such as cyclosporin A, which inhibits interleukin-2 and interferon-γ production, and an antineoplastic drug such as cyclophosphamide have been tested. Both these drugs protect against the development of MAIDS in a time-independent fashion.

The MAIDS model has broadened our understanding of retrovirus-induced immunosuppression; however, the mechanism underlying MuLV pathogenesis is different from any known HIV mechanisms. Not only does MuLV have a different viral tropism than HIV-1, but the lymphoproliferative consequences of its infection also set it apart. Most importantly, unlike HIV-1 in which the role of defective virus in pathogenesis is not clearly defined, the MuLV pathogenic effects can be directly linked to the presence of a defective genome. Lastly, the transcription regulatory mechanisms of a simple oncornavirus like MuLV might differ significantly from those of the more complex lentiviruses such as HIV-1. These differences preclude the use of MAIDS as a system for development of most anti-AIDS agents.

C. Feline Leukemia Virus

Feline leukemia virus, like the closely related MuLV, belongs to the oncovirinae subfamily of retroviruses. Since its discovery in Scotland (Jarrett *et al.*, 1964), three FeLV subtypes, FeLV-A, FeLV-B, and FeLV-C, have been identified. In addition to these subtypes, at least two defective variants, FeLV–FAIDS and FeLV-*myc*, have also been isolated. Furthermore, endogenous FeLV-like viral sequences (enFeLVs) have been identified in domestic cats and in numerous species of wildcats of Mediterranean origin; in contrast, enFeLVs have not been found in wildcats from sub-Saharan Africa, Southwest Asia, and the American continent (Benveniste *et al.*, 1975). The prevalence of enFeLVs is not limited to the Felidae species as related sequences have also been identified in rats. This observation suggests that FeLV may have been acquired from rats via transpecies infection (Benveniste *et al.*, 1975).

Each of the FeLV subtypes and variants has distinct genetic attributes and biological properties. The ecotropic FeLV-A is ubiquitous in FeLV-infected cats either as the sole dominant subtype or in conjunction with one or more FeLV subtypes, and is generally considered to be minimally pathogenic. In contrast to FeLV-A, both FeLV-B and

FeLV-C are amphotropic and are only found in association with subgroup A FeLV in naturally infected cats. Many FeLV-Bs such as the Richard strain can be found in approximately 50% of infected cats and their presence is associated with leukemias/lymphomas (Hardy *et al.*, 1976a; Jarrett *et al.*, 1978). The much rarer FeLV-C subtype is found in only 1% of the infected hosts and is frequently associated with aplastic anemia (Hardy *et al.*, 1976b; Jarrett *et al.*, 1978). FeLV-B and FeLV-C appear to result from recombination between FeLV-A and enFeLV sequences. The evolution of these viral subtypes highlights the importance of genetic recombination in the generation of viral diversity. Furthermore, recombination and transcomplementation between these nonpathogenic subtypes and enFeLV can produce a pathogenic virus. FeLV–FAIDS and FeLV-*myc* are two pathogenic strains that evolved from virus/virus complementation and virus/oncogene recombination, respectively.

Shortly following the discovery of FeLV, an association of FeLV infection with immunosuppression was observed (Anderson *et al.*, 1971; Perryman *et al.*, 1972). These early findings have been substantiated by numerous investigators, thus verifying FeLV as a potent immunosuppressive virus capable of causing a fatal immunodeficiency syndrome (Hardy, 1990). Infected cats display lymphodegenerative and lymphoproliferative signs (Hardy and Essex, 1986; Good *et al.*, 1990; Hardy, 1990; Reinacher, 1989), including thymic atrophy (Anderson *et al.*, 1971; Hardy, 1981, 1982), lymphoid depletion (Quackenbush *et al.*, 1990; Anderson *et al.*, 1971), lymphopenia (Essex *et al.*, 1975), reduced T cell-dependent humoral immune response (Quackenbush *et al.*, 1990; Pardi *et al.*, 1991), reduced cytokine production and blastogenic responses to mitogens (Tompkins *et al.*, 1989; Mathes *et al.*, 1979; Good *et al.*, 1990; Cockerell and Hoover, 1977; Cockerell *et al.*, 1976), reduced response to allograft (Perryman *et al.*, 1972), increased susceptibility to opportunistic infections (Hardy, 1981, 1982), aplastic anemia (Mackey *et al.*, 1975; Onions *et al.*, 1982), and lymphosarcoma (Dorn *et al.*, 1968). Approximately 75% of persistently infected cats die from diseases associated with immunosuppression and accompanying opportunistic infections (Reinacher, 1989). Lymphoproliferative and myeloproliferative disorders account for most remaining fatalities.

An isolate of FeLV that induces immunodeficiency after experimental inoculation has been well characterized on the molecular and pathological levels. This isolate, designated FeLV–FAIDS, is derived from the thymus of a naturally infected cat with lymphoma. It consists of a replication-competent, mildly pathogenic subgroup A virus (desig-

nated 61E) and an acutely pathogenic, replication-defective form (termed 61C). Unlike the MuLV defective genome, the minimal pathogenic determinant of this virus lies within the envelope gene. Molecular chimeras between the pathogenic and minimally pathogenic helper virus identified two domains of the gp70 env protein that contribute the pathogenic phenotype: a 7-amino-acid segment near the carboxy terminus and a 109-amino-acid region near the amino terminus. In addition, sequences in the long terminal repeat also contribute to pathogenesis (Donahue *et al.*, 1991). The presence of these pathogenic determinants correlate strongly with *in vitro* cytopathology and T cell killing in lymphocyte cell lines.

1. The Pathogenic Mechanism of Feline Leukemia Virus–Feline AIDS

The exact mechanism underlying the pathology induced by FeLV–FAIDS has not been clearly defined. Two possible mechanisms have been proposed. The first theory suggests that FeLV replication may be cytopathic for $CD4^+$ lymphocytes. The only support for this theory is an 8 to 10% decline in circulating CD4 lymphocytes in cats infected with FeLV-FAIDS (Quackenbush *et al.*, 1990). A second theory implicates the nascent envelope protein of the pathogenic FeLV-FAIDS strain in a disruption of normal receptor interference, leading to superinfection (Poss *et al.*, 1990; Donahue *et al.*, 1991). Evidence for this hypothesis includes slow processing of the envelope protein of the pathogenic strain and the accumulation of unintegrated viral DNA, a finding associated with the early events following virus infection. The accumulation of high levels of unintegrated viral DNA is frequently associated with cytopathic retroviral infections including HIV-1; however, the mechanisms associated with FeLV-induced cytopathology appear to differ significantly from those of HIV-1. A major factor in HIV-1-induced cell death, as observed *in vitro*, is the formation of syncytium, whereas FeLV-induced cell death occurs in the absence of syncytium formation; however, alternate mechanisms for HIV-1-induced cell death exist (Somasundaran and Robinson, 1987) and may be similar in these two systems.

2. Antiviral Therapy

The FeLV model has proven useful for testing antiretroviral therapies. Antiretroviral drugs can prevent the development of FAIDS and are effective in limiting FeLV replication. Administration of AZT (Hoover *et al.*, 1990; Zeidner *et al.*, 1990a,b) or human recombinant interferon-α (IFN) (Hoover *et al.*, 1990; Zeidner *et al.*, 1990a,b)

prevents infected asymptomatic cats from becoming antigenemic, prevents onset of disease, and protects cats against infectious challenge. Combined administration of AZT and interleukin-2 or IFN-α is significantly superior (25–30% *in vitro*) than administration of AZT alone (Hoover *et al.*, 1990; Zeidner *et al.*, 1990a,b). Another chain termination drug, 2′,3′-dideoxycytidine (ddC), can also inhibit FeLV replication (Zeidner *et al.*, 1989; Hoover *et al.*, 1989). As with AZT, administration of tumor necrosis factor (TNF) or IFN-α enhances ddC effectiveness (Zeidner *et al.*, 1989); however, unlike AZT, the effective dose requirement for ddC varies widely depending on the target cell type (Polas *et al.*, 1990). These antiretroviral strategies, in conjunction with vaccination (Olsen *et al.*, 1976, 1980), should curtail the spread of FeLV.

Despite contributions made by the FeLV model, it is a less than ideal model for AIDS. There are several major differences between FAIDS and AIDS that may prove significant in development of the FeLV model. These include the broad range of cellular targets for FeLV (hematopoietic, lymphoid, and epithelial) compared with HIV and the lack of syncytium formation as a cytopathic mechanism. For example, FeLV myeloid tropism leads to numerous myeloproliferative diseases not commonly associated with HIV infection. Furthermore, as syncytium formation is a major *in vitro* cytopathic mechanism in HIV, the FeLV model might not be suitable for testing therapeutic approaches directed against the cell fusion mechanism. Another important difference between FeLV and HIV-1 is the role of neutralizing antibodies in controlling virus replication. Cats that successfully reject FeLV infection always possess high-titer neutralizing antibodies (Hardy *et al.*, 1976a), whereas HIV-1-infected individuals demonstrate high titers of neutralizing antibodies. Finally, although both viruses are transmitted horizontally and vertically, FeLV horizontal transmission is primarily via saliva, whereas HIV is transmitted by sexual contact and by transfer of blood. For the present, the FeLV model provides a useful system for testing antiretroviral therapies; however, a more important future role for FeLV might be in providing insights into basic retroviral pathogenesis.

