ADVANCES IN
Immunology

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VOLUME 53

ACADEMIC PRESS, INC.
Harcourt Brace & Company
San Diego New York Boston
London Sydney Tokyo Toronto

Academic Press, Inc.
1250 Sixth Avenue, San Diego, California 92101-4311

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

Library of Congress Catalog Number: 0065-2776

International Standard Book Number: 0-12-022453-4

PRINTED IN THE UNITED STATES OF AMERICA
93 94 95 96 97 98 MM 9 8 7 6 5 4 3 2 1

ADVANCES IN IMMUNOLOGY, VOL. 53

Lymphokine and Cytokine Production by FcεRI$^+$ Cells

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I. Introduction

Lymphokines and cytokines mediate a wide range of biologic functions. They are responsible for much of the regulatory activity of T cells and have potent pro- and antiinflammatory properties. Although cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor (TNF-α), and the family of molecules that include interleukin-8 (IL-8), are produced by cells of many distinct types, the lymphokines [e.g., interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and lymphotoxin (LT, or TNF-β)] have a much more restricted range of cellular origins. With the exception of GM-CSF and IFN-γ, these molecules were, until recently, regarded as exclusive products of activated T cells.

It was therefore of considerable interest when it was recognized that a subset of the lymphokines could be produced by mast cell lines (*1–6*). In this review, we discuss the growing information on production of IL-4 and of a set of related lymphokines (IL-3, IL-5, and GM-CSF) by mast cells and other FcεRI$^+$ cells as well as production of TNF-α, IL-6, IL-1, and IL-8 and its congeners by these cells. We review the pathophysiologic conditions in which lymphokine production by FcεRI$^+$ cells is increased and the signaling mechanisms employed by FcεRI$^+$ cells that lead to lymphokine production.

II. Biology of Mast Cells and Basophils

Mast cells and basophils are cell types that have two major phenotypic properties in common (*7,8*). They both store histamine and both express high-affinity FcεR, called FcεRI. IgE binds avidly to this high-affinity receptor, with a dissociation rate of the order of days. Both cell types also contain basophilic cytoplasmic granules, consisting of negatively charged, sulfated proteoglycans, either heparin or chondroitin sulfate. Histamine is positively charged and binds electrostatically to the granules. Recently, a third cell type, Langerhans cells, also has

been shown to express FcεRI (*9,10*). The role of the FcεRI of Langerhans cells is unknown.

Mast cell and basophil secretion of histamine and other mediators, namely, prostaglandin D_2 and leukotriene C4, provides the effector arm of immediate hypersensitivity reactions. Recent experimental models suggest that mast cells and basophils have a wider role in inflammatory reactions than previously appreciated. Several inflammatory reactions are mast cell dependent, presumably due to the release of mast cell-derived mediators, including TNF-α (*11–14*). Basophils accumulate in inflammatory reactions in guinea pigs, humans, and rodents, and probably release their mediators in these reactions (*15–22*). The capacity of these cells to secrete cytokines, discussed in detail in the subsequent sections of this review, provides an explanation for their potentially important role in inflammation.

Mast cells and basophils have been used as models of signal transduction by receptors of the immunoglobulin supergene family (*7*). These cells bind specific IgE antibodies via FcεRI; multivalent antigen crosslinks the IgE, resulting in receptor crosslinking and the initiation of a rapid series of biochemical events leading to secretion of granule products, including histamine.

Although mast cells and basophils have several common characteristics and they both develop from precursors in hematopoietic tissue, the two cell types differ in characteristics such as size, nuclear morphology, histamine content, proteoglycan type, protease gene expression, and arachidonic acid metabolic pathways of membrane phospholipids. Human basophils are circulating cells that have polymorphonuclear nuclei. They express at least one cell surface glycoprotein, BSP-1, that mast cells do not (*23,24*). Basophils express very low levels of the membrane tyrosine kinase c-*kit* (*25,26*) and contain 1 pg histamine/cell (*24*). By contrast, human mast cells are larger, express high levels of c-*kit*, and contain 2–3 pg histamine/cell. Rodent basophils have not been well characterized; they have been difficult to identify because they contain very few granules and have a much lower histamine content (approximately 0.002 pg/cell) than do human basophils (*27–29*).

The phenotypic distinctions between mast cells and basophils raise the possibility that these two cell types differ in their capacity to produce cytokines. Moreover, within each cell type there are various stages of maturation that may reflect distinct function. A most important distinction is between two types of mast cells. Rodent mast cells are divided into "immature" mucosal types and "more mature" connective types (*8,30,31*). Mucosal mast cells are found in the intestinal

and other mucosae. They express specific proteases, granules with chondroitin sulfate as the principal proteoglycan, and relatively low levels of histamine. By contrast, connective tissue mast cells are found in the skin and pleural and peritoneal cavities. They express proteases distinct from those of mucosal mast cells, possess granules with heparin as the principal proteoglycan, and have a relatively high histamine content.

Mucosal mast cells are T cell dependent [e.g., they are absent in congenitally athymic (*nu/nu*) mice] (*32*), presumably because they require T cell-derived IL-3 for growth and survival. They are markedly increased in number during certain intestinal parasistic infections (*33*). Connective tissue mast cells do not require T cells for growth. It was formerly thought that connective tissue mast cells were inert, but recent results demonstrate that *steel* factor (the c-*kit* ligand) is a potent stimulus to their growth (*34,35*). Furthermore, combinations of IL-3 and IL-4 also are reported to stimulate growth of connective tissue mast cells (*36,37*).

In addition to mucosal and connective tissue mast cells, culture of bone marrow, spleen, or fetal liver in IL-3 for 3–5 weeks results in the appearance of populations of mast cells (often designated bone marrow-derived mast cells) that resemble mucosal mast cells (*38–42*). When cultured on fibroblast monolayers or when grown in the presence of *steel* factor, the cultured mast cells undergo further differentiation, to cells with some phenotypic resemblance to connective tissue mast cells (*8,43,44*), including a higher histamine content and some expression of heparin proteoglycans. Despite the apparent differences between mucosal and bone marrow-derived mast cells versus connective tissue mast cells, some cells of one type can differentiate or dedifferentiate to the other type. Thus, bone marrow-derived mast cells transferred to the peritoneum of mast cell-deficient W/W^v mice will develop a connective tissue phenotype (*45*), whereas peritoneal mast cells transferred to the stomach of mast cell-deficient mice will develop a mucosal phenotype (*46*).

Human mast cells are also heterogeneous. Thus, human mucosal mast cells are T cell dependent (e.g., they are deficient in patients with T cell deficiencies such as AIDS) (*47*) and express a pattern of proteases (tryptase but not chymase) distinct from that of connective tissue mast cells (which contain tryptase, chymase, and carboxypeptidase A) (*48–51*). However, the pattern of proteoglycans does not match the rodent pattern. Furthermore, although human basophils grow in the presence of IL-3, mature human mast cells have been reported not to express IL-3 receptors (*52*).

III. Lymphokine Production by Transformed Murine Mast Cells

In a search for cell types other than T cells that might produce IL-4, RNA from a wide range of hematopoietic lineage cell lines was examined. Among these, only transformed mast cells expressed IL-4 mRNA constitutively (Fig. 1) (*1*). A group of mast cell lines that had been transformed by infection with the Abelson murine leukemia virus (Ab-MuLV) was examined. Some of these lines had been derived by infection of factor-dependent mast cell lines and others were obtained from mice that had been directly infected with the virus (*53,54*). Both types of transformation gave rise to cell lines that expressed IL-4 mRNA and secreted IL-4. Indeed, in the initial report describing this observation (*1*), 6 of 11 AB-MuLV-transformed cell lines were found to produce IL-4 constitutively and 8 of 11 were found to produce IL-4, IL-3, or GM-CSF. Production of IL-5 by this initial group of lines was not tested.

In more recent studies of lymphokine expression by an additional set of Ab-MuLV-transformed mast cell lines, constitutive lymphokine

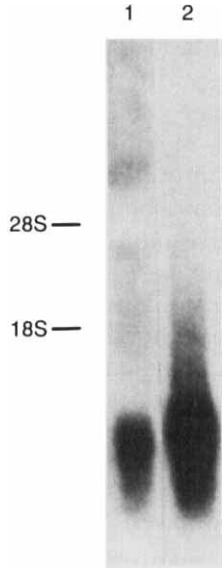

FIG. 1. IL-4 mRNA in a transformed mast cell line. Total RNA was prepared from the T cell line EL-4, which had been stimulated with phorbol myristate acetate, and from ABFTL-3 cells. Northern analysis with an IL-4 cDNA probe was carried out on EL-4 RNA (lane 1) and ABFTL-3 RNA (lane 2). (Adapted from Ref. *1*, with permission.)

production continued to be observed, although the frequency of lines producing IL-3 was greater than observed in the initial series and IL-4-production was less often observed (A. Keegan, unpublished observations, 1991). The factors that determine whether IL-4 or IL-3 is produced by a given transformed cell and whether a given cell will change its pattern of lymphokine production have not been determined. We have observed, however, that different sublines of such transformed lines can vary in their pattern of lymphokine production. Thus, an ABFTL-3 line that initially produced IL-4 and not IL-3 continues to maintain this pattern when carried over a 3-year period in one laboratory, whereas another subline of ABFTL-3 no longer produces IL-4 but now secretes IL-3 (M. Brown, personal communication, 1991; A. Keegan, unpublished observations, 1991).

Although transformed mast cell lines such as ABFTL-3 produce lymphokine constitutively, the production of IL-3 by these cells is enhanced by the addition of a calcium ionophore such as ionomycin or by cross linkage of the high-affinity FcεR that they express (55). This indicates that exogenous stimuli can enhance lymphokine production, an observation made more forcefully in the study of nontransformed mast cell lines and of FcεRI$^+$ cells freshly harvested from normal and infected mice.

Because both IL-3 and IL-4 are mast cell growth factors (56,57), the possibility was considered that constitutive secretion of these lymphokines by transformed mast cells might contribute to the transformed state of these cells. This possibility was directly examined by culturing five different transformed mast cell lines, four of which produced IL-4 but not IL-3, in the presence of a neutralizing anti-IL-4 antibody (*1*). In no case did the antibody inhibit the growth of the cells. Furthermore, IL-4 did not enhance the growth of any of these cells. Indeed, in the one case that was examined, it was shown that IL-4 did not improve the cloning efficiency of a transformed IL-4-producing mast cell line. These results thus imply that IL-4 production does not cause the transformed state of Ab-MuLV-transformed mast cells.

Indeed, it is possible that constitutive production of IL-4, IL-3, and GM-CSF by Ab-MuLV-transformed mast cells may result from the action of the v-*abl*-encoded tyrosine kinase. As is discussed later, receptor cross linkage leading to lymphokine production in mast cells, just as in T cells and NK cells, leads to activation of the *src* family of tyrosine kinases and to tyrosine phosphorylation of a series of protein substrates (58–*61*). Inhibition of tyrosine kinase activity by genestein and herbimycin blocks lymphokine production in each cell type (55,*61*–63), suggesting that these phosphorylation events are impor-

tant in inducing lymphokine production. The possibility exists that the tyrosine kinase encoded by v-*abl* and the receptor-activated tyrosine kinase(s) in mast cells induce lymphokine production by their action on similar substrates. However, it should be pointed out that three of four spontaneously transformed mast cells tested, including the widely used mastocytoma P815, expressed IL-4 mRNA constitutively (*1*). This suggests that the common phosphorylation substrate concept may not be correct. However, because little is known about the pattern of protein tyrosine phosphorylation in these transformed cell lines, no firm conclusion can be reached on this point.

IV. Lymphokine Production by Factor-Dependent Murine Mast Cell Lines

In contrast to transformed mast cells that often produce IL-4 constitutively, lines of nontransformed mast cells, generated by culture of fetal liver or bone marrow cells in IL-3 for relatively short (3–6 weeks) or for extended periods of time, fail to secrete detectable amounts of lymphokine and have little or no mRNA for IL-3, IL-4, IL-5, or GM-CSF. Stimulation of these factor-dependent mast cells by crosslinkage of their high-affinity FcεR causes them to express mRNA for several lymphokines (Fig. 2) and to secrete these factors. Two reports describing such production appeared within a short time of one another in 1989. That of Plaut *et al.* (*2*) principally dealt with long-term, IL-3-dependent mast cell lines. It was demonstrated that treatment of cells with the calcium ionophore ionomycin caused production of IL-4, IL-3, and IL-5 as well as IL-1 and IL-6. Subsequent studies of these lines also showed that they produced GM-CSF. However, no IL-2 or IFN-γ production was noted. Although phorbol esters did not by themselves induce lymphokine production, in their presence concentrations of ionomycin that were substimulatory (i.e., $<0.4\ \mu M$) resulted in such production.

These cells also produced lymphokines in response to crosslinkage of their high-affinity FcεR. Sensitization with a monoclonal IgE anti-dinitrophenyl antibody prepared the cells to secrete IL-3 and IL-4 in response to challenge with multivalent dinitrophenyl–bovine serum albumin (DNP–BSA). Lymphokine production by IgE-sensitized cells in response to DNP–BSA was inhibited by the monovalent DNP ligand, ε-DNP–L-lysine, indicating that simple binding to IgE anti-DNP was not sufficient to cause lymphokine production but, like other FcεR-mediated responses of mast cells, receptor crosslinkage was required.