D. Feline Immunodeficiency Virus

Feline immunodeficiency virus is a lentivirus isolated in 1986 from FeLV-negative, immunodeficient cats housed in a large cattery in Petaluma, California (Pedersen *et al.*, 1986). Since the initial discovery, other strains of FIV have been isolated in Britain and Japan (Harbour *et al.*, 1988; Miyazawa *et al.*, 1989; Ishida *et al.*, 1989), and natural

FIV infection can be detected in domestic cats worldwide (Harbour *et al.*, 1988; Ishida *et al.*, 1988, 1989; Yamamoto *et al.*, 1989). In addition, seroreactivity to FIV core proteins suggests that other members of the Felidae family such as African lions, tigers, Florida panthers, and bobcats harbor viruses antigenically similar to FIV (Barr *et al.*, 1989). Thus, FIV may be one member of a much larger family of feline lentiviruses similar to the primate lentivirus family (see Section IV). These data, in conjunction with the worldwide presence of FIV in domestic cats, suggest that FIV may have been present in cat populations for a long time, perhaps even before the domestication of wildcats. On the basis of cellular tropism, virion morphology, reverse transcriptase biochemical requirements, protein composition, nucleotide sequence, and genomic organization, FIV is classified as a lentivirus (Yamamoto *et al.*, 1988; Pedersen *et al.*, 1989; Olmsted *et al.*, 1989; Talbott *et al.*, 1989). FIV has a typical lentiviral genomic organization containing gag, pol, env, and four short open reading frames. One of the small open reading frames appears to encode a transactivator protein product similar to the rev protein of HIV-1 (Kiyomasu *et al.*, 1991).

FIV infection is more prevalent in older, free-roaming, male cats. The higher infection rate in this population is probably attributable to the restricted transmission mode of FIV. FIV can be efficiently transmitted via saliva by biting and, unlike FeLV, cannot be effectively transmitted either vertically by *in utero* transmission or horizontally by sexual contact, by exchange of bodily secretions, or by casual contact. Consequently, older male cats raised outdoors are more likely to be infected because they are frequently bitten by other cats during territorial fights (Yamamoto *et al.*, 1989).

Naturally infected cats remain asymptomatic for long periods. Because it is impossible to ascertain the exact time at which the naturally infected cat contracted the virus, it is difficult to determine the incubation period required for disease development; however, it is known that the median age of a healthy FIV carrier is 3 years, whereas the median age of FIV-induced fatalities ranges from 5 to 10 years (Ishida *et al.*, 1989). From these data, the FIV incubation period is estimated to be between 2 and 7 years. A more precise answer to this question requires an experimental infection approach where time and other variables can be controlled. Unfortunately, experimental infections of both normal and specific pathogen free (SPF) cats have been disappointing, as these cats do not develop fatal immunodeficiency without additional cofactors such as FeLV (Pedersen *et al.*, 1990). The requirement for coinfection with FeLV severely confounds these stud-

ies because, as described earlier, FeLV alone causes significant immunosuppression.

Six clinical phases comparable to the five phases of HIV infection (Pedersen and Barlough, 1991) have been described in naturally infected cats: an actue phase 1; an asymptomatic phase 2; clinical phases 3 and 4 similar to AIDS-related complex in HIV-1 infection; phase 5 or AIDS; and a syndrome of neurological disorders and renal dysfunction (phase 6). The acute stage is characterized by fever, diarrhea, neutropenia, and lymphadenopathy. These clinical signs are more severe in the cats coinfected with FeLV (Ishida *et al.*, 1989; Hosie *et al.*, 1989; Grindem *et al.*, 1989; Moraillon, 1990). This is followed by an asymptomatic phase. Abnormalities in immunological parameters can be detected within 1.5 to 2 years postinfection, although cats remain clinically healthy. These abnormalities include reduction in the number of $CD4^+$ T cells, decreased CD4/CD8 ratio, inability to mount a humoral immune response to T-dependent antigens, depressed mitogenic response by T cells, and development of hypergammaglobulinemia (Ackley *et al.*, 1990a; Novotney *et al.*, 1990; Barlough *et al.*, 1991; Torten *et al.*, 1991). Only the acute and asymptomatic phases have been observed after experimental infection unless cats are coinfected with FeLV and reductions in CD4 count and CD4/CD8 ratio are more pronounced in FIV/FeLV dual-infected cats (Pedersen *et al.*, 1990). In nature, FIV-infected cats can remain in the asymptomatic phase for long periods without notable health problems.

The third through fifth phases of clinical development involve the onset of disease. During the third phase, disease signs not accompanied by either secondary or opportunistic infections are observed (Ishida *et al.*, 1989; Yamamoto *et al.*, 1989; Hopper *et al.*, 1989), similar to the persistent lymphadenopathy phase of HIV-1 infection (PGL) (Ishida and Tomoda, 1990; Shelton *et al.*, 1990). Secondary infections, primarily bacterial in origin, of the oral cavity and urinary tract emerge during the fourth phase of infection, similar to AIDS-related complex (ARC) of HIV-1 infection (Ishida and Tomoda, 1990; Shelton *et al.*, 1990). Some naturally infected cats (<10%) eventually develop a clinical profile similar to that of AIDS (fifth phase). Signs include weight loss and opportunistic infections at multiple body sites. Peripheral blood lymphocytes completely fail to respond to mitogenic stimulation (Taniguchi *et al.*, 1990). Cats that survive the fifth phase eventually develop other FIV-related disorders including kidney disease, cancers, and neurological abnormalities not typically found in AIDS. As no analogous phase in AIDS exists, a sixth phase of FIV disease development is proposed to cover this group of symptoms.

This "miscellaneous FIV-related disorders" phase is an all inclusive-category that covers all pathological manifestations not included in the other phases.

1. Use of Feline Immunodeficiency Virus to Study Antiviral Therapy and Vaccines

FIV infection of cats is particularly important in the study of antiviral drugs and the development of vaccine strategies. For example, FIV infection can be dampened by antiretroviral interventions. *In vitro* studies reveal that FIV reverse transcriptase, like that of HIV, will incorporate various chain termination agents including AZT and PMEA (North *et al.*, 1989). Even though these antiviral drugs do not halt the infection completely, it appears that at least AZT and PMEA have promising clinical effects (Smyth *et al.*, 1990; Egberink *et al.*, 1990). In addition, certain FIV mutants identified *in vitro* were found to be resistant not only to AZT but to 2′,3′-dideoxyuridine and 2′,3′-dideoxyguanosine as well (Remington *et al.*, 1991). Obviously, these findings suggest that the FIV system is a viable model for testing antiretroviral drugs and perhaps can even be used to study mechanisms of viral drug resistance.

FIV vaccines are in an early stage of development. There have been very few studies and only tentative conclusions can be drawn from these findings. Recent studies by Yamamoto *et al.* (1991b) show that animals immunized with paraformaldehyde-fixed whole virions or cell-associated virus can induce high-titer neutralizing antibodies to both FIV core and envelope proteins (Yamamoto *et al.*, 1991a; Gardner, 1991) and these antibodies appear to protect against low-dose challenges. Furthermore, it has been shown that high-titer antibodies against the FIV core antigen alone do not protect vaccinated cats from infection (Hosie *et al.*, 1990). A detailed understanding of the immunological components involved in combating FIV infection can aid the development of vaccines for HIV.

In terms of logistical and practical considerations, cats fulfill the desired handling, housing, and availability criteria for an effective AIDS model. A large pool of naturally infected, asymptomatic cats can be acquired for large-scale studies and there is an abundant supply of SPF cats for experimental infection studies to help delineate the role cofactors may play in disease development. Because of its overall advantages, experimental infection with FIV is fast becoming a leading nonprimate animal model for AIDS. First and foremost, FIV is a lentivirus capable of causing AIDS-like immunodeficiency syndrome in naturally infected hosts. It is thought that a model using the natural

virus and host system should more accurately mirror the natural interactions between HIV and its human host. Second, the FIV-induced pathology is similar to AIDS not only in terms of clinical signs but also in the time required to develop disease. Unlike the SIV model where the AIDS-like symptoms appear relatively soon after infection, FIV-infected cats develop such symptoms only after a prolonged asymptomatic state analogous to that seen in AIDS. The similarity to AIDS includes the synergistic impact of cofactors in the development of disease. For example, cats dually infected by both FIV and FeLV develop a more severe disease course (Pedersen *et al.*, 1990) in a manner similar to that found in AIDS patients who are coinfected with HTLV-I virus (Bartholomew *et al.*, 1987). Third, FIV-infected cats (both naturally and experimentally infected) develop neurological lesions similar to the encephalitis seen in AIDS patients (Hoover *et al.*, 1990). Lastly, FIV is one of the few viruses that causes a decreased CD4 cell count in an infected host while leaving the CD8 count unaffected. Taken together, these favorable clinical profiles make the FIV system a promising AIDS model.

Certain shortcomings make the FIV system a less than perfect model. A minor difficulty is that FIV is not exclusively CD4 tropic; sorted CD4 and CD8 cell populations were shown to be equally susceptible to FIV infection (Brown *et al.*, 1991). This finding is supported by a recent isolation of interleuken-2-independent $CD4^+$ and $CD8^+$ cell lines chronically infected with FIV (Yamamoto *et al.*, 1991b). This evidence clearly establishes that $CD8^+$ cells can be infected and contradicts the fact that the $CD8^+$ cell population did not decline in infected animals. These findings suggest that more than one viral receptor may be involved in FIV infection. An additional problem in the use of FIV for pathogenesis studies is the long latent period for disease and the need for cofactors for immunodeficiency.

More serious problems are the paucity of basic information concerning the cat immune system and the lack of reagents for its study; however, steady progress is being made toward solving these problems. For instance, the development of CD4 and CD8 cell lines prompted recent development of monoclonal antibodies specific for cat T cell markers (Ackley *et al.*, 1990b; Dean *et al.*, 1991). These antibodies have already contributed significantly toward a better understanding of the cat immune system and FIV tropism. The development of new reagents should continue, considering the rapidly increasing importance of the FIV model. Furthermore, in our society where cats are accorded the status of honorary family members, the demand for diagnostic tools for veterinary use should ensure support for their development.

IV. Simian Immunodeficiency Virus Infection of Macaques

A. Introduction and Phylogeny of Nonhuman Primate Lentiviruses

Simian immunodeficiency virus infection of macaques is the best available animal model for pathogenesis of human AIDS. SIV shares with HIV a cell tropism for $CD4^+$ lymphocytes and macrophages, and induces a syndrome of opportunistic infections, immunodeficiency, central nervous system disease (encephalitis), and wasting that is indistinguishable from human AIDS as induced by HIV-1 infection; however, as SIV is a diverse group of related lentiviruses with distinct properties and pathogenicity, it is important to review the origins and classifications of the nonhuman primate lentiviruses.

The first experience with SIV was in association with an unusual clustering of lymphomas and immunodeficiency disorders noted in the early 1980s among a colony of captive macaques at the New England Regional Primate Research Center (Hunt *et al.*, 1983; Letvin *et al.*, 1983a,b). Clinical and pathological observations ultimately led to the isolation of a new group of T cell-tropic retroviruses, now called simian immunodeficiency viruses, or more specifically, SIVmac (Daniel *et al.*, 1985; Kanki *et al.*, 1985). Experimental inoculation of rhesus macaques (*Macaca mulatta*) with SIVmac resulted in immunodeficiency and death from opportunistic infections, thus establishing the SIV model for AIDS (Letvin *et al.*, 1983b, 1985). Shortly thereafter, an immunodeficiency syndrome was noted in macaques inoculated with tissues from sooty mangabey monkeys at the Yerkes and Delta Regional Primate Research Centers (Murphey-Corb *et al.*, 1986; Fultz *et al.*, 1986a). This led to the isolation and characterization of SIVsm from seropositive captive mangabeys. Subsequent molecular analyses demonstrated that SIVmac and SIVsm are closely related. As extensive serosurveys have demonstrated that macaques (an Asian species) are not naturally infected with SIV in the wild, SIVmac infection in captive animals in North American primate centers probably resulted from cross-species transmission from sooty mangabeys, a species for which feral infection has been demonstrated (Hirsch *et al.*, 1989b; Marx *et al.*, 1991).

In contrast to the lack of evidence for SIV infection of Asian primate species, a large number of African primates, including sooty mangabeys, appear to harbor related lentiviruses. Distinct SIV strains have been isolated from a number of African nonhuman primate species, and many more species are apparently infected as judged by serological tests (Johnson *et al.*, 1992). None of these viruses is associated with immunodeficiency in their natural host species. To date, four

major African subgroups have been defined on the basis of sequence comparisons (as reviewed in Johnson *et al.*, 1992): (1) sooty mangabeys (*Cercocebus atys*, SIVsm); (2) African green monkeys (*Cercopithicus aethiops*, SIVagm); (3) mandrills (*Papio sphinx*, SIVmnd); (4) chimpanzees (*Pan troglodytes*, SIVcpz). These four subgroups are approximately equidistant from each other (about 50 percent sequence identity in *gag*) when nucleotide sequences are subjected to phylogenetic tree analyses (Fig. 2). Our current understanding of the relationship between these nonhuman primate lentiviruses and the human immunodeficiency viruses (HIV-1 and HIV-2) suggests that the ancestors of the human viruses were probably members of a large family of lentiviruses resident in African nonhuman primates for many centuries (Johnson *et al.*, 1992; Desrosiers, 1990). Two of these groups are closely related to human lentiviruses: SIVsm to HIV-2 (Hirsch *et al.*, 1989a) and SIVcpz to HIV-1 (Huet *et al.*, 1990).

The present discussion focuses on isolates belonging to the SIVmac/SIVsm subgroup because only viruses from this subgroup consistently cause AIDS in an experimental setting (Letvin and King, 1990; McClure *et al.*, 1989; Zhang *et al.*, 1988; Putkonen *et al.*, 1989; Baskin *et al.*, 1988). Although SIVagm will infect macaques, the infection is most often asymptomatic and virus is difficult to isolate at later time points. No data concerning experimental infection of nonhuman primates with HIV-2, SIVmnd, or SIVcpz are available. Thus, as a model for AIDS, isolates of SIVmac and SIVsm have proven the most useful. Recent advances and current understanding of the pathogenesis of AIDS induced by SIV in macaques emphasizing possible parallels with human AIDS are emphasized here.

B. Pathogenesis of Simian Immunodeficiency Virus Infection of Macaques

1. *Natural History of Simian Immunodeficiency Virus Infection of Macaques*

As summarized in Table II, SIV infection of macaques resembles HIV-1 infection of humans in many respects. One notable difference is in the latency periods of the two viruses. The time between infection and onset of immunodeficiency in adult humans can be 10 years or longer and is rarely shorter than 6 months. By contrast, the nonclinical phase in SIV-infected macaques is compressed to months in the majority of cases. In this respect, SIV infection resembles the typical course of pediatric AIDS; however, such comparisons must consider that the SIV studies normally use a viral inoculum selected for its ability to

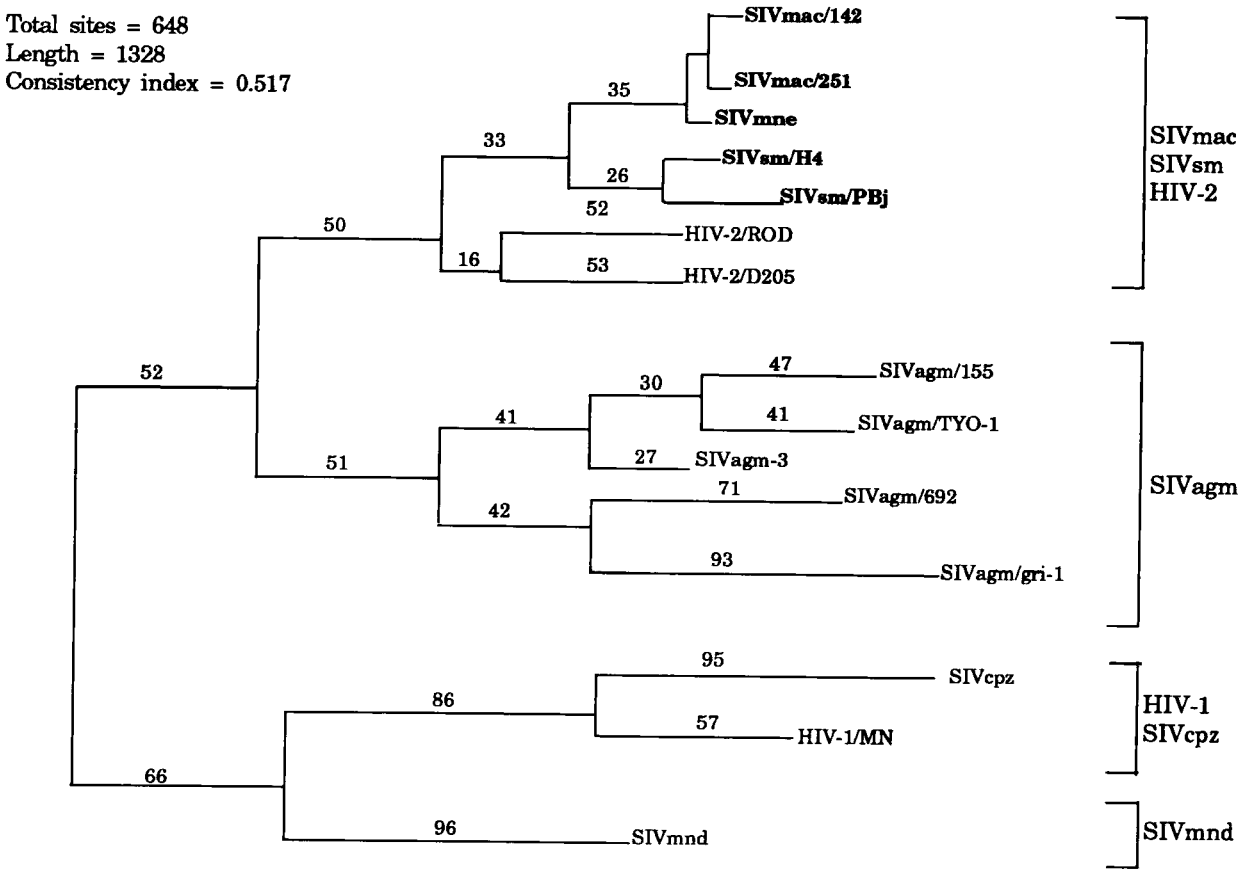

Total sites = 648
Length = 1328
Consistency index = 0.517
SIVmac/142
SIVmac/251
SIVmne
SIVsm/H4
SIVsm/PBj
HIV-2/ROD
HIV-2/D205
SIVagm/155
SIVagm/TYO-1
SIVagm-3
SIVagm/692
SIVagm/gri-1
SIVcpz
HIV-1/MN
SIVmnd
35
33
26
52
50
16
53
52
47
30
41
41
27
51
71
42
93
95
86
57
66
96
SIVmac
SIVsm
HIV-2
SIVagm
HIV-1
SIVcpz
SIVmnd

TABLE II
SIMIAN IMMUNODEFICIENCY VIRUS (SIV) INFECTION OF MACAQUES FOR HUMAN AIDS

Characteristics of virus and disease	SIV/macaques	HIV-1/humans
Tropism for CD4	Yes	Yes
Macrophage tropic variants	Yes	Yes
Syncytium formation	Yes	Yes
Decline in CD4 cells with onset of disease	Yes	Yes
Antigenemia	Yes	Yes
gag antibodies decline with disease onset	Yes	Yes
High virus load terminally	Yes	Yes
V3 as neutralizing epitope	No	Yes
CD8 viral suppression	Yes	Yes
Rapid generation of variants (viral swarm)	Yes	Yes
High proportion of unintegrated viral DNA	Yes	Yes
Opportunistic infections		
Cytomegalovirus	Yes	Yes
Candida	Yes	Yes
Pneumocystis	Yes	Yes
Mycobacterium	Yes	Yes
Lymphoma	Yes	Yes
Kaposi's sarcoma	No	Yes
Central nervous system disease	Yes	Yes
Wasting	Yes	Yes
Variable clinical progression	Yes	Yes
Perinatal maternal transmission	Probable	Yes
Sexual transmission	Unknown	Yes

induce AIDS reproducibly in a time frame suitable for experimental study.