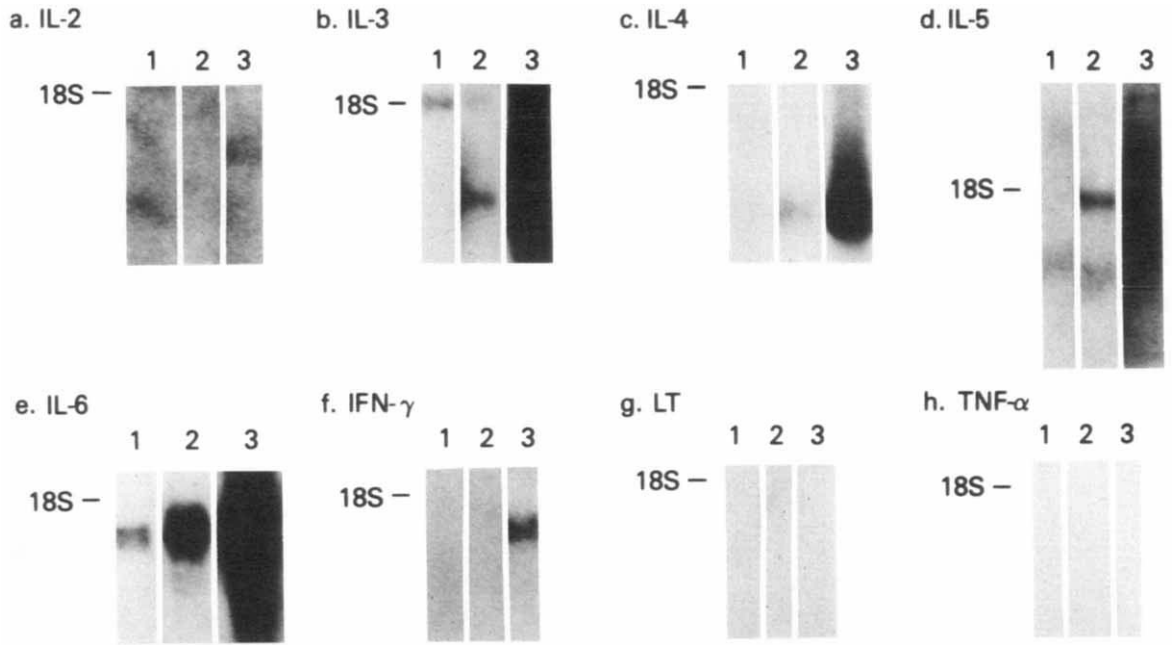

FIG. 2. Northern analysis of cytokine mRNA in a factor-dependent mast cell line. Poly(A^+) RNA was prepared from CFTL-12 cells that had been incubated with IgE anti-DNP antibody and then without antibody (lane 1), DNP–BSA (lane 2), ionomycin (lane 3) for 2.5 hr. Northern analysis was carried out with probes for IL-2, IL-3, IL-4, IL-5, IL-6, IFN-γ, lymphotoxin (LT), and TNF-α. Exposure times were IL-2, 24 hr; IL-3, 6 hr, IL-4, 6 hr; IL-5, 24 hr; IL-6, 12 hr; IFN-γ, 63 hr; LT, 96 hr; TFN-α, 96 hr. (Reproduced from Ref. 2, with permission.)

Wodner-Filipowicz and colleagues (*3*) reported that lymphokine production could be obtained in bone marrow cells that had been cultured in IL-3 for approximately 3 weeks. Such cells are highly enriched in $Fc\varepsilon R^+$ cells that possess granules that stain with Alcian blue and have other morphologic features characteristic of mast cells. They showed that such cells produced IL-3 and GM-CSF in response to calcium ionophores or to $Fc\varepsilon R$ crosslinkage. In these studies, phorbol esters were shown to increase strikingly lymphokine production stimulated by ionomycin. The supernatants of the stimulated cells were not tested for IL-4 or IL-5 content.

As noted, mast cell lines produce IL-1, IL-6, and members of the IL-8 family in response to receptor crosslinkage or to ionomycin. This point was emphasized by studies of Burd *et al.* (*6*) on IL-3-dependent mast cell lines. However, mast cell lines had been known to produce TNF-α or a "TNF-like" factor based on initial reports by Djeu and colleagues (*4,64*) that had demonstrated that mouse mast cell lines and rat basophil leukemia cells killed the same spectrum of target cells as "natural cytotoxic" (NC) cells, and that the cytotoxic activity was inhibited by an anti-TNF antibody. These same investigators demonstrated that antigen (after IgE sensitization), anti-IgE, and anti-IgE receptor antibody all induced rapid augmentation of the NC activity of mast cell lines and rat basophil leukemia cells.

Subsequently, Young and colleagues (*5*) demonstrated that freshly isolated mouse peritoneal mast cells had no spontaneous NC activity, but that stimulation with combinations of lipopolysaccharide and phorbol myristate acetate (PMA) or with concanavalin A and PMA induced NC activity, associated with the secretion of TNF-like molecules. This mast cell TNF differed from macrophage TNF in certain physical properties.

Gordon and Galli (*65,66*) followed up these studies by demonstrating that mast cell lines and peritoneal mast cells express mRNA for TNF-α. Furthermore, some mast cell TNF secretion occurred in the presence of inhibitors of mRNA synthesis. In addition, evidence was obtained to indicate that some preformed TNF-α existed in these cells. The capacity of the mast cell to store TNF-α apparently explains the rapid secretion of TNF-α crosslinkage of $Fc\varepsilon R$.

The range of lymphokines produced by mast cells is thus sufficiently broad for these cells to play potentially important roles in allergic-type inflammatory responses as well as in the acute-phase reactions and the more general type of inflammatory responses induced by IL-1, IL-6, and TNF-α. Furthermore, the observation that factor-dependent mast cell lines produce IL-4, IL-5, IL-3, and GM-CSF but not IL-2, IFN-γ,

or LT on stimulation (2) suggests that they may have immunoregulatory properties similar to those of CD4$^+$ T cell clones of the T_{H2} type (67), which produce the same pattern of lymphokines.

V. Lymphokine Production by Murine Peritoneal, Splenic, and Bone Marrow FcεRI$^+$ Cells

The recognition that both transformed and factor-dependent mast cell lines could produce lymphokines led to the question of whether freshly isolated mast cells and other FcεRI$^+$ cells expressed this capacity and what physiologic significance such lymphokine production might have.

Crosslinkage of FcεR on peritoneal mast cells resulted in secretion of IL-3, IL-6 (2; M. Plaut, unpublished, 1990), an unidentified growth-promoting cytokine (68), and TNF-α (65,66). TNF-α was secreted from both stored and newly synthesized pools. The full spectrum of cytokines that are produced by peritoneal mast cells has not been determined.

An FcεRI$^+$ cell was identified in spleen and bone marrow and was shown to be a potent producer of IL-4 (69). This cell was identified by a search for non-T cells that produced lymphokines in response to crosslinking of Fc receptors. Spleen cell populations from which cells expressing Thy-1, CD4, CD8, B220, and class II MHC molecules had been removed produced IL-4 when incubated on culture dishes coated with IgE (Fig. 3). Because neither purified B nor T cells showed

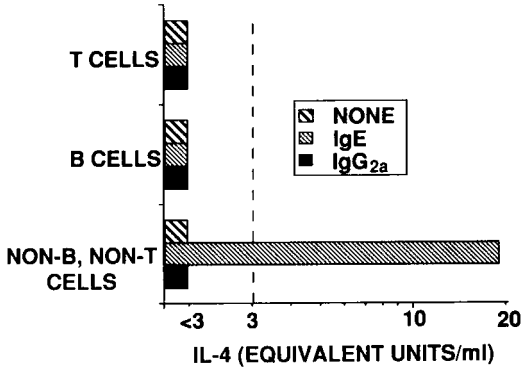

FIG. 3. Non-B, non-T cells produce IL-4 in reponse to stimulation with immobilized IgE. Non-B, non-T cells purified from spleens of normal BALB/c mice were cultured on dishes without coating or coated with IgE or IgG_{2a}. IL-4 production was measured using a CT.4S coculture assay. (Reproduced from Ref. 69, with permission.)

this property, it seems highly unlikely that contamination of the "non-B, non-T" cell preparations with either B or T cells could have been responsible for their IL-4 producing capacity. Bone marrow cell suspensions also contained cells that secreted IL-4 in response to stimulation with immobilized IgE but thymus and lymph node cell preparations lacked activity (*70*).

Although both splenic non-B, non-T cells and bone marrow cells produced IL-4 in response to stimulation with immobilized IgE, this production was relatively modest. However, IL-4 production by splenic and bone marrow cells from normal donors in response to FcεR cross-linkage could be strikingly enhanced by coculturing these cells with IL-3, but not with other lymphokines or cytokines (Fig. 4) (*71*). Furthermore, costimulation with IL-3 revealed that crosslinkage of FcγRII/FcγRIII with immobilized IgGs, including IgG_1, IgG_{2a}, and IgG_{2b}, also stimulated IL-4 production. As noted above, addition of IL-3 at the time of FcεRI crosslinkage or growth of mast cell lines in high concentrations of IL-3 strikingly enhances lymphokine production by these cells (*55*).

Freshly isolated splenic and bone marrow $FcεRI^+$ cells produced IL-4 in substantial amounts in response to immobilized IgE plus IL-3 or to treatment with ionomycin, but secreted little IL-3 (*69*;

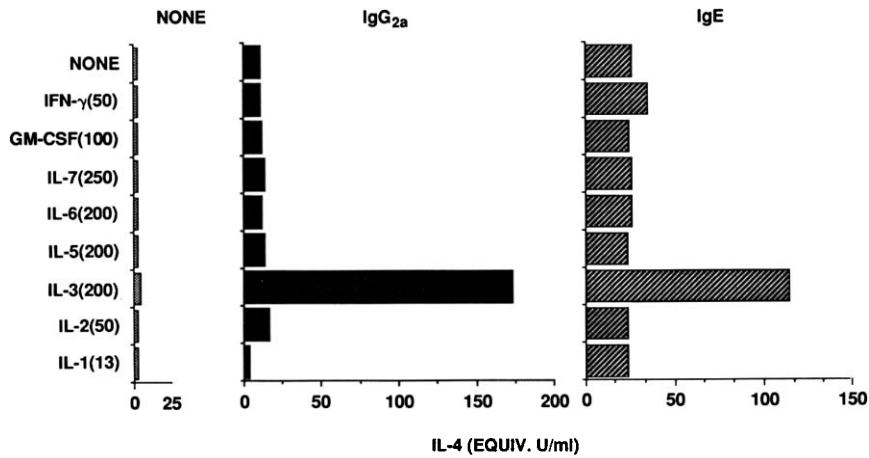

FIG. 4. Among lymphokines, only IL-3 augments IL-4 production. Non-B, non-T cells from spleen were cultured on dishes without coating or coated with IgE or IgG_{2a} alone or in the presence of the indicated factors (units/milliliter indicated in parentheses). IL-4 production was measured in a CT.4S coculture assay. (Reproduced from Ref. *71*, with permission.)

S. Ben-Sasson, unpublished observations, 1991). In this respect, they differed from factor-dependent mast cell lines, which produced substantial amounts of IL-3. Furthermore, freshly isolated non-B, non-T cells produced lymphokine in response to crosslinkage of either their high-affinity FcεR or FcγRII/III if IL-3 was present, whereas factor-dependent mast cell lines did not appear to produce lymphokine in response to immune complexes consisting of antigen and IgG_1 or IgG_{2a} antibody.

In order to characterize the IL-4 producing cells among the non-B, non-T cells from spleen and bone marrow, cells in these populations expressing high-affinity FcεR were quantitated and isolated. Because no antibody to the murine FcεRI is available, the measurement of numbers of cells presumed to express this receptor was achieved by incubating cells with IgE (2–10 μg/ml), followed by biotinylated anti-IgE and then streptavidin–phycoerythrin. This resulted in staining of 1–2% of the cells among splenic non-B, non-T cells (72). Two-color analysis revealed that these cells were CD23 (FcεRII) negative, implying that their FcεR was FcεRI. In the analysis of the frequency of such cells in bone marrow cell suspensions, from which B cells had not been removed, two-color analysis was carried out to determine the frequency of FcεR$^+$, CD23$^-$ cells, which were presumed to be FcεRI$^+$ cells. In general, these cells constituted 1–2% of total bone marrow cells.

Interestingly, the frequency of cells stained with biotinylated anti-IgE and streptavidin–phycoerythrin when IgE was omitted from the staining mixture was similar to that obtained when IgE was added, although the intensity of staining was somewhat lower (72). This implies that most FcεRI$^+$ cells in spleen and bone marrow have some of their receptors already occupied with IgE, even in individual mice that have very low serum levels of IgE (often 0.1 μg/ml or less). This observation is consistent with the binding of IgE to the high-affinity FcεR, FcεRI.