Macaques infected with SIV and humans infected with HIV-1 have similar clinical manifestations. Experimental inoculation of macaques with isolates of SIVmac or SIVsm leads to a persistent infection eventually characterized by immunodeficiency, opportunistic infections, and death. As in AIDS patients, the terminal stages of disease are

FIG. 2. Phylogenetic tree of the primate lentiviruses. The tree was generated and calibrated as described (Smith *et al.*, 1988). Briefly, a minimum evolutionary tree based on gag nucleotide sequences was constructed using the PAUP algorthim with the global branch swapping option MUL-PARB. Time is implied, flowing from left to right. The total number of sites examined is 1328 (of which 648 were variable) and the consistency index was 0.517. Lengths of horizontal lines are proportional to the minimum number of single nucleotide substitutions required to generate the observed variation (shown above each branch). The length of the vertical lines is for clarity only. All sequences were taken from the Los Alamos HIV Database.

heralded by a marked reduction in circulating $CD4^+$ lymphocytes, loss of antibody reactivity to viral gag proteins, and antigenemia. In addition, infected macaques develop a similar spectrum of opportunistic infections with agents such as cytomegalovirus (CMV), *Pneumocystis,* and *Candida.* Other frequent observations are a wasting syndrome and CNS disease that are pathologically indistinguishable from those seen in human AIDS. Interestingly, Kaposi's sarcoma has never been described in SIV-infected macaques (Letvin and King, 1990; McClure *et al.,* 1989; Zhang *et al.,* 1988).

In most animals, lymphadenopathy, viremia, antigenemia, and a decrease in circulating $CD4^+$ peripheral blood mononuclear cells (PBMCs) can be documented within the first few weeks of inoculation (Letvin and King, 1990; McClure *et al.,* 1989; Zhang *et al.,* 1988; Putkonen *et al.,* 1989). Thereafter, three distinct clinical courses have been observed (Letvin and King, 1990); McClure *et al.,* 1989; Zhang *et al.,* 1988). About one-third of cases develop persistent viremia in the absence of a systemic SIV-specific antibody response; these animals usually die in the first few months after infection. Another one-third to one-half of the animals develop a persistent veremia in the face of a strong systemic SIV-specific antibody response. This group generally survives 1 to 3 years, with clinical demise characterized by weight loss, a decrease in titer of antibodies reactive with SIV gag proteins, and persistently low numbers of circulating $CD4^+$ cells. Finally, a small subset of infected macaques remain persistently infected for years. SIV is not readily (if at all) recovered from PBMCs after the first several months, but a vigorous SIV-specific antibody response is sustained and SIV-specific nucleic acid sequences in PBMCs are easily detected by polymerase chain reaction (PCR) amplification. It is not known how long these animals survive, but some continue to thrive 4 to 5 years after innoculation.

Under ideal conditions, knowledge of the course and ultimate distribution of the causative agent in the infected host is a necessary basis for understanding viral pathogenesis. Unfortunately, many details of SIV infection of macaques remain obscure; however, it is clear that once infection is established and early replication (1 to 2 weeks) is underway, SIV antigenemia is easily detected in plasma, and virus can be recovered from most animals by cocultivation of PBMCs (Zhang *et al.,* 1988; Letvin and King, 1990). An early manifestation of SIV infections is an erythematous maculopapular skin rash that histologically contains perivascular infiltration (primarily $CD8^+$ cells) in apposition to Langerhans cells (Letvin and King, 1990). Generation of $CD8^+$, major histocompatibility complex (MHC) class I-restricted, SIV gag-

specific cytotoxic T cell (CTL) clones from these infiltrates suggests a central role of SIV-specific CTL in the immunopathogenesis of AIDS-associated skin rashes (N. Letvin, personal communication). Another common early (1 to 2 months) clinical manifestation is peripheral lymphadenopathy which results primarily from follicular hyperplasia. Immunohistochemical studies of biopsies removed from lymph nodes show that SIV antigens can usually be found in a network of follicular dendritic cells (Letvin and King, 1990).

After these initial events, the majority of SIV-infected macaques proceed to develop AIDS and die over the ensuing 3 to 36 months; however, the nature and sequence of events that lead to death are not known. At autopsy, end-stage disease is often characterized by widespread distribution of SIV (Letvin and King, 1990; McClure *et al.*, 1989; Baskin *et al.*, 1988; Hirsch *et al.*, 1991). This can vary considerably from one animal to the next. As one might expect, primary lymphoid tissues associated with other organ systems (e.g., lung and intestine) are also common targets. In fact, gut-associated lymphoid tissue is nearly uniformly infected and, as such, may account for the intractable diarrhea seen in most terminally ill macaques. Surprisingly, SIV is also frequently detected in nonlymphoid tissues such as the kidneys (Hirsch *et al.*, 1991). In such organs, SIV antigens do not appear to be in parenchymal cells; rather, immunostaining is limited to resident or inflitrating tissue macrophages and occasionally lymphocytes.

Levels of viral DNA in tissues of terminally ill, SIV-infected macaques are sufficiently high to be detected not only by PCR amplification but also by Southern blot analysis. Levels of viral DNA appear to correlate with expression of SIV antigens in tissues. A recent study indicates that much of the viral DNA in tissues taken at autopsy is unintegrated (Hirsch *et al.*, 1991). This finding is similar to observations made in other nonhuman primate lentivirus infections (Haase, 1986; Narayan and Clements, 1989). Given that the predominant infected cells in these macaques were macrophages, the high levels of unintegrated viral DNA may simply reflect the presence of a large viral burden in the end stage of disease similar to that observed in the brains of HIV-infected patients (Pang *et al.*, 1990; Narayan and Clements, 1989). The role of unintegrated viral DNA in pathogenesis is not known. It is interesting to note that unintegrated viral DNA does not appear to be a suitable template for efficient viral transcription (Stevenson *et al.*, 1990). Therefore, it may be that intracellular accumulation of viral DNA has other untoward effects that remain to be described.

Another characteristic of SIV DNA in tissues is that each genome

appears to be unique (Hirsch *et al.*, 1991). This is similar to the quasispecies observed in HIV-1-infected individuals (Meyerhans *et al.*, 1989; Goodenow *et al.*, 1989). Given that SIV is a retrovirus containing an RNA genome, this is not surprising; however, the biological and pathogenic consequences of multiple unique SIV genomes within the host are unknown. For other RNA viruses, genome plasticity confers great advantage to the virus as it infects and reinfects the host (Steinhauer and Holland, 1986). Newly arising viral genomes may confer properties of altered cellular tropism or may allow virions to escape neutralization by the host immune system.

2. *Use of Simian Immunodeficiency Virus Molecular Clones to Define Viral Determinants of Pathogenesis*

A powerful feature of the SIV model system is the availability of molecular clones of proviral DNA that, after transfection into susceptible cells in culture, give rise to infectious virions. SIV derived in this manner can be used for experimental inoculation of macaques. This approach can yield key functional information if macaques inoculated with virions derived from cloned DNA develop AIDS. Several molecular clones of SIVmac/sm have been characterized in this fashion (reviewed in Johnson *et al.*, 1992, and summarized in Table III). Of this group, SIVmac/239 appears to offer the most promise in terms of inducing AIDS in a time frame suitable for experimental investigation (Kestler *et al.*, 1990); other clones can induce AIDS but usually after a

TABLE III
INFECTIVITY AND PATHOGENICITY OF SIVmac AND SIVsm MOLECULAR CLONES[a]

Clone	Source	Infectious *in vivo*	Induction of AIDS
SIVmac/251	Rhesus macaque	Yes	No
SIVmac/142	Rhesus macaque	No	No
SIVmac/239	Rhesus macaque	Yes	Yes[b]
SIVmac/251(1A11)	Rhesus macaque	Yes	No
SIVmne/Cl 8	Pig-tailed macaque	Yes	Yes[b]
SIVsm/H3	Sooty mangabey	Yes	Yes[b]
SIVsm/H4	Sooty mangabey	Yes	Yes[b]

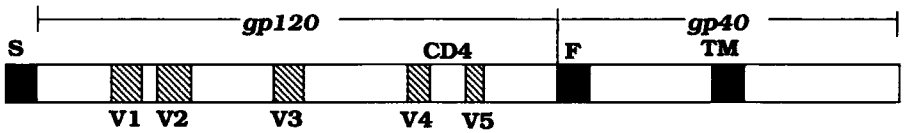
[a] SIVmac, simian immunodeficiency virus from macaque; SIVsm, simian immunodeficiency virus from sooty mangabey.

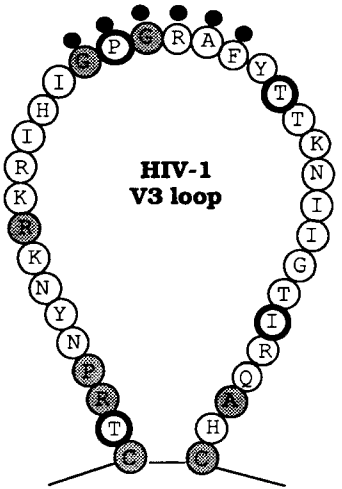
[b] Macaques infected with simian immunodeficiency virus-derived from the 239 clone usually develop AIDS 6 to 12 months after inoculation. Macaques infected with the other clones that cause AIDS (Cl 8, H3, and H4) tend to survive longer (beyond 1 year).

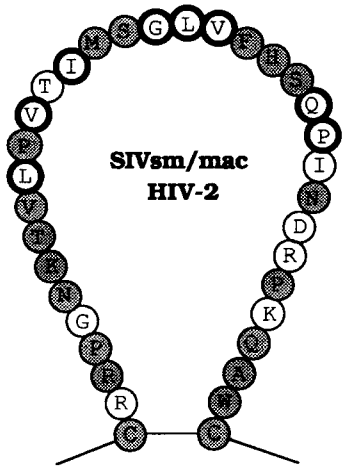
longer latent period (in the animals studied to date). Using molecular clones, several experimental approaches are available to identify viral determinants potentially involved in the pathogenesis of AIDS. First, the biologically active cloned DNA can be manipulated to study the function of specific genes *in vivo*. Second, chimeric clones can be constructed by exchanging fragments between pathogenic (AIDS-inducing) and nonpathogenic clones. Finally, genetic variation of the original clone can be followed over time in infected macaques that develop AIDS.

The strength of the SIV system is clearly demonstrated in a recent study of the *nef* gene of SIVmac (Kestler *et al.*, 1991). The original SIVmac/239 molecular clone had a premature in-frame stop codon in the *nef* gene, but remained infectious for macaque PBMCs in culture. Additional studies showed no significant differences between clones with a truncated and those with a full-length *nef* gene with regard to replicative capacity in cultured cells. Virions derived in culture (after transfection of the original clone into CEMX174 cells) were infectious when injected into macaques; however, when clones of the *nef* gene were subsequently isolated from infected macaques, it was discovered that the premature stop codon had universally reverted to a sense codon. These data indicated a strong *in vivo* selection for genomes with the full-length *nef* gene. Further studies demonstrated that the *nef* gene was required for high levels of replication in infected animals and for full pathogenic potential. Macaques infected with clones containing either the full-length or prematurely truncated *nef* gene developed AIDS with the expected frequency and demonstrated high levels of virus replication. In contrast, macaques infected with a clone containing a large deletion in *nef* did not develop AIDS and had a much lower viral burden. Thus, although the specific function of *nef* remains unknown, it has now been shown to be required for the induction of AIDS and to be important for the *in vivo* replication of SIV. As in the case of *nef*, other SIV genes can be evaluated for *in vivo* relevance for viral replication and AIDS. These studies may have great importance in the quest for novel antiviral strategies including drug and vaccine development.

The availability of molecular clones of SIV that do and do not cause AIDS also makes possible the generation of DNA clones that represent chimeric molecules. Thus, by exchanging fragments between pathogenic and nonpathogenic clones, it might be possible to identify particular sequences important for the induction of AIDS. Studies using this approach are underway in a few laboratories, but to date no definitive results have been reported.







North American	**HIV/LAI**	CTRPNYNKRKRIHI..GPGRAFYTTKNIIGTIRQAHC
	HIV/MN	-----N-T--S-R-QR------V-IGK-.-NM-----
	HIV/SF2	-----N-T--S-Y-..------H--GR---D--K---
	HIV/RF	-----N-T--S-TK..----VR-A-GQ---D--K---
African	**HIV/ELI**	-A--YQ-T-Q-TP-..-L-QSL---R.SRSI-G----
	HIV/MAL	----GNNT-RG--F..---Q-L---G.IVGD--R-Y-

SIVmac/sm	**SIVsmH4**	CRRPENKTVLPVTIMSGLVFHSQP.INDRPKQAWC
	SIVmac251	----G----------.----------
	SIVmac142	----G----------A-------.V-E-------
	SIVmneCL8	----G----------.----------
HIV-2	**HIV-2/NIH**	-K--G------I-F---FK-----V--KK-R----
	HIV-2/ST	---------V-I-L---RR---KI.--KK-R----

3. *Genetic Drift of Simian Immunodeficiency Virus Molecular Clones in Vivo*

Several recent studies have examined the genetic variation of SIV during the course of experimental infection of macaques. Four different clones were used in these studies: SIVmac/239 (Burns and Desrosiers, 1991), SIVsm/H3, SIVsm/H4 (Johnson *et al.*, 1991) and SIVmne/CL8 (Overbaugh *et al.*, 1988). All three studies analyzed clones of gp120 derived directly from PBMCs of infected macaques by PCR amplification; one study also examined gp40 and the integrase domain of the *pol* gene (Johnson *et al.*, 1991). Despite the use of three different clones, a number of common features emerge from these studies. It is now clear that the SIV *env* gene can undergo rapid and dramatic variation during the course of infection in macaques ($\sim 10^{-2}$ to 10^{-3} substitutions per site per year). The average frequency of substitutions in the integrase domain of the *pol* gene is approximately an order of magnitude lower than the frequency of substitutions in the *env* gene (Johnson *et al.*, 1991). This finding is consistent with sequence comparisons among viruses (HIV or SIV) isolated from different individuals in which the internal structural genes (*gag* and *pol*) are more conserved among isolates than the *env* gene.

Within gp120, variation occurs in regions previously defined as variable domains by analogy to HIV-1 (Fig. 3). These include V1/V2 domains near the amino terminus and the V4/V5 domains that bound the CD4 binding domain. Variation also occurs in the gp40 subunit of the SIV env protein, and surprisingly, the cytoplasmic tail appears to be a region of considerable variation in some clones. It seems unlikely that this variation is due to selection by humoral antibodies as the cytoplasmic tail presumably is not externally exposed. The function of the cytoplasmic tail is not known for SIV (or HIV), but it clearly plays a role

FIG. 3. Variation in the envelope glycoproteins of simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV). Top: The variable regions for the SIV and HIV-1 envelope are shown schematically at the top of the figure, with variable regions (V1–V5) indicated as shaded boxes. Other structural features are indicated in black including the signal peptide (S), the fusion domain (F), and the transmembrane region (TM). Middle: Schematic representation of the V3 loops of HIV-1 and SIV from macque (SIVmac); the amino acid shown in the circle is that most frequently occurring at the position, highly variable residues are shown as open circles, highly conserved residues are shown as shaded circles, and semiconserved residues are shown as circles with a heavy line. Small black circles indicate residues critical for binding of a monoclonal antibody that neutralizes HIV-1 infectivity. Bottom: Alignments of representative V3 loop sequences for various HIV and SIV isolates; data were taken from the Los Alamos HIV Database.

in virus infectivity as demonstrated in previous studies (Hirsch *et al.*, 1989a; Kodama *et al.*, 1989).

The precise role of genetic variation in the pathogenesis of AIDS in macaques is not known. At present, too few animals have been studied to draw any meaningful conclusions. The importance of particular domains can now be examined in detail (e.g., V1/V2 and V4/V5). In addition, *env* clones derived from infected animals can be inserted into biologically active clones of viral DNA to examine further the properties of sequences selected by *in vivo* passage.

C. The Simian Immunodeficiency Virus/Macaque Model for Testing Vaccines

SIV infection of macaques is also a highly relevant animal model for the testing of potential AIDS vaccine strategies. As shown in Table IV, a number of strategies have been applied in this system including whole inactivated virus (WIV) with various inactivation regimens (Carlson *et al.*, 1990; Desrosiers *et al.*, 1989; Murphey-Corb *et al.*, 1989; Putkonen *et al.*, 1991b); fixed infected cells (Stott *et al.*, 1990); vaccinia-expressed antigens (Hu *et al.*, 1991); and recombinant antigens, mostly env proteins. In summary, vaccine trials have demonstrated that a protective immune response can be elicited by WIV vaccines and that this protective effect is broad (protecting against challenge with viruses that diverge up to 20% in *env*) (Johnson *et al.*, 1992) and is apparently mediated by humoral factors. The role of humoral immunity has been suggested by transfer of protection on adminstration of plasma from an infected macaque (Putkonen *et al.*, 1991a). Protection appears to correlate well with the presence of broadly neutralizing antibody. Attenuated live SIV was found to protect macaques from disease but not from infection with a highly pathogenic SIV strain (Marthas *et al.*, 1989). Success with recombinant approaches has been far less rewarding but these investigations are preliminary. Recent reports (Stott, 1991) suggest that anticell antibodies may be a possible mediator of protection elicited by killed whole cell vaccines rather than a specific antiviral immunity. This issue is still controversial and requires further study, particularly of the mediators of protection from WIV vaccines. In addition, viral challenge that more closely mimics the route of HIV-1 infection (cell-associated virus and vaginal or rectal routes of administration) requires investigation; however, SIV is clearly the best animal model for preliminary vaccine trials.

A potential area of concern for the relevancy of SIV vaccine trials is the structure of the env protein. A notable difference between SIV and

TABLE IV
SIMIAN IMMUNODEFICIENCY VIRUS (SIV) AS A MODEL FOR VACCINES

Type of vaccine	Inactivation/adjuvant	Challenge strain	Dose (MID_{50})	Protection [% (total)]
Whole inactivated SIV (WIV)				
Nonclonal SIV				
SIVsm/B670 (DRPRC)[a]	Formalin/MDP	SIVsm/B670	10	88 (9)
SIVmac/251 (NERPRC)	Formalin/MDP	SIVmac/251	10–100	33 (6)
SIVmac/251 (CRPRC)	Psoralen and UV/MDP	SIVmac/251	200–1000	0 (4)
	BPl/MDP	SIVmac/251	0.1 TCID	100 (3)
Clonal SIVsm/H4 (NIH)	Psoralen and UV/MDP	SIVsm/E660	50	100 (6)
	Psoralen and UV/MDP	SIVmac251/32H	50	100 (4)
Attenuated live SIV				
SIVmac251/1A11clone	None	SIVmac/251	100–1000	0 (3)
Recombinant antigens				
Vaccinia gp160 + rgp160	MDP	SIVmne	10	100 (4)
Vaccinia gag + env + WIV	Formalin/MDP	SIVmac/251	200	0 (6)
Passive protection				
Plasma (9 ml/kg)	n.a.	SIVsm/H56	10–100	75 (4)

[a] MDP, muramyl dipeptide; UV, ultraviolet light; BPl, β-propriolactone; SIVmac, SIV from macaque; SIVsm, SIV from sooty mangabey; SIVmne, SIV from pig-tailed macaque; rgp160, recombinant gp160; DRPRC, Delta Regional Primate Research Center; NERPRC, New England Regional Primate Research Center, CRPRC, California Regional Primate Research Center; NIH, National Institutes of Health; n.a., not applicable.