FcεR$^+$ cells among non-B, non-T cells of spleen or in bone marrow cell suspensions were purified by fluorescence-activated cell sorting. The purified FcεR$^+$ cells contained all of the capacity of the starting cell population to produce IL-4 in response to immobilized IgE, as anticipated (*61*). Somewhat unexpectedly, these cells also possessed all of the capacity of the starting cell population to produce IL-4 in response to stimulation with immobilized IgG or with ionomycin, implying that all IL-4-producing capacity of non-B, non-T cells in spleen and of bone marrow cells in response to FcR crosslinkage or to elevation of intracellular free calcium concentration was the property of FcεR$^+$ (presumably FcεRI$^+$) cells.

In order to obtain an estimate of the proportion of the purified cell population that was capable of producing IL-4 in response to FcεR crosslinkage, a limiting dilution assay capable of detecting IL-4 production by a single cell was employed (73). Using this approach, it was verified that only FcεR$^+$ cells had the capacity to produce IL-4 in response to immobilized IgE (Fig. 5). However, the highest frequency of IL-4 producing cells that was observed among purified FcεR$^+$ cells was 1/5, and that was seen only in cell populations derived from animals that had been treated with stimulants that markedly enhance IL-4 production by FcεRI$^+$ cells (i.e., infection with *Nippostrongylus brasiliensis* or injection of anti-IgD antibody) (72).

Morphologic analysis by transmission election microscopy of the purified bone marrow or splenic FcεR$^+$ cells revealed that 30–50% were granulated (74). Based on granule and nuclear morphology, the great majority of the granulated cells were classified as basophils or basophilic myelocytes. Very few if any mast cells were observed. These results raised the possibility that basophils rather than mast cells might be the major producers of IL-4 among FcεR$^+$ cells freshly isolated from spleen and bone marrow of normal donors and of donors that had been

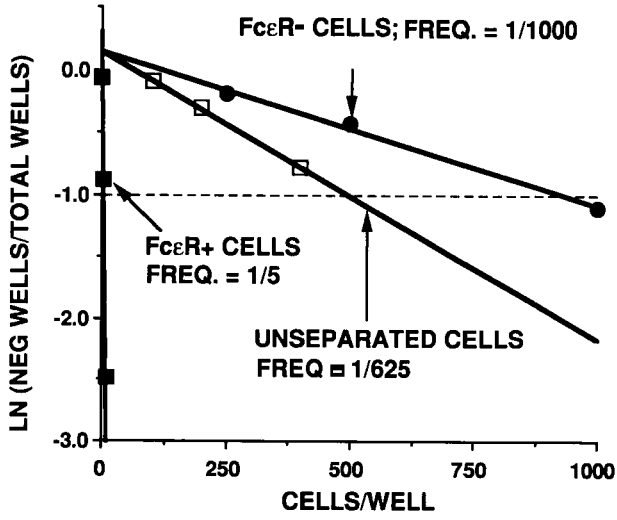

FIG. 5. IL-4 production in response to immobilized IgE is found in FcεR$^+$ cells. Bone marrow cells from anti-IgD-injected mice were stained for FcεR and separated by fluorescence-activated cell sorting. The frequency of cells capable of producing IL-4 in response to immobilized IgE, in the presence of IL-3, was measured by limiting dilution analysis.

infected with *N. brasiliensis* or injected with anti-IgD. However, 50% or more of the cells that were purified based on their expression of FcεR could not be classified. It therefore remained a possibility that these cells, not the basophilic cells, were the major producers of IL-4 in response to immobilized IgE.

Because basophils are difficult to obtain in high purity and in large numbers from mice, the issue of whether they were responsible for IL-4 production in response to FcR crosslinkage or to treatment with ionomycin remained unresolved. To gain further insight into the character of these cells, an analysis of the IL-4-producing potential of bone marrow cells cultured for short periods in IL-3 or in IL-3 plus *steel* factor was undertaken. Indeed, a previous observation by Le Gros *et al.* (75) had shown that very short-term (5–6 days) cultured bone marrow cells, grown in IL-3, produced several lymphokines in response to IgG immune complexes. In our experience, cultures of that duration, although enriched in FcεRI$^+$ cells compared to the original bone marrow cell suspension, are still quite heterogeneous. The authors did not determine whether the lymphokine-producing cells expressed FcεRI nor whether IgE immune complexes would also cause lymphokine production.

In our studies, (R. A. Seder, M. Plaut, and W. E. Paul), manuscript in preparation), bone marrow cells were cultured for 7 to 10 days in IL-3, resulting in a cell population containing 30–70% FcεR cells. The FcεR$^+$ cells are also capable of further subdivision. Approximately half of these cells express the membrane protein tyrosine kinase c-*kit*. When FcεR$^+$ cells were separated into c-*kit*$^+$ and c-*kit*$^-$ (or dull) cells, it was found that, on immediate stimulation, IL-4 production was observed almost exclusively in the c-*kit* group. The FcεR$^+$, c-*kit*$^+$ cells appear to be a homogeneous population of mast cells whereas cells with basophilic morphology are concentrated in the FcεR$^+$, c-*kit*$^-$ (or dull) population. These results further strengthen the concept that among freshly isolated cells and cells cultured for very short periods, basophils, rather than mast cells, may be the major producers of IL-4. However, the lack of complete purity of this cell population and the possibility that the FcεR$^+$, c-*kit*$^-$ cells may include mast cell precursors leave the possibility open that a mast cell precursor may be a major IL-4 producer in response to FcεR crosslinkage. Additional evidence that the FcεR$^+$, c-*kit*$^+$ and the FcεR$^+$, c-*kit*$^-$ cells have distinct functional properties resides in their histamine content and capacity to release histamine. The histamine content of the c-*kit*$^+$ population was high; the cells had low unstimulated (spontaneous) secretion of histamine, but high secretion in response to antigen (after sensitization

with IgE antibody) or ionomycin. In contrast, the c-*kit*$^-$ cells had a low histamine content, relatively high spontaneous histamine secretion, but low antigen- and ionmycin-stimulated secretion. Thus, these two populations are markedly divergent in their capacity to secrete histamine as well as in their capacity to secrete IL-4.

VI. Lymphokine Production by Human FcεR$^+$ Cells

Thus far, most of the work on lymphokine production by FcεR$^+$ cells has employed cell populations prepared from mice. Several investigators have demonstrated that human mast cells secrete TNF-α. Walsh *et al.* (*76*) have reported that skin mast cells contain stored TNF-α; secretion of TNF-α was induced *in vitro* by morphine sulfate within 45 minutes, and was induced *in vivo* during contact hypersensitivity reactions to dinitrochlorbenzene, within 4–6 hours. However, mRNA for TNF-α was expressed at 48 hours, suggesting that the mRNA was expressed only when cytokine was resynthesized. Gordon *et al.* (*77*) have found that lung mast cells secrete TNF-α rapidly and express TNF-α mRNA; it is possible that lung mast cells have both preformed and mRNA-dependent forms of TNF-α.

Several other groups have investigated whether human FcεR$^+$ cells secrete IL-4. Piccinni *et al.* (*78*) performed experiments with human bone marrow cells similar to those performed with mouse bone marrow cells. They found that, in the presence of IL-3, a non-B, non-T FcεR$^+$ cell from human bone marrow expressed mRNA for IL-4 on crosslinkage of FcεR or FcγR; the cells also expressed mRNA for IL-5 but not for IL-2, IFN-γ, or IL-6. Some but not all of the cell preparations secreted IL-4. The preparations contain both basophils and mast cells, so either of these cell types might be the source of IL-4. In contrast, peripheral blood FcεR$^+$ cells did not express IL-4 mRNA. Although these results are comparable in some respects to results with mouse bone marrow, they differ in that the levels of secreted human IL-4 are relatively low, and in that IL-4 and IL-4 mRNA peaked at 48 hours, much later than for mouse FcεR$^+$ cells. The significance of these findings awaits additional data. The failure of peripheral blood cells to express IL-4 mRNA reflects either a failure of basophils to produce IL-4 or a requirement for a local factor in the bone marrow to facilitate IL-4 production.

A second report of an IL-4-producing FcεR$^+$ cell is that of Arock *et al.* (*79*). They found that a patient with chronic myelogenous leukemia (CML) in relapse had large numbers of circulating basophils and basophilic myelocytes; the serum of this patient had mildly elevated IL-4

levels (135 pg/ml, using an assay with a detection limit of 50 pg/ml). A preparation of highly enriched basophil lineage peripheral blood cells cultured for 18 hours produced IL-4 and expressed IL-4 mRNA constitutively. These cells produced more IL-4 when cultured with IL-3, with IgE followed by anti-IgE, or with the calcium ionophore, A23187. By *in situ* hybridization, a substantial fraction of the cells was positive for IL-4 mRNA, suggesting that the basophil lineage cells expressed IL-4 mRNA. These investigators also cultured bone marrow cells from normal donors for 3–4 weeks in IL-3, then purified the basophil lineage cells and cultured them for 18 hours. With IgE followed by anti-IgE or with A23187, these cells also secreted IL-4, expressed IL-4 mRNA, and consisted of a substantial subset of cells positive by *in situ* hybridization for IL-4 mRNA. These results indicate that normal bone marrow-derived basophils produce IL-4. It is somewhat surprising that the fraction of cells expressing IL-4 mRNA is very high whereas the level of secreted IL-4 is relatively low. Because no time course of IL-4 production is provided, it is difficult to compare these results to those of Piccinni *et al.* (*78*).

Church and Holgate and their colleagues (*80*; M. Church and S. Holgate, personal communication, 1992) have examined lung mast cells from patients with allergic asthma. These cells release IL-4 within 1 hour in response to FcεR crosslinking, achieved by challenge with anti-IgE. The authors interpreted their data to indicate that lung mast cells from allergic individuals contained stored IL-4. The relationship of these findings to those of Piccinni *et al.* and Arock *et al.* (*78,79*) await additional studies.

VII. IL-4 Production by FcεR$^+$ Cells in Infected or Immunized Mice

Relatively little information exists regarding the significance of lymphokine production by FcεR$^+$ cells in pathophysiological processes. It has been shown that in mice infected with the helminth *N. brasiliensis*, a striking increase occurs in the capacity of their splenic non-B, non-T cells and their bone marrow cells to produce IL-4 in response to receptor crosslinkage (*70*). Indeed, by 9 days after infection, the cells displayed a 10 to 50-fold increase, on a per cell basis, in IL-4-producing capacity in response to immobilized IgE. Moreover, spleens of *N. brasiliensis*-infected mice increase in size substantially and the non-B, non-T cell proportion among spleen cells also increases. This results in an increase of more than 100-fold in the overall capacity of splenic FcεR$^+$ cells to produce IL-4 in response to immobilized IgE.

Not only is there an increase in the frequency of IL-4-producing

cells among the splenic non-B, non-T cells of infected mice, these cells appear much less dependent on IL-3 in their production of IL-4 in response to FcεR crosslinkage (*70*). This could be explained by the production of substantial amounts of IL-3 in the course of the immune response to the parasite, resulting in the differentiation of the $Fc\varepsilon R^+$ cells to efficient IL-4 producers or in synergy with receptor cross linkage in signaling lymphokine production. Indeed, intraperitoneal injection of IL-3 into normal mice increases the capacity of peritoneal non-B, non-T cells to produce IL-4 in response to immobilized IgE (*71*).

Particularly interesting was the observation that splenic non-B, non-T cells from *N. brasiliensis*-infected mice were stimulated to produce IL-4 by an antigenic extract of *N. brasiliensis*. This indicates that antigen-mediated crosslinkage of Fc receptors, that had bound antibody specific for *N. brasiliensis* antigens, triggered the cells to produce IL-4 (Fig. 6) (*70*). In these studies, it was not established whether the antigen induced its effect by cross-linking FcεR that had bound antigen-specific IgE or by crosslinking FcγR that had bound antigen-specific IgG.

Williams *et al.* (submitted for publication) have recently observed a similar process in mice infected with *Schistosoma mansoni*. Previous studies of BALB/c mice infected with this trematode had shown that in the early phases of the infection, challenge of spleen cells with worm-derived antigens (SWAP) led to the production of IL-2 and IFN-γ, but little IL-4 or IL-5 (*81*). However, after 6–8 weeks of infection, at which time the worms commence laying eggs, a striking change occurs and antigen preparations [SWAP or the highly crossreactive schistosoma egg antigens (SEA)] elicit the production of IL-4 and IL-5 from spleen cells with concomitant reduction in the production of IL-2 and IFN-γ. Careful analysis indicates that in response to antigenic challenge, non-B, non-T $Fc\varepsilon R^+$ cells produce large amounts of IL-4, suggesting that such cells rather than T cells are predominant IL-4-producers in this infection. By contrast, IL-5 appears to be produced mainly by T cells in this infection.