HIV is variation in the V3 cysteine loop in gp120. In HIV-1, this loop is the principal neutralizing domain in the env protein and is highly variable from isolate to isolate (see Fig. 3), perhaps indicating immune selection. In contrast, minimal variation is observed in the SIV "V3 loop" homolog in isolates of SIVmac/SIVsm (see Fig. 3). Furthermore, sequential clones taken from animals inoculated with SIV derived from cloned DNA also demonstrate limited variation in this region of gp120. Limited studies with SIV suggest that the V3 loop analog of this virus is not a neutralizing epitope; in contrast, the majority of neutralizing activity in the plasma of SIV-immunized or infected macaques appears to be directed to conformational epitopes (Haigwood *et al.*, 1992b). In addition, neutralizing antibody generated to the V3 loop of a specific HIV-1 strain will neutralize only highly related strains. Therefore, antibodies directed to this epitope are unlikely to explain the broad neutralizing range of sera from infected patients (Steimer *et al.*, 1991) or the broad reactivity that the sera of immunized baboons and chimpanzees develop after repeated immunizations. Because of the intense interest in the SIV model as a vaccine development tool for HIV-1, this observation clearly has important implications. If the envelope structures of SIV and HIV-1 differ in a significant way regarding elicitation of protective immune responses after immunization (or infection), then conclusions regarding vaccine trials with SIV in macaques may not be directly applicable to HIV-1 vaccine development. It will be important to resolve this question so that data from SIV vaccine studies can be properly interpreted with respect to HIV-1. Ideally, the HIV-1/chimpanzee model should be used to verify the results of SIV/macaque vaccine trials.

In summary, SIV infection of macaques is the best available animal model for the study of pathogenesis as the disease spectrum observed so closely mimics human AIDS. An additional important contribution of this model will also be in the development of unique vaccine strategies and testing of antiviral drugs.

V. Animal Infection with HIV-1

Despite past success and future promise of AIDS models using related viruses, it remains obvious that any vaccine or therapeutic agent intended to combat HIV-1 infection and its consequences must be effective against HIV-1 itself. Subtle and not so subtle differences in structural and regulatory genes are seen between HIV-1 and its close relatives, SIV and HIV-2. In addition, patterns of variations in the

neutralizing epitopes in env proteins of SIV/HIV-2 isolates differ from those seen for HIV-1 isolates, precluding generalizations concerning vaccine targets. These differences, along with variation in cell tropism among different viruses and in immune mechanisms among species, require that all proposed agents or strategies be tested against HIV-1 at some stage of their development.

At the present time there are several nonhuman species for which HIV-1 infection has been reported; these include the chimpanzee, the rabbit, and the SCID mouse reconstituted with human immunocompetent cells. As with all models considered, each has positive and negative features and there are clues concerning possible improvements for future use of these models for HIV-1 infection.

A. Infection of the Chimpanzee with HIV-1

As the closest extant phylogenetic relative to humans, the chimpanzee (*Pan troglodytes*) has the potential to play a critical role in studies of HIV-1 infection and the progression to AIDS. Among the reasons the chimpanzee can be an excellent model for this disease is the near identity of the CD4 receptors in human and chimpanzee T lymphocytes (Camerini and Seed, 1990). In addition, there is much known about the immune system of the chimpanzee (Zarling *et al.*, 1990) and nearly every indicator shows it to be very similar to the human immune system. It was shown very early (Alter *et al.*, 1984) that the chimpanzee could support infection with HIV-1. This has been confirmed using a variety of infection routes (Fultz *et al.*, 1986b, 1987) and various HIV-1 isolates (Nara *et al.*, 1989); however, of the over 100 chimpanzees experimentally infected with HIV-1, until recently (Fultz *et al.*, 1991) only two had shown any sign of disease that may be analogous to human AIDS (Nara *et al.*, 1990). Other factors limiting utilization of the chimpanzee include its status as an endangered species and the high cost of obtaining and maintaining chimpanzees. The use of this species for study of infectious agents such as HIV-1 may be prohibitively expensive for most laboratories engaged in basic research.

1. Pathogenesis of HIV-1 Infection in the Chimpanzee

Over 100 hundred chimpanzees have been infected with HIV-1 and none have developed opportunistic infections. In rare cases, a decline in the number of CD4-bearing lymphocytes or lymphadenopathy was observed. It is frequently difficult to isolate virus from the peripheral blood cells of infected chimpanzees. Therefore, the overall picture is one of persistent infection without overt progression to disease in the

infected animals. Several explanations have been advanced for the failure of HIV-infected chimpanzees to progress to AIDS (Fultz, 1991). First, the animals maintained for these experiments are generally kept free from infectious agents that may be considered cofactors for the development of disease. In a similar cohort of HIV-1-infected human patients, 13 to 27% of the group would be predicted to develop AIDS within 8 years; however, the fact that the chimpanzees are kept in relative isolation from most infectious agents, may considerably lengthen that term. It therefore may be that, with more time, AIDS-like disease will be observed in HIV-1-infected chimpanzees. Disease may also be hastened by administration of agents known to serve as cofactors in human AIDS. A report (Lusso *et al.*, 1990) that human herpesvirus-6 (HHV-6) can infect chimpanzee T cells and cause accelerated cytopathicity of HIV-1 suggests that cofactors may play a role in disease development.

A second factor that may limit disease onset in chimpanzees is that well-defined, tissue culture-grown virus has been used in most reported experiments; these strains of HIV-1 may be attenuated to the point that they do not readily cause disease. Questions concerning the virulence of the laboratory strains of HIV-1 in the chimpanzee were approached in a recent study (Fultz 1991) in which a chimpanzee was infected with three diverse strains of HIV-1. This animal developed hematological and immunological abnormalities that have been seen in both HIV and SIV infections, as well as a number of symptoms that may be equated to AIDS. The HIV-specific antibody titers decreased 10-fold within the course of the infection, the animal stopped gaining weight, CD4 cells decreased to new low values, recurrent lymphopenia (less than 3000 lymphocytes per microliter) was documented, and thrombocytopenia developed and persisted up to 57 months after inoculation with the last virus (Fultz, 1991). In addition, complement C4 levels decreased and autoimmune antibodies to histone H-2B were detected. Testing of the lymphocytes indicated that the proliferative response to T cell mitogens declined significantly. All of these signs are compatible with HIV-1 infection; however, the direct effect of the virus cannot be proven from this single experiment. In other experiments, chimpanzees infected with HIV-1 for greater than 4 years had persistently abnormal low numbers of platelets beginning at various times after the initial infection.

In addition to the factors mentioned above that may limit onset of disease in chimpanzees, a third possibility is that HIV-1 is not pathogenic in this species. There is some indication that the chimpanzee may be the natural host for a virus that is extremely similar to HIV-1. A

lentivirus similar to HIV-1 and referred to as SIV_{cpz} was isolated from two wild-born, pet chimpanzees in Gabon in west equatorial Africa (Peeters *et al.*, 1989). Sera from the animals cross-reacted with all HIV-1 proteins including the envelope glycoproteins. Molecular cloning and sequence analysis of an infectious clone of SIV_{cpz} were carried out and showed that the genetic organization is identical to that of HIV-1 (Huet *et al.*, 1990). Although the sequence is more divergent than most strains of HIV-1 reported in humans, this virus is more closely related to HIV-1 than any characterized SIV or HIV-2 strains. Sequence identity of SIV_{cpz} to HIV-1 isolates ranges from 84% identity in amino acid sequence for the *pol* gene and 75% for the *gag* gene to a low of 36% identity between the genes for the regulatory protein vpu.

The findings of SIV_{cpz} in a wild-born chimpanzee raises questions concerning the origin of HIV-1 and its spread in the human population, as well as questions concerning natural immunity to HIV-1 in this species; however, the data must still be viewed with caution because even with extensive serosurveys (particularly of captive animals) these are the only seropositive chimpanzees to be described to date. Such immunity would obviously have impact on the effect of HIV-1 in the experimental trials. Several features of the chimpanzee infection may be relevant to this possibility. First of all, the CD8 cells from infected and about 50% of uninfected animals had a profound negative effect on HIV-1 infection *in vitro* (Castro *et al.*, 1991). This may indicate that there is a surveillance by cytolytic cells that prevents spread of the virus throughout the animal. The *in vitro* studies of HIV-1 in the chimpanzee indicate that the cytopathic affect of HIV-1 is limited to certain cell types and is not as widespread as observed in the infection of human cell cultures; however, in contrast to initial reports indicating that HIV infection is limited to T cells, a recent study using HIV-1 passaged *in vivo* in chimpanzees showed that this virus will infect macrophages and is cytopathic for CD4 cells (Watanabe *et al.*, 1991b), even though it did not cause disease in the host animal. Although the occurrence of natural immunity in chimpanzees would provide an explanation for the lack of HIV-1 disease, further data are needed to verify it.

2. *The Chimpanzee Model for Testing HIV-1 Vaccines*

Despite shortcomings related to HIV-1 pathogenesis that may limit the utility of the chimpanzee as a model for AIDS, there is great value in the chimpanzee as a model in which to test HIV-1 vaccines for potential use in humans. The fact that chimpanzees become chronically infected on challenge with HIV-1 makes this an excellent

experimental tool to test the efficacy of vaccines to prevent or retard human HIV-1 infection. A number of studies have been carried out and protection against challenge with isolated HIV-1 stocks of known titer (Arthur *et al.*, 1989) has been demonstrated in some cases (Girard *et al.*, 1991). In addition, immunization of animals already infected resulted in inability to isolate virus in peripheral blood cells from certain animals (Gibbs *et al.*, 1991). The majority of chimpanzee vaccine studies have used envelope proteins gp120 or gp160 but the *gag* protein, p55, was tried as a vaccine with little success (Emini *et al.*, 1990a). The regulatory protein *nef* was also studied (Bahraoui *et al.*, 1990) and found to raise an immune response in chimpanzees.

The chimpanzee infection model allows examination of time-related factors in HIV-1 immunity. For example, it was shown that antibody formed early in chimpanzee infection may have an enhancing rather than a neutralizing effect on *in vitro* infection with HIV-1 (Robinson *et al.*, 1989). Another group using multiple isolates and antisera from bleedings taken at various intervals from chimpanzees showed that antibody may aid in selection of variants resistant to neutralization (Nara *et al.*, 1990). Serum taken early in infection could neutralize the HIV-1 used in the inoculum but not that isolated from the animals at later times. Examination of the resistant virus showed that it no longer reacted with antibodies directed against the principal neutralizing determinant (the V3 region of *env*).

At the present time, the number of animals used in any one chimpanzee vaccine trial precludes definitive conclusions; however, it appears that immunization with whole intact virus and with various recombinant proteins including *gag*, *nef*, and *vif* as well as use of peptides derived from the neutralizing V3 loop may have a positive effect in either preventing or slowing HIV-1 infection in the chimpanzee (Girard *et al.*, 1991). On the other hand, similar protocols in other reports, using recombinant gp160, have not given significant levels of protection (Berman *et al.*, 1990). When envelope protein was administered in a vaccinia vector, no evidence for neutralizing antibody was obtained, leading to the conclusion that no protective immunity had been elicited (van Eendenburg *et al.*, 1989) by this immunization protocol. An earlier study (Hu *et al.*, 1987) using recombinant *env* in a vaccinia construct indicated that this immunization did not prevent infection on challenge of the animals. The fact that antibody can elicit protection against challenge was shown by an experiment (Emini *et al.*, 1990b) in which challenge virus was treated with anti-HIV-1 antibody or with IgG from nonimmune animals prior to its introduction into the chimpanzees. It was shown that polyclonal neutralizing anti-

body destroyed the ability of the HIV-1 to infect, whereas nonimmune IgG had no effect.

3. *Use of the Chimpanzee Model to Test Therapies Based on CD4*

It is well established that the surface marker for the helper T cell subset in humans, CD4, binds to the env protein gp120 of HIV-1 with high affinity and that this interaction plays a role in HIV-1 pathogenesis (Capon and Ward, 1991; McDougal *et al.*, 1991). Demonstration of CD4/gp120 binding along with the fact that helper T cells are selectively depleted in AIDS suggests that this is the preferred receptor for HIV-1. Because of this interaction between CD4 and the HIV env gp120, CD4 has been proposed as a basis for antiviral agents (Fischer, 1991). Recombinant soluble CD4 and modified forms of it have been used as a means to block CD4 interaction with HIV and to target toxins to HIV-1-infected cells (Chaudhary *et al.*, 1988). In addition, recent reports describe use of CD4 immunization to raise antibodies against self CD4 to block interactions with gp120 (Watanabe *et al.*, 1992).

Animals models used to test CD4-based therapies require similarity to human CD4 in those regions that bind to gp120 (Clayton *et al.*, 1988). Comparison of the first two domains of the CD4 sequences of human, chimpanzee, rhesus monkey, and mouse is depicted in Fig. 4. This comparison shows that chimpanzee CD4 has closest similarity to human CD4; only five amino acid differences are found between them and four of these are in domain 1. Although it binds to gp120 with an affinity comparable to that of human CD4, chimpanzee CD4 does not promote the formation of syncytia in *in vitro* HIV-1 infection as does human CD4. It was shown that a single amino acid substitution (glutamic acid at position 87 to glycine in chimpanzee) mediates this functional difference (Camerini and Seed, 1990). This difference does not impair the ability of chimpanzee CD4 cells to bind gp120 nor to support HIV-1 infection (McClure *et al.*, 1987).

The chimpanzee HIV-1 infection model has been used to test the effect of CD4 immunoadhesin (a chimeric molecule with the two amino-terminal domains of CD4 linked to an IgG_1 Fc region) on HIV-1 infection (Ward *et al.*, 1991). It was shown that two animals given the CD4-IgG molecule remained seronegative 47 weeks after HIV-1 challenge, whereas an infected but untreated control was seropositive 7 weeks postchallenge.

This conservation of CD4 in primates was exploited in studies of anti-CD4-based therapy (Watanabe *et al.*, 1992). These studies involved immunization of chimpanzees with recombinant soluble CD4

	10		20		30		40		50		60
Human	KKVVL	GKKGD	TVELT	CTASQ	KKSIQ	FHWKN	SNQIK	ILGNQ	GSFLT	KGPSK	LNDRA	DSRRS
Chimp	-----	-----	-----	-----	-----	-----	---T-	-----	-----	-----	----V	-----
RhMac	-----	-----	-----	-----	--NT-	-----	-----	---I-	-L---	-----	-S---	---K-
Mouse	-TL--	--E-E	SA--P	-ES--	--ITV	-T--F	-D-R-	---QH	K -GV-I	R-G-P	F SQ--F	--KKG

	70		80		90		100		110		120
Human	LWDQG	NFPLI	IKNLK	IEDSD	TYICE	VEDQK	EEVQL	LVFGL	TANSD	THLLQ	GQSLT	LTLES
Chimp	-----	--T--	-----	-----	-----	-G---	-----	-----	-----	-----	-----	-----
RhMac	-----	C-SM-	-----	-----	-----	--NK-	---E-	-----	-----	----E	-----	-----
Mouse	A-EK-	S----	-NK--	M---Q	-----	L-NR-	---E-	W--KV	-FSPG	-S---	-----	N ---D-

	130		140		150		160		170		180	
Human	PPGSS	PSVQC	RSPRG	KNIQG	GKTLS	VSQLE	LQDSG	TWTCT	VLQNQ	KKVEF	KIDIV	VLAFQ
Chimp	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	----->
RhMac	-----	---K-	---G-	-----	-R-I-	-P---	R----	-----	-S-D-	-T---	-----	----->
Mouse	SKV-N	-LTE-	KHKK-	-VVS-	S-V--	M-N-R	V---D	F-N--	-TLD-	--NW-	GMTLS	--G-->

in Freund's incomplete adjuvant and assessment of anti-HIV activity of the resulting antibodies. It was found that serum samples from CD4-injected chimpanzees (tested versus antiovalbumin controls) blocked *in vitro* infection of human PBMCs with HIV and, further, that PBMCs from the CD4-injected chimpanzees would not support HIV infection. Importantly, there is no evidence of diminished or impaired immune function in the chimpanzees making anti-CD4. These chimpanzees have not been challenged *in vivo* with HIV because it is not certain what parameters of infection would be useful to follow. In parallel experiments, however, rhesus monkeys that had been immunized to make antiself CD4 showed protection to challenge with SIV (Watanabe *et al.*, 1991a).

The recent results concerning CD4-based therapies together with the information from vaccine studies indicate that the chimpanzee model for HIV-1 infection will be invaluable in the study of means to combat HIV-1 infection. Although the chimpanzee model is limited by the absence of immunodeficiency along with HIV-1 infection, there is some hope that use of different viral strains or *in vivo* serial passage of virus (Gendelman *et al.*, 1991) may foster reproducible onset of disease in the future (Moore and Weiss, 1991). This would greatly enhance the value of the chimpanzee in the study of pathogenesis and development of agents to limit HIV-1 disease.

B. Infection of Rabbits with HIV-1

It has been known for a number of years that the laboratory rabbit (*Oryctolagus cuniculus*) is readily infected with the human retrovirus HTLV-1 (Miyoshi *et al.*, 1985; reviewed by Sawasdikosol and Kindt, 1992). Studies with the rabbit model have led to new information about the action and transmission of this virus including proof that virus may be passed on mother's milk. Leukemia-like disease may be demonstrated in certain instances of rabbit HTLV-1 infection (Seto *et al.*, 1987). This model is being used to test drug and vaccine strategies against HTLV-I infection that may have eventual application to HIV-1 infection. More to the point of the present topic, infection with a second human retrovirus, HIV-1, has been demonstrated in rabbits.

Despite early reports to the contrary (Morrow *et al.*, 1987), data

FIG. 4. Alignment of amino acid sequences for the two amino-terminal domains of CD4 from human, chimpanzee, rhesus monkey, and mouse. The dashed line indicates identity to human sequence; residues written above the line represent insertions introduced to maintain optimal alignment.

accumulating from various sources (Filice *et al.*, 1988; Kulaga *et al.*, 1989) indicate that the rabbit supports HIV-1 infection. Although early attempts to establish infection by injection of partially purified virus or by injection of blood samples from human AIDS patients (Morrow *et al.*, 1987) resulted in no signs of infection, a number of different laboratories now report success in infecting rabbits with HIV-1 (reviewed by Varnier and Kindt, 1992). Rabbits have been infected by intraperitoneal injection of cell-free virus following pretreatment with agents that cause activation of peritoneal macrophages or, alternatively, by intravenous injection of human lymphoid cells that are infected with HIV-1. Although no reproducible clinical consequences of infection have been noted, early studies of HIV-1 infection in rabbits infected with HTLV-1 yielded sporadic episodes of weight loss and transient neurological impairment (Kulaga *et al.*, 1989). More recently, data showing the activation of HIV-1 in infected rabbits by superinfection with syphilis have been reported (Tseng *et al.*, 1991). Similarly, coinfection of HIV-1-infected rabbits with HTLV-1 accelerates the appearance of viral transcripts in peripheral blood cells (Truckenmiller *et al.*, 1989, 1992).