A third situation in which a striking increase in the IL-4-producing potential of splenic non-B, non-T cells occurs is in mice that have been injected with anti-IgD antibodies. It is known that this strong polyclonal stimulant favors the appearance of $CD4^+$ T cells that produce IL-4 on challenge (*70,73*). Immunoglobulin isotypes whose expression is controlled by IL-4 (IgG_1 and IgE) show striking increases in their serum concentration in mice treated with anti-IgD (*82,83*). These increases in IgE levels are inhibited by treating the mice with anti-IL-4

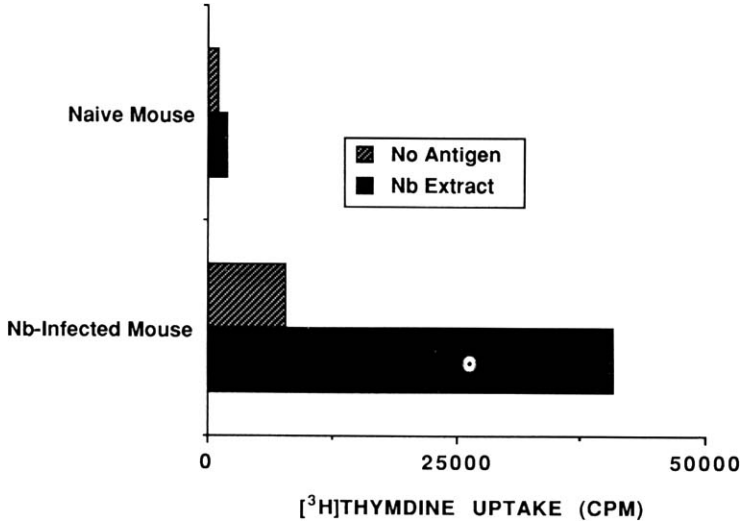

FIG. 6. Non-B, non-T cells from mice infected with *Nippostrongylus brasiliensis* produce IL-4 in response to antigen. Non-B, non-T cells were prepared from spleens of normal mice and of mice that had been infected with *N. brasiliensis* 9 days earlier. The cells were cultured in microtiter wells without coating or coated with an extract of *N. brasiliensis* (Nb)-IL-4 production was measured using a CT.4S coculture assay. (Reproduced from Ref. 70, with permission.)

antibodies (84). Analysis of FcεR$^+$ cells from these mice reveals that these cells also show a striking increase in IL-4-producing capacity in response to immobilized IgE, immobilized IgG, or calcium ionophore (70,72).

The possibility that FcεR$^+$ cells may play a role in disease processes is suggested by the observations of Piccinni *et al.* (78), of Hunt *et al.* (80), and of M. Church and S. Holgate (personal communication, 1992). As alluded to above, only FcεR$^+$ cells or mast cells derived from human donors with allergic disorders produce IL-4. Piccinni *et al.* demonstrated that IL-4 production by FcεR$^+$ cells from bone marrow was most striking in atopic individuals whereas bronchial mast cells from asthmatic but not normal individuals contained IL-4 in the studies reported by Hunt *et al.*

VIII. Does IL-4 Production by FcεRI$^+$ Cells Play an Important Role in Determination of Lymphokine-Producing Phenotype of CD4$^+$ T Cells?

It has recently been shown that a major (perhaps the dominant) factor determining whether CD4$^+$ T cells will differentiate into IL-4-

producers or into IFN-γ producers is whether IL-4 is present at the time of priming (*85–88*). Indeed, if IL-4 is added to cultures at the time of priming of T cells from mice transgenic for genes encoding a cytochrome *c*-specific T cell receptor, $CD4^+$ T cells develop into IL-4 producers and the appearance of IFN-γ producers is markedly suppressed (*87*). By contrast, if IL-4 is not present, than the dominant lymphokines produced by the primed $CD4^+$ T cells in response to the cytochrome c peptide are IL-2 and IFN-γ. Furthermore, treatment of mice with monoclonal anti-IL-4 antibody at the time of priming with a conventional antigen (*88a*), or infection with *Leishmania major* (*89*) or with *Candida albicans* (*90*), suppresses the appearance of specific T cells that produce IL-4 on subsequent *in vitro* challenge with antigen but does not diminish, or actually enhances, the appearance of IFN-γ-producing cells.

It is therefore tempting to speculate that production of IL-4 by $Fc\varepsilon RI^+$ cells may determine the particular lymphokine-producing phenotype that T cells adopt during the *in vivo* priming process. This could operate at the initiation of the immune response and provide the IL-4 that is critical for the initial commitment of T cells to become IL-4-producers. Alternatively (or in addition), IL-4 production by $Fc\varepsilon RI^+$ cells may result in the reinforcement of the lymphokine-producing phenotype of $CD4^+$ T cells in primed individuals. Thus, once some production of IL-4 is initiated by T cells, leading to class switching for IgE and IgG_1, subsequent antigenic challenge could elicit striking IL-4 production by $Fc\varepsilon RI^+$ cells and thus could bias the differentiation of newly emerging T cells into the IL-4-producing phenotype as these cells are primed by antigen. Furthermore, it has not yet been determined whether the capacity of previously primed $CD4^+$ T cells to produce IL-4 is "fixed" *in vivo* or depends on the continued presence of IL-4. If IL-4 is required to maintain the IL-4-production phenotype of $CD4^+3$ T cells, IL-4-producing $Fc\varepsilon RI^+$ cells could be an important source of the lymphokine for this purpose.

There is some reason to doubt that production of IL-4 by $Fc\varepsilon RI^+$ cells is the initiation stimulus for T cells from naive donors to become IL-4 producers. Thus, in the anti-IgD system, described above, the increase in IL-4-producing capacity of $Fc\varepsilon R^+$ cells occurs after rather than before the increase in $CD4^+$ T cells that can produce IL-4, suggesting that, in this instance, such a mechanism is not operative.

The requirement for crosslinkage of $Fc\varepsilon RI$ or $Fc\gamma RII/III$ to induce IL-4 production by $Fc\varepsilon R^+$ cells requires the existence of IgE or IgG antibody specific for antigenic determinants on the immunogen to elicit such a response. That would also appear to be inconsistent with

IL-4 produced as a result of cross-linkage of FcεRI on mast cells and basophils being critical to the priming of T Cells to produce IL-4. The production of the IgE and IgG needed to sensitize FcεRI or FcγRII/III depends on immunoglobulin class switching, which is a T cell-dependent event. Indeed, for the induction of IgE or IgG_1, two immunoglobulins well suited to act through FcR to elicit lymphokine production, IL-4 plays a critical role in the switching process. However, it has not yet been determined whether other, non-FcR-dependent, physiologic mechanisms exist that could result in these cells being a source of IL-4 and related lymphokines at the very outset of immune responses.

IX. Signaling Mechanisms Leading to IL-4 Production

There is a large body of data demonstrating that the stimulation of release of preformed mediators by mast cells and basophils has a minimal requirement for the crosslinkage of two FcεRI molecules. While such a detailed analysis for lymphokine production has not been carried out, it is clear that FcεR crosslinkage is also essential for this response. Alluded to above is the observation that ε-DNP–L-lysine at 10^{-5} *M* inhibits lymphokine production in response to multivalent DNP–protein conjugates by cells whose FcεRI have been sensitized with monoclonal IgE anti-DNP antibody.

Although receptor crosslinkage is essential for lymphokine production, it seems clear that involvement of a large number of receptors in the crosslinks or higher order crosslinkage leads to a diminution in lymphokine production. This has been demonstrated by the analysis of events associated with the well-known suppression of histamine release and of lymphokine production that occurs when high concentrations of ligand are employed. Dose–response curves relating lymphokine production by FcεRI$^+$ cells, sensitized with IgE anti-DNP antibody, to antigen concentration typically show a maximum between 3 and 30 ng/ml of DNP–protein (2). At higher concentrations, such as 100–300 ng/ml, substantially less lymphokine is generally produced. In principle, this decrease in lymphokine production might reflect either inhibition due to "excessive" signaling or to a diminution in signaling because sufficiently large amounts of ligand were used so that each molecule of ligand bound to only one IgE molecule, and thus no crosslinkage occurred. The latter has been excluded by showing that the addition of ε-DNP–L-lysine at 10^{-6} to 10^{-7} *M*, concentrations below those that fully inhibit, actually increases lymphokine production at high ligand concentration (M. Plaut and W. E. Paul, unpub-

lished observations). This can be explained by postulating that the competition of the monovalent ligand diminishes the degree of crosslinkage and thus induces a signal that is either in quantitative terms more favorable for stimulation or is qualitatively different.

Even more direct confirmation of the conclusion that high-concentration inhibition results from excessive rather than diminished crosslinkage comes from studies of protein tyrosine phosphorylation of various substrates in response to stimulation with multivalent DNP–protein conjugates of FcεRI$^+$ cells sensitized with IgE anti-DNP. In those instances, high ligand concentrations that lead to diminished lymphokine production cause substantially greater protein tyrosine phosphorylation than do concentrations that are optimally stimulatory (A. Keegan, unpublished observations, 1991).

Lymphokine production requires continued stimulation through FcεR. In general, secretion of IL-4 is not observed for ~1 hour after cross-linkage and continues in increasing amounts for 4–6 hours (2). Interruption of crosslinkage at any time, by the addition of 10^{-5} *M* ε-DNP–L-lysine, blocks lymphokine production as quickly as can be measured. FcεRI crosslinkage leading to cytokine secretion can be obtained by antigen or anti-IgE (after sensitization by IgE antibody), or by incubation with plate-bound IgE. Most of the careful kinetic data are based on antigen stimulation. IL-4 production in response to plate-bound IgE has been studied only with fresh FcεR$^+$ populations of bone marrow and spleen and with FcεR$^+$ cells obtained from 7 to 14-day cultures of bone marrow cells in IL-3. IL-4 production in response to plate-bound IgE is somewhat greater than that to antigen, but its kinetics are considerably slower (24–48 hours) than the time course observed in response to antigen (4–6 hours). Plate-bound IgE failed to stimulate detectable cytokine secretion from long-term factor-dependent mast cell lines. However, prolonged culture of these cell lines, especially in IL-3, results in disappearance (either through uptake or destruction) of cytokines, so that IL-4 produced at 24 or 48 hours might not be detectable.

The signaling process of FcεRI$^+$ cells in response to FcR crosslinkage has several features similar to that employed by T cells, B cells, and NK cells in response to signaling through their comparable receptors (*91*). This is of particular interest because these processes lead to release of granule contents and to secretion of newly synthesized lymphokines by FcεRI$^+$ cells, T cells, and NK cells. For that reason, it is of some importance to point out similarities in receptor structure and in the signaling cascade among these cells, insofar as it is known.

FcεRI is a multicomponent receptor consisting of three distinct polypeptides, designated α, β, and γ (92). The α chain is a member of the immunoglobulin gene superfamily, has a large extracellular domain, and contains the binding site for the Fc portion of IgE. It is noncovalently associated with the β chain, which, based on its distribution of hydrophobic and hydrophilic domains, has been postulated to span the membrane four times and is, in this regard, relatively unique among the multichain immune recognition receptors (MIRRs) (91) utilized by each of the cell types under discussion. The γ chain has a short extracellular domain and a cytosolic domain containing three copies of a consensus sequence that also occurs in the ζ chain of the T cell receptor. Furthermore, the γ chain is utilized by FcγRIII, together with an Fcγ-specific α chain (FcγRIIIα), on both mast cells and basophils and on NK cells. Recent studies have demonstrated that chimeric molecules containing the γ or ζ cytosolic domain and an extracellular domain such as that of the α chain of the IL-2 receptor (93), of CD8 (94), or of CD4 (95) can, on crosslinkage, signal lymphokine production in T cells and the release of preformed mediators in mast cells.

These results suggest that the crosslinkage of the cytosolic domain of γ or ζ is a key to the induction of the signals that cause both granule exocytosis and lymphokine production. However, they do not speak to the roles played by other non-ligand-binding receptor polypeptides such as FcεRIβ or the γ, δ, and ε components of the T cell CD3 complex. It should be noted that these molecules also contain the γ/ζ cytosolic motif. It seems most likely that these other chains modulate or amplify the response, or possibly mediate other responses that occur on receptor crosslinkage.

There is a growing body of evidence that crosslinkage of these receptors leads to a complex process of tyrosine dephosphorylation and phosphorylation mediated, respectively, by phosphatases such as CD45 found on each of these cells, and by protein tyrosine kinases, presumably members of the *src* family. Efforts to follow the reaction from the cell surface crosslinking event to the proximal activation of kinases and to the eventual transcriptional regulatory events are now well underway for the three major cell types described here.

The effect of IL-3 in regulating the magnitude of lymphokine production by FcεRI$^+$ cells stimulated through FcR crosslinkage may depend on its capacity to alter the pattern of tyrosine phosphorylation induced by the crosslinking event. Keegan *et al.* (55) have shown that coculture or prior culture of factor-dependent mast cell lines with IL-3 both enhances phosphorylation of substrates normally tyrosine phos-

phorylated by FcεRI crosslinkage and leads to the phosphorylation of certain substrates that are not phosphorylated in response to either IL-3 or FcεRI crosslinkage alone. Although decisive evidence has not been obtained that these phosphorylation events mediate the effect of IL-3, it has been shown that transfection of receptors with endogenous tyrosine kinase activity, such as the platelet-derived growth factor, epidermal growth factor, or colony-stimulating factor-1 receptors, into a factor-dependent mast cell line prepares cells of that line to express enhanced lymphokine production on stimulation by FcεRI crosslinkage if the cognate growth factor is added to the culture system (55).

Relatively little is known about the molecular regulation of lymphokine production by FcεRI$^+$ cells. Interestingly, Wodner-Filipowicz and Moroni (*96*) reported that, in response to ionomycin, GM-CSF production by bone marrow cells that had been cultured for ~3 weeks in IL-3 was not due to new transcription but rather could be accounted for by stabilization of GM-CSF mRNA. This raises the possibility that regulation of lymphokine production by T cells, in which transcriptional regulation plays a major role, and mast cells may be quite different. On the other hand, it is known that lymphokine production in factor-dependent mast cell lines is strikingly inhibited by cyclosporine A (*2,6*), suggesting that transcriptional regulation may be important. Recently, efforts to study the regulation of transcription of IL-4 by mast cells have been initiated. Transient transfection analysis of Ab-MuL V-transformed mast cells has revealed that a DNase I-hypersensitive site in the second intron of the IL-4 gene has prototypic enhancer activity in both transformed mast cells and in stimulated IL-3-dependent mast cell lines (*97*). Interestingly, this intronic enhancer was inactive in EL-4 cells, a T cell line that produces IL-4 on stimulation with phorbol esters. This suggests that the intronic enhancer is mast cell specific in its action on IL-4 transcription and that the regulation of IL-4 transcription in mast cells and T cells may be distinct.

X. Conclusion

Recent work has established that transformed mast cells, factor-dependent mast cells, and freshly isolated FcεRI$^+$ cells all have the capacity to produce lymphokines and cytokines. In particular, the set of molecules produced by these cells, including IL-4, IL-3, GM-CSF, and IL-5, as well as IL-1, IL-6, TNF-α, and IL-8, could have very important roles in the regulation of inflammatory responses. It is particularly striking that the lymphokines produced all have potential

roles in immunologically important responses, most notably in orchestrating the events associated with allergic inflammation.

Work remains to be done to establish unambiguously the character of the cell in the freshly isolated spleen and bone marrow cell populations that produces IL-4 in response to FcR crosslinkage. In particular, the question of whether this cell is a basophil or a basophilic myelocyte needs to be answered. Because basophilic lineage cells are the majority population in the FcεR$^+$, c-kit$^-$ population in short-term bone marrow cultures, which are excellent IL-4 producers, basophilic lineage cells are good candidates to be IL-4 producers.

A second major issue is to determine the physiologic significance of production of IL-4 and other lymphokines and cytokines by FcεR$^+$ cells. The observation that the amount of IL-4 produced by the FcεR$^+$ cell population strikingly increases in certain parasitic infections suggests that these cells will prove to have important functions, but direct approaches to establish the nature of such functions will be needed.

Nonetheless, the fact that stimulation of FcεRI$^+$ cells by receptor crosslinkage leads to the release of lymphokines and cytokines that can have broad impact on immunologically mediated responses, especially allergic inflammation, provides a strong impetus for further study of this phenomenon.

Acknowledgments

We thank our colleagues, Shlomo Ben-Sasson, Melissa Brown, Dan Conrad, Ann Dvorak, Fred Finkelman, Stephen Galli, Joan Hanley-Hyde, James Ihle, Anne Kagey-Sobotka, Achsah Keegan, Graham Le Gros, Richard Nordan, Jacalyn Pierce, Saul Sharkis, and Cynthia Watson, whose contributions have been central to the work from our group on lymphokine production by FcεRI$^+$ cells. The editorial assistance of Ms. Shirley Starnes is gratefully acknowledged.

References

1. Brown, M. A., Pierce, J. H., Watson, C. J., Falco, J., Ihle, J. N., and Paul, W. E. (1987). B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. *Cell (Cambridge, Mass.)* **50,** 809.
2. Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P., and Paul, W. E.(1989). Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature (London)* **339,** 64.
3. Wodnar-Filipowicz, A., Heusser, C. H., and Moroni, C. (1989). Production of the haemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. *Nature (London)* **339,** 150.
4. Okuno, T., Takagaki, Y., Pluznik, D. H., and Djeu, J. Y. (1986). Natural cytoxic (NC) cell activity in basophilic cells release on NC-specific cytotolic factor by IgE receptor triggering. *J. Immunol.* **136,** 4652.

5. Young, J. D.-E., Liu, C.-C., Butler, G., Cohn, Z. A., and Galli, S. J. (1987). Identification, purification and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9175.
6. Burd, P. R., Rogers, H. W., Gordon, J. R., Martin, C. A., Jayaraman, S., Wilson, S. D., Dvorak, A. M., Galli, S. J., and Dorf, M. E. (1989). Interleukin-3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J. Exp. Med.* **170,** 245.
7. Metzger, H., Alcaraz, G., Hohman, R., Kinet, J.-P., Priluba, V., and Quarto, R. (1986). The receptor with high affinity for immunoglobulin E. *Annu. Rev. Immunol.* **4,** 419.
8. Galli, S. J., and Lichtenstein, L. M. (1988). Biology of mast cells and basophils. *In* "Allergy: Principles and Practice" (E. Middleton, Jr., C. E. Reed, N. F. Adkinson, Jr., and J. W. Yunginger, eds.), 3rd Ed., p. 71. C. V. Mosby, St. Louis.
9. Bieber, T., de la Salle, H., Wollenberg, A., Hakimi, J., Chizzonite, R., Ring, J., Hanau, D., and de la Salle, C. (1992). Human epidermal Langerhans cells express the high affinity receptor for immunoglobulin E (FcεRI). *J. Exp. Med.* **175,** 1285.
10. Wang, B., Rieger, A., Kilgus, O., Ochiai, K., Maurer, D., Fodinger, D., Kinet, J.-P., and Stingl, G. (1992). Epidermal Langerhans cells from normal human skin bind monomeric IgE via FcεRI. *J. Exp. Med.* **175,** 1353.
11. Takizawa, H., Ohta, K., Hirai, K., Misaki, Y., Horiuchi, T., Kobayashi, N., Shiga, J., and Miyamoto, T. (1989). Mast cells are important in the development of hypersensitivity pneumonitis. A study with mast-cell-deficient mice. *J. Immunol.* **143,** 1982.
12. Matsuda, H., Kawakita, K., Kiso, Y., Nakano, T., and Kitamura, Y. (1989). Substance P induces granulocyte infiltration through degranulation of mast cells. *J. Immunol.* **142,** 927.
13. Yano, H., Wershil, B. K., Arizono, N., and Galli, S. J. (1989). Substance P-induced augmentation of cutaneous vascular permeability and granulocyte infiltration in mice is mast cell dependent. *J. Clin. Invest.* **84,** 1276.
14. Wershil, B. K., Wang, Z. S., Gordon, J. R., and Galli, S. J. (1991). Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent. Partial inhibition of the reaction with antiserum against tumor necrosis factor-alpha. *J. Clin. Invest.* **87,** 446.
15. Askenase, P. W. (1977). Role of basophils, mast cells, and vasoamines in hypersensitivity reactions with a delayed time course. *Prog. Allergy* **23,** 199.
16. Brown, S. J., Galli, S. J., Gleich, G. J., and Askenase, P. W. (1982). Ablation of immunity to *Amblyomma americanum* by anti-basophil serum: Cooperation between basophils and eosinophils in expression of immunity to ectoparasites (ticks) in guinea pigs. *J. Immunol.* **129,** 790.
17. Charlesworth, E. N., Hood, A. F., Soter, N. A., Kagey-Sobotka, A., Norman, P. S., and Lichtenstein, L. M. (1989). Cutaneous late-phase response to allergen: Mediator release and inflammatory cell infiltration. *J. Clin. Invest.* **83,** 1519.
18. Ogilvie, B. M., Askenase, P. W., and Rose, M. E. (1980). Basophils and eosinophils in three strains of rats and in athymic (nude) rats following infection with the nematodes *Nippostronglyus brasiliensis* or *Trichinella spiralis*. *Immunology* **39,** 385.
19. Steeves, E. B. T., and Allen, J. R. (1990). Basophils in skin reactions of mast cell-deficient mice infested with *Dermacenter variabilis*. *Int. J. Parasitol.* **20,** 655.
20. Hurtado, I., and Urbina, C. (1983). Presence of basophils in PHA-reacted skin sites of mice. An electron microscopic study. *Int. Arch. Allergy Appl. Immunol.* **72,** 116.
21. Naclerio, R. M., Proud, D., Togias, A. G., Adkinson, N. F., Jr., Meyers, D. A., Kagey-Sobotka, A., Schulman, E. S., Plaut, M., Norman, P. S., and Lichtenstein, L. M. (1985). Inflammatory mediators in late antigen-induced rhinitis. *N. Engl. J. Med.* **313,** 65.

22. Iliopoulos, O., Baroody, F. M., Naclerio, R. M., Bochner, B. S., Kagey-Sobotka, A., and Lichtenstein, L. M. (1992). Histamine-containing cells obtained from the nose hours after antigen challenge have functional and phenotypic characteristics of basophils. *J. Immunol.* **148,** 2223.
23. Bodger, M. P., Mounsey, G. L., Nelson, J., and Fitzgerald, P. H. (1987). A monoclonal antibody reacting with human basophils. *Blood* **69,** 1414.
24. Lichtenstein, L. M., and Bochner, B. S. (1991). The role of basophils in asthma. *Ann. N.Y. Acad. Sci.* **629,** 48.
25. Columbo, M., Horowitz, E. M., Botana, L. M., MacGlashan, D. W., Jr., Gillis, S., Zsebo, K. M., Galli, S. J., and Lichtenstein, L. M. (1992). The effect of recombinant human *c-kit* ligand on mediator release from human cutaneous mast cells and peripheral blood basophils. *J. Immunol.* **149,** 599.
26. Lerner, N. B., Nocka, K. H., Cole, S. R., Qiu, F., Strife, A., Ashman, L. K., and Besmer, P. (1991). Monoclonal antibody YB5.B8 identifies the human *c-kit* protein product. *Blood* **77,** 1876.
27. Urbina, C., Ortiz, C., and Hurtado, I. (1981). A new look at basophils in mice. *Int. Arch. Allergy Appl. Immunol.* **66,** 158.
28. Dvorak, A. M., Nabel, G., Pyne, K., Cantor, H., Dvorak, H. F., and Galli, S. J. (1982). Ultrastructural identification of the mouse basophil. *Blood* **59,** 1279.
29. Seder, R. A., Paul, W. E., Dvorak, A. M., Sharkis, S. J., Kagey-Sobotka, A., Niv, Y., Finkelman, F. D., Barbieri, S. A., Galli, S. J., and Plaut, M. (1991). Mouse splenic and bone marrow cell populations that express high-affinity Fcε receptors and produce interleukin 4 are highly enriched in basophils. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2835.
30. Galli, S. J., Dvorak, A. M., Marcum, J. A., Ishizaka, T., Nabel, G., Der Simonian, H., Pyne, K., Goldin, J., Rosenberg, R. D., Cantor, H., and Dvorak, H. F. (1982). Mast cell clones: A model for the analysis of cellular maturation. *J. Cell Biol.* **95,** 435.
31. Dayton, E. T., Pharr, P., Ogawa, M., Serafin, W. E., Austen, K. F., Levi-Schaffer, F., and Stevens, R. L. (1988). 3T3 fibroblasts induce cloned interleukin-3-dependent mouse mast cells to resemble connective tissue mast cells in their granular constituency. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 569.
32. Ruitenberg, E. J., and Elgersma, A. (1976). Absence of intestinal mast cell response in congenitally athymic mice during *Trichinella* spiral infection. *Nature (London)* **164,** 258.
33. Nawa, Y., and Miller, H. R. P. (1979). Adoptive transfer of the intestinal mast cell response in rats infected with *Nippostronglylus brasiliensis*. *Cell. Immunol.* **42,** 225.
34. Zsebo, K. M., Wypych, J., McNiece, I. K., Lu, H. S., Smith, K. A., Karkare, S. B., Sachdev, R. K., Yuschenkoff, V. N., Birkett, N. C., Williams, L. R., Satyagal, V. N., Tung, W., Bosselman, R. A., Mendiaz, E. A., and Langley, K. E. (1990). Identification, purification, and biological characterization of hematopoietic stem cell factor from Buffalo rat liver-conditioned medium. *Cell (Cambridge, Mass.)* **63,** 195.
35. Williams, D. E., Eisenman, J., Baird, A., Rauch, C., van Ness, K., March, Ĉ. J., Park, L. S., Martin, U., Mochizuki, D. Y., Boswell, H. S., Burgess, G. S., Cosman, D., and Lyman, S. D. (1990). Identification of a ligand for the c-kit proto-oncogene. *Cell (Cambridge, Mass.)* **63,** 167.
36. Hamaguchi, Y., Kanakuram, Y., Fujitam, J., Takedam, S., Nakanom, T., Taruim, S., Honjo, T., and Kitamura, Y. (1987). Interleukin 4 as an essential factor for *in vitro* clonal growth of murine connective tissue mast cells. *J. Exp. Med.* **165,** 268.
37. Tsuji, K., Nakahata, T., Takagi, M., Kobayashi, T., Ishiguro, A., Kikuchi, T., Naganuma, K., Koike, K., Miyajima, A., Arai, K., and Akabana, T. (1990). Effects of interleukin-3 and interleukin-4 on the development of "connective tissue-type"

mast cells: Interleukin-3 supports their survival and interleukin-4 triggers and supports their proliferation synergistically with interleukin-3. *Blood* **75,** 421.