Rabbits infected with HIV-1 produce antibody to HIV-1 proteins within 10 weeks of administration of HIV-1. HIV-1 has been detected by PCR, *in situ* hybridization, and virus isolation from rabbit cells and organs for periods up to 2 years after infection (Truckenmiller *et al.*, 1989; Varnier *et al.*, 1990). Reports from several laboratories suggest that the brain may be a preferential target of HIV-1 infection in rabbits (Sawyer *et al.*, 1990). Although there is no consistent evidence for clinical disease, few infected animals have been closely observed for long periods. Immunosuppression is not obvious in infected animals, but diminished cellular responses to heat-killed *Mycobacterium bovis* were observed in rabbits given antigen 7 days prior to virus injection (Gordon *et al.*, 1991).

Despite strong evidence for persistent HIV-1 infection, there are several shortcomings that render the rabbit a less than ideal model for testing of antiviral agents or vaccines that are aimed at combating human AIDS and HIV-1 infection. First, relatively large doses of virus are required for infection. This has been shown in both *in vitro* and *in vivo* studies (Kulaga *et al.*, 1988, 1989; Filice *et al.*, 1988). Second, virus is not readily isolated and the infection, although it persists, does not cause overt disease within the time frame in which infected rabbits have been observed. An additional problem is that the rabbit CD4 homolog had not been characterized until quite recently. As a consequence, there are no antibodies against the rabbit CD4 receptor avail-

able to measure possible differences in T cell subpopulations on infection (Wilkinson, 1988).

The precise role of a CD4-like molecule in rabbit infection has not been clearly described. Recent studies of the role of CD4 in rabbit cell infection have indicated that transfection of rabbit cell lines with human CD4 renders them more susceptible to infection with HIV-1 (Hague *et al.*, 1992; Yamamura *et al.*, 1991). The use of recombinant soluble human CD4 to inhibit *in vitro* infection of rabbit cell lines suggests that this molecule could play a role in the infection process. Structural comparison of human and rabbit CD4 (Hague *et al.*, 1992) show that the rabbit homolog is more distant than those from primates. On the basis of the available data it is possible that construction of a rabbit with a human CD4 transgene may provide an improved HIV-1 infection model.

There is a good reservoir of knowledge concerning the metabolism of the rabbit from toxicology studies and this species has been used extensively as a model for various human diseases of viral and bacteriological etiology. Recently reported infections of rabbits with bovine leukemia virus (BLV) (Altanerova *et al.*, 1989) and bovine immunodeficiency-like virus (BIV) (Gonda *et al.*, 1990a) promise to provide new tools for study of these and similar viral agents. Although there are promising aspects to such a small animal model for infection with HIV-1, the value of the rabbit model remains potential. Studies showing viral DNA and RNA by PCR and *in situ* hybridization indicate that there is infection and it persists for years; however, until means to exacerbate the course of infection and/or demonstrate the presence of disease are available, this animal model remains interesting but of limited use for development of anti HIV-1 agents.

C. The SCID-hu Mouse

The C.B-17 SCID/SCID mouse was first reported (Bosma *et al.*, 1983) to carry a spontaneous recessive mutation that gives rise to a severe combined immunodeficiency. This strain has neither functional T cells nor B cells; therefore the defect presumably involves a step in the lymphoid development lineage at the step of the enzymes that dictate recombination and rearrangement of the T cell receptor in immunoglobulin genes (Schuler, 1990). Because of this immune defect, the so-called SCID mouse may be engrafted with tissue from various sources with no possibility of rejection. This ability has made it possible to develop an animal model for the study of HIV infection of human lymphoid tissue engrafted onto the SCID mouse (Mosier, 1991; McCune *et al.*, 1991). In addition to direct measurements of HIV

infection in the human lymphoid grafts, certain immune functions carried out by the grafted tissue may also be monitored for evidence of deficiency caused by the virus (Mosier *et al.*, 1991). There are two major variations in experiments using the SCID mouse for a model for HIV infection. One involves reconstitution of the SCID mouse by the injection of human peripheral blood cells taken from donors negative for the Epstein–Barr virus (PBL-SCID-hu mice) (Mosier *et al.*, 1988); the other involves surgical engraftment of fetal thymus or lymph node tissue in the SCID mouse (McCune *et al.*, 1988).

It is reasonably certain that the infection observed in the SCID-hu mice is HIV-1. The possibility that pseudotypes (i.e., phenotypic mixtures of xenotropic murine leukemia viruses and HIV-1) are the cause of infection in the SCID mouse has been raised (Marx, 1990). Viral pseudotypes incorporating components of murine leukemia virus would be expected to infect mouse cells *in vivo* and to have an expanded host range *in vitro* as well. This would diminish the value of SCID-hu models for the study of human infection; however, subsequent data indicate that the occurrence of pseudotypes is improbable because no evidence was seen for spread of HIV-1 in the normal organs of the mouse, nor was the virus recovered from infected SCID-hu mice infectious in any cell other than human $CD4^+$ T lymphocytes.

Infection of the PBL-SCID-hu mouse with HIV-1 results in changes in lymphocyte function consistent with those seen in HIV-infected individuals (Mosier *et al.*, 1991). Injection of as little as 10 tissue culture infectious doses ($TCID_{50}$) of HIV could cause infection. In these experiments, virus was administered intraperitoneally and a variety of assays were used to detect the virus. These included culture of virus from peritoneal cavity, spleen, peripheral blood, or lymph nodes, and in addition, viral sequences were detected by *in situ* hybridization and by amplification with PCR. A number of viral strains including LAV/Bru, IIIb, MN, SF2, and SF13 were successfully used in these studies. Infection of the PBL-SCID-hu mice appeared to be optimum when animals that has been engrafted 2 weeks previously were injected. The use of animals 8 weeks postengraftment led to a significantly diminished number of infected animals.

Immunoglobulin levels in the sera of the PBL-engrafted, HIV-1-infected mice were measured. A sharp increase in levels of human immunoglobulin was found at about 2 weeks following HIV-1 infection. This was followed at weeks 4–8 by diminished levels of immunoglobulin. This phenomenon is similar to that seen in certain AIDS patients. Along with the rise and subsequent decrease in immunoglobulin levels, a similar decrease in CD4 cells was seen in the infected

mice as compared with the control animals that were engrafted but not infected.

In the variation on the SCID-hu model for HIV infection pioneered by McCune and his co-workers, fetal human lymphoid tissue is surgically engrafted into the SCID mouse (Kaneshima *et al.*, 1991). Thymus, lymph node, and fetal liver tissue have been used in this fashion. It has been shown that 95% of SCID-hu mice prepared with lymph nodes from 18- to 23-week-old fetuses may be infected by intravenous injection of HIV-1 and, this infection results in plasma viremia (Kaneshima *et al.*, 1991). Detection of HIV sequences in the plasma of infected animals by the PCR can be used to follow the infection. This system has been used to demonstrate efficacy of AZT and 2′,3′-dideoxyinosine in infected mice (McCune *et al.*, 1990; Shih *et al.*, 1991). The detection of virus by PCR as well as by *in situ* hybridization in human tissue recovered from SCID-hu mice was significantly diminished, as compared with controls, in animals given doses of these drugs similar to those used in human patients (McCune *et al.*, 1990). In addition to prevention of infection in animals treated with AZT prior to viral challenge, it was shown that viremia sharply decreased in mice infected with HIV-1 previous to administration of AZT as well.

Although both variations of the SCID-hu mouse have been used to obtain data concerning HIV-1 infection, certain features distinguish the two models (Mosier, 1991). First, only primary clinical isolates of HIV-1 replicate in the engrafted human organs; tissue culture isolates commonly used in the laboratory are not infectious in this setting, although they will infect PBL-SCID mice. In addition, larger doses of virus are needed to infect by the intravenous route used by McCune and his co-workers. It is not clear at the present time which of the variations of this model is most useful. Whichever system is used to prepare the animals, infection studies with the SCID-hu mouse have the potential to provide at least part of the linkage between *in vitro* experiments showing the efficacy of a given drug or immune mediator and its use in human patients.

To date there are no reports concerning the use of SCID-hu mice to test anti-HIV-1 vaccines. Several possibilities for this use of these models may be envisioned, especially as progress is made toward reconstitution of an entire human immune system in the SCID mouse. For example, direct immunization of immunocompetent, reconstituted mice with inactivated virus or proteins and peptides derived from it may be attempted. Passive immunization studies may also provide information concerning antibody specificities most effective against cell–cell spread of *in vivo* infection.

VI. Conclusions

Several animal models for HIV-1 infection and for AIDS have been reported. Currently, there is no obvious consensus concerning which of these proposed models represents the best model for human AIDS. Each has areas of applicability, but none is perfect. As discussed above the choice of model must depend on the goals of the study. The chimpanzee infection with HIV-1 may represent the best test of an HIV-1 vaccine, but has little value for therapeutic agents because no disease follows infection. The immunodeficiency seen in the SIV and FIV models mimics AIDS and these serve as models for pathogenesis; however, structural differences in the viruses preclude a direct test of anti-HIV vaccines in these models. Further work is needed on the present models and new approaches are required to construct the ideal system. The pressing need to develop effective prophylactic and therapeutic agents to combat the AIDS epidemic should provide the impetus for this development.

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