38. Nagao, K., Yokoro, K., and Aaronson, S. A. (1981). Continuous lines of basophil/mast cells derived from normal mouse bone marrow. *Science* **212,** 333.
39. Schrader, J. W., Lewis, S. J., Clark-Lewis, I., and Culvenor, J. G. (1981). The persisting (P) cell: Histamine content, regulation by a T cell-derived factor, origin from a bone marrow precursor, and relationship to mast cells. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 323.
40. Razin, E., Cordon-Cardo, C., and Good, R. A. (1981). Growth of a pure population of mouse mast cells *in vitro* with conditioned medium derived from concanavalin A-stimulated splenocytes. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2559.
41. Tertian, G., Yung, Y.-P., Guy-Grand, D., and Moore, M. S. (1981). Long-term *in vitro* culture of murine mast cells. I. Description of a growth-factor dependent culture technique. *J. Immunol.* **127,** 788.
42. Nabel, G., Galli, S. J., Dvorak, A. M., Dvorak, H. F., and Cantor, H. (1981). Inducer T lymphocytes synthesize a factor that stimulates proliferation of clones mast cells. *Nature (London)* **291,** 332.
43. Fujita, J., Nakayama, H., Onoue, H., Ebi, Y., Kanakura, Y., Kuriu, A., and Kitamura, Y. (1988). Failure of W/W^v mouse-derived cultured mast cells to enter S phase upon contact with NIH/3T3 fibroblasts. *Blood* **72,** 463.
44. Tsai, M., Shih, L. S., Newlands, G. F., Takeishi, T., Langley, K. E., Zsebo, K. M., Miller, H. R., Geissler, E. N., and Galli, S. J. (1991). The rat c-kit ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells *in vivo*. Analysis by anatomical distribution, histochemistry, and protease phenotype. *J. Exp. Med.* **174,** 125.
45. Otsu, K., Nakano, T., Kanakura, Y., Asai, H., Katz, H. R., Austen, K. F., Stevens, R. L., Galli, S. J., and Kitamura, Y. (1987). Phenotypic changes of bone marrow-derived mast cells after intraperitoneal transfer into W/W^v mice that are genetically deficient in mast cells. *J. Exp. Med.* **165,** 615.
46. Kobayashi, T., Nakano, T., Nakahata, T., Asai, H., Yagi, Y., Tsuji, K., Komiyama, A., Akabane, T., Kojima, S., and Kitamura, Y. (1986). Formation of mast cell colonies in methylcellulose by mouse peritoneal cells and the differentiation of these cloned cells in both the skin and the gastric mucosa of W/W^v mice: Evidence that a common precursor can give rise to both "connective tissue-type" and "mucosal" mast cells. *J. Immunol.* **13,** 1378.
47. Irani, A. M., Craig, S. S., DeBlois, G., Elson, C. O., Schechter, N. M., and Schwartz, L. B. (1987). Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T lymphocyte function. *J. Immunol.* **138,** 4381.
48. Irani, A. A., Schechter, N. M., Craig, S. S., DeBlois, G., and Schwartz, L. B. (1986). Two types of human mast cells that have distinct neutral protease compositions. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 4464.
49. Reynolds, D. S., Gurley, D. S., Stevens, R. L., Sugarbaker, D. J., Austen, K. F., and Serafin, W. E. 1989. Cloning of cDNAs that encode human mast cell carboxypeptidase A, and comparison of the protein with mouse mast cell carboxypeptidase A and rat pancreatic carboxypeptidases. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9480.
50. Miller, J. S., Westin, E. H., and Schwartz, L. B. (1989). Cloning and characterization of complementary DNA for human tryptase. *J. Clin. Invest.* **84,** 1188.
51. Irani, A. A., Goldstein, S. M., Wintroub, B. U., Bradford, T., and Schwartz, L. B. (1991). Human mast cell carboxypeptidase. Selective localization to MC$_{TC}$ cells. *J. Immunol.* **147,** 247.

52. Valent, P., Besemer, J., Sillaber, C., Butterfield, J. H., Eher, R., Majdic, O., Kishi, K., Klepetko, W., Eckersberger, F., Lechner, K., and Bettelheim, P. (1990). Failure to detect IL-3-binding sites on human mast cells. *J. Immunol.* **145,** 3432.
53. Pierce, J. H., Di Fiore, P. P., Aaronson, S. A., Potter, M., Pumphrey, J., Scott, A., and Ihle, J. N. (1985). Neoplastic transformation of mast cells by Abelson-MuL V: Abrogation of IL-3 dependence by a nonautocrine mechanism. *Cell (Cambridge, Mass.)* **41,** 685.
54. Barsumian, E. L., McGivney, A., Basciano, L., and Siraganian, R. (1985). Establishment of four mouse mastocytoma cell lines. *Cell. Immunol.* **90,** 131.
55. Keegan, A. D., Pierce, J. H., Artrip, J., Plaut, M., and Paul, W. E. (1991). Ligand stimulation of transfected and endogenous growth factor receptors enhances cytokine production by mast cells. *EMBO J.* **10,** 3675.
56. Fung, M. C., Hapel, A. J., Ymer, S., Cohen, D. R., Johnson, R. M., Campbell, H. D., and Young, I. G. (1984). Molecular cloning of cDNA for murine interleukin-3. *Nature (London)* **307,** 233.
57. Mosmann, T. R., Bond, M. W., Coffman, R. L., Ohara, J., and Paul, W. E. (1986). T-cell and mast cell lines respond to B-cell stimulatory factor 1. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 5654.
58. Benhamou, M., Gutkind, J. S., Robbins, K. C., and Siraganian, R. P. (1990). Tyrosine phosphorylation coupled to IgE receptor-mediated signal transduction and histamine release. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 5327.
59. Eiseman, E., and Bolen, J. B. (1992). Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature (London)* **355,** 78.
60. Paolini, R., Jouvin, M-H., and Kinet, J-P. (1991). Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement. *Nature (London)* **353,** 855.
61. Benhamou, M., Stephan, V., Robbins, K. C., and Siraganian, R. P. (1992). High affinity IgE receptor-mediated stimulation of rat basophilic leukemia (RBL-2H3) cells induces early and late protein-tyrosine phosphorylations. *J. Biol. Chem.* **267,** 7310.
62. June, C. H., Fletcher, M. C, Ledbetter, J. A., Schieven, G. L., Siegel, J. N., Phillips, A. F., and Samelson, L. E. (1990). Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 7722.
63. Mustelin, T., Coggeshall, K. M., Isakov, N., and Altman, A. (1990). T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* **247,** 1584.
64. Richards, A. L., Okuno, T., Takagaki, Y., and Djeu, J. Y. (1988). Natural cytotoxic cell-specific cytotoxic factor produced by IL-3-dependent basophilic/mast cells. Relationship to TNF. *J. Immunol.* **141,** 3061.
65. Gordon, J. R., and Galli, S. J. (1990). Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature (London)* **346,** 274.
66. Gordon, J. R., and Galli, S. J. (1991). Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epislon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *J. Exp. Med.* **174,** 103.
67. Mosmann, T. R., and Coffman, R. L. (1989). TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7,** 145.
68. Takagi, M., Nakahata, T., Koike, K., Kobayashi, T., Tsuji, K., Kojima, S., Hirano, T.,

Miyajima, A., Arai, K., and Akabane, T. (1989). Stimulation of connective tissue-type mast cell proliferation by crosslinking of cell-bound IgE. *J. Exp. Med.* **170,** 233.

69. Ben-Sasson, S. Z., Le Gros, G., Conrad, D. H., Finkelman, F. D., and Paul, W. E. (1990). Cross-linking Fc receptors stimulate splenic non-B, non-T cells to secrete interleukin 4 and other lymphokines. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1421.
70. Conrad, D. H., Ben-Sasson, S. Z., Le Gros, G. Finkelman, F. D., and Paul, W. E. (1990). Infection with *Nippostrongylus brasiliensis* or injection of anti-IgD antibodies markedly enhances Fc-receptor-mediated IL-4 production by non-B, non-T cells. *J. Exp. Med.* **171,** 1497.
71. Le Gros, Ben-Sasson, S. Z., Conrad, D. H., Clark-Lewis, I., Finkelman, F. D., Plaut, M., and Paul, W. E. (1990). Interleukin-3 promotes production of interleukin-4 by splenic non-B, non-T cells in response to Fc receptor cross-linkage. *J. Immunol.* **145,** 2500.
72. Seder, R. A., Plaut, M., Barbieri, S., Urban, J., Jr., Finkelman, F. D., and Paul, W. E. (1991). Purified $Fc\varepsilon R^+$ bone marrow and splenic non-B, non-T cells are highly enriched in the capacity to produce IL-4 in response to immobilized IgE, IgG2a, or ionomycin. *J. Immunol.* **147,** 903.
73. Seder, R. A., Le Gros, G., Ben-Sasson, S. Z., Urban, J., Jr., Finkelman, F. D., and Paul, W. E. (1991). Increased frequency of interleukin 4-producing T cells as a result of polyclonal priming. Use of a single-cell assay to detect interleukin 4-producing cells. *Eur. J. Immunol.* **21,** 1241.
74. Seder, R. A., Paul, W. A., Dvorak, A. M., Sharkis, S. J., Kagey-Sobotka, A., Niv, Y., Finkelman, F. D., Barbieri, S. A., Galli, S. J., and Plaut, M. (1991). Mouse splenic and bone marrow cell populations that express high-affinity Fcε receptors and produce interleukin 4 are highly enriched in basophils. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2835.
75. Le Gros, G., Le Gros, J., and Watson, J. D. (1987). The induction of lymphokine synthesis and cell growth in IL-3 dependent cell lines using Ag-Ab complexes. *J. Immunol.* **134,** 422.
76. Walsh, L. J., Trinchieri, G., Waldorf, H. A., Whitaker, D., and Murphy, G. F. (1991). Human dermal mast cells contain and release tumor necrosis factor α, which induces leukocyte adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4222.
77. Gordon, J. R., Post, T. J., Schulman, E. S., and Galli, S. J. (1991). Characterization of mouse mast cell TNF-α induction *in vitro* and *in vivo,* and demonstration that purified human lung mast cells contains TNF-α. *FASEB J.* **5,** A1009 (abst).
78. Piccinni, M. P., Macchia, D., Parronchi, P., Giudizi, M. G., Bani, D., Alterini, R., Grossi, A., Ricci, M., Maggi, E., and Romagnani, S. (1991). Human bone marrow non-B, non-T cells produce interleukin-4 in response to cross-linkage of Fc epsilon and Fc gamma receptors. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 8656.
79. Arock, M., Dalloul, A. H., Dugas, B., Fourcade, C., Ouaaz, F., Merle-Beral, H., Schmitt, C., Binet, J. L., Michel, L., Mencia-Huerta, J. M., Debre, P., and Mossalayi, M. D. (1992). Interleukin-4 production by normal and leukemic human basophils. Submitted for publication.
80. Hunt, T. C., Heusser, C., Mueller, R., Bullock, G., Okayama, Y., Ashman, L. K., and Church, M. K. (1992). Human lung mast cells produce interleukin-4. *J. Allergy Clin. Immunol.* **89,** 245 (abstr.).
81. Pearce, E. J., Caspar, P., Grzych, J. M., Lewis, F. A., and Sher, A. (1991). Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni. J. Exp. Med.* **173,** 159.
82. Finkelman, F. D., Scher, I., Mond, J. J., Kessler, S., Kung, J. T., and Metcalf, E. S. (1982). Polyclonal activation of the murine immune system by an antibody to IgD. II.

Generation of polyclonal antibody production and cells with surface IgG. *J. Immunol.* **129,** 638.

83. Finkelman, F. D., Snapper, C. M., Mountz, J. D., and Katona, I. M. (1987). Polyclonal activation of the murine system by a goat antibody to mouse IgD. IX. Induction of a polyclonal IgE response. *J. Immunol.* **138,** 2826.
84. Finkelman, F. D., Katona, I. M., Urban, J. F., Jr., Snapper, C. M., Ohara, J., and Paul, W. E. (1986). Suppression of *in vivo* polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor-1. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 9675.
85. Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D., and Paul, W. E. (1990). Generation of interleukin 4 (IL-4)-producing cells *in vivo* and *in vitro:* IL-2 and IL-4 are required for *in vitro* generation of IL-4-producing cells. *J. Exp. Med.* **172,** 921.
86. Swain, S. L., Weinberg, A. D., English, M., and Huston, G. (1990). IL-4 directs the development of TH2-like helper effectors. *J. Immunol.* **135,** 3796.
87. Seder, R. A., Paul, W. E., Davis, M. M., and Fazekas de St. Groth, B. (1992). The presence of IL-4 during *in vitro* priming determines the lymphokine-producing potential of CD4⁺ T cells from T cell receptor transgenic mice. *J. Exp. Med.* **176,** 1091.
88. Hsieh, C.-S., Heimberger, A. B., Gold, J. S., O'Garra, A., and Murphy, K. M. (1992). Differential regulation of T helper phenotype development by interleukins 4 and 10 in an ab T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6065.

88*a*. Gross, A., Ben-Sasson, S., and Paul, W. (1993). *J. Immunol.* (in press).

89. Sadick, M. D., Heinzel, F. P., Holaday, B. J., Pu, R. T., Dawkins, R. S., and Locksley, R. M. (1990). Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. *J. Exp. Med.* **171,** 115.
90. Romani, L., Mencacci, A., Grohmann, U., Mocci, S., Mosci, P., Puccetti, P., and Bistoni, F. (1992). Neutralizing antibody to interleukin 4 induces systemic protection and T helper type 1-associated immunity in murine candidiasis. *J. Exp. Med.* **176,** 19.
91. Keegan, A. D., and Paul, W. E. (1992). Multichain immune recognition receptors: similarities in structure and signaling pathways. *Immunol. Today* **13,** 63.
92. Ravetch, J. V., and Kinet, J. P. (1991). Fc receptors. *Annu. Rev. Immunol.* **9,** 457.
93. Letourner, F., and Klausner, R. D. (1991). T-cell and basophil activation through the cytoplasmic tail of T-cell-receptor zeta family proteins. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 8905.
94. Irving, B. A., and Weiss, A. (1991). The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell (Cambridge, Mass.)* **64,** 891.
95. Romeo, C., and Seed, B. (1991). Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell (Cambridge, Mass.)* **64,** 1037.
96. Wodnar-Filipowicz, A., and Moroni, C. (1990). Regulation of interleukin 3 mRNA expression in mast cells occurs at the posttranscriptional level and is mediated by calcium ions. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 777.
97. Henkel, G., Weiss, D. L., McCoy, R., Deloughery, T., Tara, D., and Brown, M. A. (1992). A DNase I hypersensitive site in the second intron of the murine interleukin 4 gene defines a mast cell specific enhancer. *J. Immunol.* **149,** 3239.

This article was accepted for publication on 23 September 1992.

ADVANCES IN IMMUNOLOGY, VOL. 53

The Leukemia Inhibitory Factor and Its Receptor

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I. Introduction

Many protein factors that affect the growth and differentiation of mammalian cells have been characterized and are collectively termed growth factors or cytokines. The path to discovery for each cytokine has often been based on assumptions that the cytokine is unique and specific in its action: colony-stimulating factors and erythropoietin have positive growth regulatory activity, interleukins communicate between leukocytes, and tumor necrosis factor, leukemia inhibitory factor, and oncostatins have negative effects on cell growth. However, since cDNAs for the various cytokines were first cloned and expressed it has become obvious that few are specific to one target cell and fewer still are unique in function. For some, the original effect on one cell type, e.g., inhibition of proliferation, could be paralleled by the opposite action, e.g., induction of proliferation, on another cell target. For others, the original action, e.g., that of inhibiting the growth of tumor cells, was unlikely to correspond to an endogenous response by the body to reject tumor cells. More likely the effect on tumor cells reflected some function on normal cells. The subject of this review, the leukemia inhibitory factor (LIF), exemplifies many features of cytokine biology. The gene encoding LIF has been cloned and its product characterized extensively; LIF binds to receptors on responsive cells with high and low affinity and the molecular basis for the two types of receptors has been determined.

II. Purification and Molecular Cloning of the Leukemia Inhibitory Factor

LIF has been studied in various laboratories for about a decade, although many have known it under different guises. As a leukemia inhibitory factor, LIF was characterized on the basis of its capacity to induce the differentiation and death of a mouse myeloid leukemic cell line, M1 (Metcalf *et al.*, 1988). Murine LIF was first purified to homogeneity in 1987 (Hilton *et al.*, 1988a,b), its amino acid sequence was determined (Simpson *et al.*, 1988), and a cDNA encoding active material was isolated (Gearing *et al.*, 1987). Because a variety of bio-

chemical fractionation procedures had been applied to characterize other molecules active on M1 differentiation [e.g., D-factor, granulocyte colony-stimulating factor (G-CSF), and MGI-2B], it was not clear at the time whether LIF was a novel molecule, but LIF was later shown to be identical to D-factor (Tomida *et al.*, 1984; Lowe *et al.*, 1989). Purification of native murine LIF was achieved by sequential ion-exchange chromatography on DEAE–Sepharose, affinity chromatography on lentil–lectin Sepharose, ion-exchange on CM-Sepharose, and reverse-phase high-performance liquid chromatography (HPLC) (Hilton *et al.*, 1988a,b).

Purified mouse LIF is a disulfide-linked monomeric glycoprotein of M_r 58,000 that can be degylcosylated to M_r 20,000 (Hilton *et al.*, 1988b), the size predicted by the cDNA (Gearing *et al.*, 1987, 1988). cDNAs encoding human and rat LIFs were later identified (Moreau *et al.*, 1988; Yamamori *et al.*, 1989). Genomic clones encoding human, mouse, rat, pig, and sheep LIFs were identified using the murine and human LIF cDNA sequences as hybridization probes (Gough *et al.*, 1988; Lowe *et al.*, 1989; Stahl *et al.*, 1990; Willson *et al.*, 1992). The LIF gene is located on human chromosome 22q12 and mouse chromosome 11 (Budarf *et al.*, 1989; Sutherland *et al.*, 1989; Kola *et al.*, 1990). Expression of human, mouse, rat, and sheep LIFs has been reported using various bacterial, yeast, insect, and mammalian expression systems (Gearing *et al.*, 1987, 1989a; Gough *et al.*, 1988; Willson *et al.*, 1992; Metcalf and Gearing, 1989a,b; Lowe *et al.*, 1989; Moreau *et al.*, 1988). The specific activities of the various LIFs, where reported, have been similar ($\sim 10^8$ units/mg protein), suggesting that glycosylation is not critical for activity. Deletion of eight amino acids from the C-terminus of mouse LIF resulted in complete loss of biological activity (Gearing *et al.*, 1987).

The Leukemia Inhibitory Factor Is a Polyfunctional Cytokine *in Vitro*

The history of LIF is one of discovery and rediscovery. Its differentiation-inducing activity on myeloid leukemic cells gave rise to two other names, D-factor and differentiation-inducing factor (DIF) (Tomida *et al.*, 1984; Abe *et al.*, 1989). From laboratories working in unrelated areas, LIF was known by other pseudonyms. Thus, the differentiation-inhibiting activity (DIA)/differentiation-retarding factor (DRF) of embryonic stem cells (Williams *et al.*, 1988; Smith *et al.*, 1988), the cholinergic nerve differentiation factor (CDF) (Fukada, 1985; Yamamori *et al.*, 1989), the melanoma-derived lipoprotein lipase inhibitor (MLPLI) (Mori *et al.*, 1989), the human interleukin for

DA cells (HILDA) (Moreau *et al.*, 1988; Godard *et al.*, 1988; Gascan *et al.*, 1989), and the hepatocyte-stimlating factor III (HSFIII) (Baumann and Wong, 1989) were all purified to homogeneity or molecularly cloned and found to be identical to LIF. Many of these names are still in use, and the variety of names reflects quite different functions. LIF is at once a differentiation-inducing and differentiation-inhibiting factor, a mitogenic factor, and a positive or negative cellular activation factor. Due to its availability, recombinant LIF demonstrated expanded activity on adult and embryonal systems, blood cells and vessels, nerves, liver, muscle, gonads, placenta, kidneys, fat, and bone.

III. The Leukemia Inhibitory Factor in Development

The first indication that LIF was important in development was the confirmation of its identity with the DIA/DRF activity that inhibited the differentiation of totipotent mouse embryonic stem (ES) cells (Williams *et al.*, 1988; Smith *et al.*, 1988). This effect contrasted with the induction of differentiation in the M1 leukemic cells. ES cells are derived from the pluripotent founder tissue of the mouse embryo, the inner cell mass (Evans and Kaufman, 1981; Martin, 1981). Such cells rapidly differentiate in the absence of an exogenous factor (DIA/LIF) or a feeder layer of fibroblasts, but in the presence of either they can be maintained in culture and reintroduced into a host blastocyst, where they can contribute to all cell types of the developing mouse. Novel ES cell lines have been derived directly in LIF-containing media and are totipotent (Nichols *et al.*, 1990; Pease *et al.*, 1990). A regulatory loop was suggested from experiments of differentiating ES cells, in which the differentiated progenies release a burst of LIF to prevent the remaining totipotent ES cells from losing their developmental capacity (Rathjen *et al.*, 1990a). Genetic manipulation of ES cells in culture provided the basis for the "knockout mouse" gene targeting technology currently in widespread use (Capecchi, 1989), in which defined alterations of the ES genome can be achieved via homologous recombination.

In studying the mechanism of action of LIF on mouse embryonic stem cells it was observed that cell-to-cell contact could substitute for the addition of LIF to the culture media, and the presence of a cell-associated factor was postulated. An alternatively spliced variant of the mouse LIF cDNA was isolated and found to encode an extracellular matrix-localized form of LIF (Rathjen *et al.*, 1990a). Splicing of a novel signal sequence apparently resulted in the formation of an

identical LIF protein. The biochemical rationale for the two forms of LIF, one soluble, the other immobilized, has been discussed (Rathjen *et al.*, 1990b; Smith *et al.*, 1992) and the suggestion made that the immobilized LIF may be deposited as a topological or temporal signal in the developing embryo (Smith *et al.*, 1992). The mRNAs encoding the soluble and immobilized LIFs appear to be separately regulated (Rathjen *et al.*, 1990a,b), and when expressed from a strong promoter, the matrix cDNA form can also direct the synthesis of the soluble form of LIF (Heath, 1992). The matrix-associated form of LIF might be unique to the mouse, because neither the human, sheep, nor pig LIF genes encode the alternate exon found in the mouse (Willson *et al.*, 1992). The relevance of the matrix LIF to embryological development in other species is currently debated.

The corollary of the ES cell survival studies *in vitro* is that the embryo must produce or receive LIF *in vivo* for the maintenance of the inner cell mass. At some point in time LIF expression would need to stop or other factors would become dominant for normal development to proceed. Consistent with this, LIF expression was observed in preimplantation blastocysts, but not in the postimplantation blastocyst (Conquet and Brulet, 1990; Murray *et al.*, 1990). The extraembryonic tissue of postimplantation mouse embryos continued to make LIF (Conquet and Brulet, 1990). The mouse uterus produces a burst of LIF at the time of implantation, which is under maternal control and always precedes implantation (Bhatt *et al.*, 1991). Together, these results suggest that LIF may regulate the growth and initiate the implantation of blastocysts.

LIF expression also fluctuates in different tissues during development, there being more in adult brain than in embryonic brain, and more in day 1 postnatal heart than in adult heart (Patterson and Fann, 1992). LIF expression is also evident in the neonatal gut, kidney, lung, and thymus, in embryonic skeletal muscle and liver, and in day 11 postnatal spleen. The presence of LIF mRNA in embryonic muscle and kidney is especially interesting because LIF causes increased proliferation of myoblasts (Austin and Burgess, 1991), can inhibit Na^+-dependent hexose transport in renal epithelial cells (Tomida *et al.*, 1990), and can inhibit the induction of differentiation in kidney development (Bard and Ross, 1991).

The development of primordial germ cells is also affected by LIF. A combination of LIF and steel factor (mast cell growth factor/stem cell factor) improves primordial germ cell survival and proliferative capacity *in vitro* (Godin *et al.*, 1991; Matsui *et al.*, 1991; De Felici and Dolci, 1991). By contrast, when overexpressed *in vivo*, LIF in-

hibits spermatogenesis and oogenesis. In these mice there are also profound changes in the fatty tissues that may also affect the hormonal balance, so it is not clear if this action is a direct one. The summation of activities of LIF on normal development has led Smith and colleagues to suggest that the primary role of LIF is as a generalized stem cell factor (Smith *et al.*, 1992). This is obviously an attractive conclusion, but LIF has other activities that may not fit this role. In the presence of LIF, steel factor, and basic fibroblast growth factor (bFGF), primordial germ cells have been reported to convert to ES cells (Matsui *et al.*, 1992). Because bFGF is associated with the extracellular matrix, bFGF might account in part for the enhanced survival of ES cells on feeder cells and provide an alternative to matrix LIF.

A. The Leukemia Inhibitory Factor in Hematopoiesis

Despite the discovery of LIF on the basis of its action on myeloid leukemic cells, its action on normal hematopoietic cells has been harder to define. The mouse myeloid leukemic cell line M1 differentiated into macrophage-like cells when treated with LIF (Metcalf *et al.*, 1988), and human myeloid leukemic cell lines could be suppressed by combinations of LIF and G-CSF or granulocyte/macrophage colony-stimulating factor (GM-CSF) (Maekawa and Metcalf, 1989; Maekawa *et al.*, 1990). It was logical, therefore, to assess the role of LIF in the development of the monocyte/macrophage lineage. Cells of this lineage were observed to display LIF receptors (Hilton *et al.*, 1988c). LIF has proliferative actions on the mouse myeloid cell line DA-1 (Moreau *et al.*, 1988) and, in combination with interleukin-3 (IL-3), can potentiate the proliferation of *myc*-transformed mouse erythroid cell lines (Cory *et al.*, 1991); to date, however, no proliferative, differentiative, or activation function has been assigned to LIF on normal monocytes or macrophages. As a single agent *in vitro*, LIF is also inactive as a colony-stimulating factor (Metcalf *et al.*, 1988).

Nevertheless, LIF might have a role to play in the production of blood cells. In particular, primitive progenitor cells and the megakaryocyte lineage are affected by LIF. The first suggestions of a role in the development of progenitor cells were from experiments designed to enhance the infection rate of mouse bone marrow, enriched for progenitors by 5-fluorouracil treatment, that had been incubated with a retrovirus (Fletcher *et al.*, 1990, 1991). As a measure of infection of primitive cells, the infection rate of CFU-S cells was assessed. Bone marrow incubated with recombinant LIF (from COS cells) had

a much higher frequency of CFU-S infection than control marrows. Because retroviral infection is thought to require the host cell to transit the cell cycle, it was inferred that LIF either induced CFU-S proliferation or enhanced their survival. In separate studies, LIF has been shown to augment the proliferation of human hematopoietic stem cells in synergy with IL-3 (Leary *et al.*, 1990; Verfaillie and McGlave, 1991).

Megakaryopoiesis is influenced by LIF *in vivo* and *in vitro*. Administration of LIF to mice results in an increase of spleen megakaryocytes and ciculating platelets (Metcalf *et al.*, 1990) and, in combination with IL-3, LIF potentiates megakaryocyte production in cell culture (Metcalf *et al.*, 1991). The stimulation of platelet production following thrombocytopenia provides a potential use for LIF in clinical applications (Metcalf *et al.*, 1992).

A role for LIF in lymphocyte biology can be inferred from the production of LIF by the thymic stroma (Le *et al.*, 1990; Sakata *et al.*, 1992), the disappearance of the thymic cortex when LIF is overexpressed *in vivo* (Metcalf and Gearing, 1989a,b), and the presence of LIF receptors on a subpopulation of circulating lymphocytes (Hilton *et al.*, 1988c). However, to date, there have been no accounts of LIF action on lymphocyte function.

B. Leukemia Inhibitory Factor and Bone Metabolism

The observations that LIF is active in the metabolism of bone and calcium have derived from studies *in vitro* and *in vivo*. First accounts suggested that LIF could enhance the release of calcium from rat calvaria (Abe *et al.*, 1986) and so enhance bone resorption. This was later confirmed using recombinant LIF and was shown to be prostaglandin dependent and associated with an increase in the number of osteoclasts (Reid *et al.*, 1990). By contrast, LIF inhibited resorption in a fetal rat long bone system (Lorenzo *et al.*, 1990). The contrasting activities were suggested to be the result of an indirect action of LIF. Osteoblasts, but not osteoclasts, display LIF receptors (Allan *et al.*, 1990) and bone resorption is associated with contaminating osteoblasts (Martin *et al.*, 1992). The action of LIF on bone is therefore explained primarily via the osteoblast. LIF was shown to induce proliferation of primary rat calvarial osteoblasts as well as certain osteoblastic cell lines (Reid *et al.*, 1990; Lowe *et al.*, 1991; Noda *et al.*, 1990). The ability of osteoblasts to make LIF suggests a potential autocrine regulatory mechanism stimulated by tumor necrosis factor (TNF). This production was stimulated by TNF-α (Allan *et al.*, 1990). The enhanced production of osteopontin in the osteoblastic MC3T3-

E1 cells suggests LIF has a role in the differentiation stimulus for osteoblasts (Noda *et al.*, 1990).

Support for the action of LIF on bone came from experiments in which LIF was administered to mice *in vivo*. In one study, in which LIF levels were chronically maintained at massive levels, trabecular bone accumulated to such an extent that the femoral marrow was totally excluded (Metcalf and Gearing, 1989a,b). Calcium plaques were also observed at various tissue sites. In a separate study, in which LIF was injected intraperitoneally, serum calcium levels were elevated but bone sections were apparently normal (Metcalf *et al.*, 1990).

IV. Roles for LIF in Disease: Inflammation and Cachexia?

There have been two clinically interesting systems in which LIF has been demonstrated to be active: inflammation and cachexia. Serum LIF levels are elevated in mice treated with bacterial lipopolysaccharide (Metcalf, 1988), and LIF has been demonstrated to be identical in structure and function to hepatocyte-stimulating factor III (Baumann *et al.*, 1989; Baumann and Wong, 1989), which causes the release of acute-phase plasma proteins (APPs). Together these results suggest a role for LIF as part of the host response to inflammation. The production of APPs by LIF is also modulated by glucocorticoids. An acute-phase response follows LIF administration to mice: chronic administration led to an increase in serum haptoglobin (H. Baumann, W. Munger, and D. Gearing, unpublished work, 1992), a decrease in serum albumin levels, and an increased erythrocyte sedimentation rate (Metcalf *et al.*, 1990).

A role in cachexia was developed from studies of overproduction of LIF *in vivo*. Mice in which LIF was produced chronically developed a syndrome of rapid weight loss due to depletion of all observable fat stores (Metcalf and Gearing, 1989a,b). Mice injected with LIF developed a similar syndrome (Metcalf *et al.*, 1990). The mechanism for this weight loss is best explained by the observation that a melanoma-derived lipoprotein lipase inhibitor is identical to LIF (Mori *et al.*, 1989). Furthermore, melanoma cell lines that secreted LIF caused cachexia in nude mice, while those that produced no detectable LIF did not. The action of LIF as a cachectic agent is similar to that of TNF-α, which is also known as a cachectin (Oliff *et al.*, 1987). Considering that TNF can induce LIF production in osteoblasts (Allan *et al.*, 1990), part of the action of TNF might be indirect via LIF production, should the induction in osteoblasts be generalized to other sites of LIF production.

A novel use for LIF has been observed in protection against oxygen-induced lung toxicity (Tsan *et al.*, 1992). This effect of LIF appears to be mediated by increased Mn superoxide dismutase and is enhanced with TNF.

V. Molecular Characterization of the Leukemia Inhibitory Factor Receptor

Equilibrium analyses of the binding of radioiodinated LIF to the surface of responsive and nonresponsive cells has led to the classification of two receptor types, high affinity and low affinity. The high-affinity receptor type has a dissociation constant in the range of 10–200 p*M* (Yamamoto-Yamaguchi *et al.*, 1986; Hilton *et al.*, 1988c; Williams *et al.*, 1988; Rodan *et al.*, 1990; Tomida *et al.*, 1990) and the low-affinity receptor type has an affinity constant in the range of 1–3 n*M* (Hilton *et al.*, 1991). The presence of the high-affinity receptors correlates with biological activity. The difference between the high- and low-affinity receptor types has been attributed to a higher rate of dissociation from the low-affinity receptor (Hilton and Nicola, 1992). The size of the LIF receptor has been measured by affinity cross-linking and was found to be approximately 250 kDa (Godard *et al.*, 1992). Several reports have documented some of the intracellular consequences of LIF binding to responsive cells. Using M1 leukemic cells, these include tyrosine phosphorylation of a 160-kDa protein, induction of interferon regulatory factor 1, and phosphorylation of heat-shock protein hsp27 (Lord *et al.*, 1991; Abdollahi *et al.*, 1991; Michishita *et al.*, 1991). Within hepatoma cells, several transcription factors, including JunB and members of the C/EBP family, appear to be induced following LIF (and IL-6) stimulation (Baumann *et al.*, 1992). Similarly, M1 leukemic cells and sympathetic neurons respond to LIF with enhanced transcription of c-*fos* and *JunB* genes (Lord *et al.*, 1991; Yamamori, 1991)

Using an expression cloning strategy in mammalian cells based on the use of radioiodinated ligand (Sims *et al.*, 1988) and a sensitive screening method (Gearing *et al.*, 1989b), a cDNA encoding a LIF receptor was identified from a human placental cDNA library (Gearing *et al.*, 1991). The cloned LIF receptor encoded a 200-kDa glycoprotein that specifically bound human, but not murine, LIF with low affinity (1–2 n*M*). The human LIF receptor cDNA encodes a 1097-amino acid residue preprotein with a 44-residue signal sequence, a 789-amino acid residue extracellular domain, a 26-residue transmembrane domain, and a 238-amino acid cytoplasmic domain. The human LIF receptor cDNA is a member of the hematopoietin, or cytokine,

receptor family, the common feature of which is an extracellular structure of approximately 200 amino acid residues that can be split into two, approximately equal, domains. The first domain typically contains a group of four regularly spaced cysteine residues, of which the second is followed, two residues downstream, by a tryptophan residue. The second domain resembles a fibronectin type III repeat and contains the hallmark sequence Trp-Ser-X-Trp-Ser, where X is any amino acid (Bazan, 1990; Cosman *et al.*, 1990; Gearing *et al.*, 1989b). Receptor subunits for interleukins 2, 3, 4, 5, 6, 7, and 9, GM-CSF, G-CSF, growth hormone, prolactin, and ciliary neurotrophic factor have also been shown to be members of this family (reviewed in Gearing and Ziegler, 1993). The LIF receptor extracellular domain contains two hematopoietin receptor (HR) domains, one immunoglobulin-like (Ig) domain, and three fibronectin type III (FNIII) domains arranged in the order HR-Ig-HR-3(FNIII).

Two alternatively spliced versions of the mouse LIF receptor have been identified: one, very similar (75% identity) to the human receptor, is a type I membrane protein, and the other encodes a soluble form of the LIF receptor, in which the third FNIII, transmembrane, and cytoplasmic domains have been deleted (Gearing *et al.*, 1991; D. Gearing, M. Comeau, and S. Gimpel, unpublished observations, 1992). Recombinant mouse LIF receptor can block LIF binding to the cellular receptor *in vitro* (Gearing *et al.*, 1991). The soluble LIF receptor might act as an antagonist, agonist, or carrier protein, roles that have been observed for soluble versions of other hematopoietin receptor family members (reviewed in Gearing and Ziegler, 1993). Normal mouse serum apparently contains large amounts of a truncated form of this soluble LIF receptor (Layton *et al.*, 1992). The LIF receptor genes are on human chromosome 5p12–13 and mouse chromosome 15, clustered with the genes for three other receptor family members, the IL-7 receptor, growth hormone receptor, and prolactin receptor (N. Jenkins, N. Copeland, K. Huebner, J. Overhauser, and D. Gearing, unpublished results). It is not clear whether there is physiological relevance to this gene cluster.

The cloned LIF receptor is structurally most similar to the IL-6 receptor affinity-converting/signal-transducing subunit, gp130, and to the G-CSF receptor, except that these receptor subunits have the structure Ig-HR-3(FNIII). The cytoplasmic domain of the LIF receptor was most similar to that of gp130. The structural resemblance to gp130 immediately presented a possible explanation for the redundancy of action of LIF and IL-6: common cytoplasmic signal-transducing molecules associating directly with the LIF receptor and

L: **Met** Lys **Val**
O: **Met** Gly **Val**

L: **Leu** Ala Ala Gly Val Val Pro **Leu** Leu **Leu** **Val** **Leu** His Trp Lys
O: **Leu** Leu Thr Gln Arg Thr Leu **Leu** Ser **Leu** **Val** **Leu** ... ...

L: His Gly Ala Gly*__Ser__ Pro Leu Pro Ile Thr Pro Val Asn Ala Thr
O: Ala Leu Leu Phe Pro Ser Met Ala Ser*__Met__ Ala Ala Ile Gly Ser

L: **Cys** Ala Ile Arg His Pro Cys His Asn Asn Leu Met Asn **Gln** Ile
O: **Cys** Ser ... Lys Glu Tyr Arg Val Leu Leu Gly Gln Leu **Gln** Lys

L: Arg Ser Gln **Leu** Ala **Gln** Leu Asn Gly Ser Ala Asn Ala **Leu** Phe
O: Gln Thr Asp **Leu** Met **Gln** Asp Thr Ser Arg ... ... **Leu** Leu

L: Ile Leu **Tyr** Tyr Thr Ala **Gln** **Gly** Glu Pro Phe **Pro** Asn Asn **Leu**
O: Asp Pro **Tyr** Ile Arg Ile **Gln** **Gly** Leu Asp Val **Pro** Lys ... **Leu**

L: Asp Lys Leu **Cys** Gly Pro Asn Val Thr Asp **Phe** **Pro** Pro Phe His
O: Arg Glu His **Cys** Arg Glu Arg Pro Gly Ala **Phe** **Pro** Ser Glu Glu

L: Ala Asn ... **Gly** Thr Glu Lys Ala Lys Leu Val Glu Leu Tyr Arg
O: Thr Leu Arg **Gly** Leu Gly Arg Arg Gly Phe Leu Gln Thr Leu Asn

L: Ile Val Val Tyr Leu Gly Thr Ser ... **Leu** Gly Asn Ile Thr Arg
O: Ala Thr Leu Gly Cys Val Leu His Arg **Leu** Ala Asp Leu Glu Gln

L: Asp Gln Lys Ile Leu Asn Pro Ser Ala Leu **Ser** Leu His Ser ...
O: Arg Leu Pro Lys Ala Gln Asp Leu Glu Arg **Ser** Gly Leu Asn Ile

L: ... ... **Lys** **Leu** Asn Ala Thr Ala Asp Ile Leu Arg **Gly**
O: Glu Asp Leu Glu **Lys** **Leu** Gln Met Ala Arg Pro Asn Ile Leu **Gly**

L: **Leu** Leu Ser **Asn** Val Leu **Cys** Arg Leu Cys Ser Lys Tyr ... ...
O: **Leu** Arg Asn **Asn** Ile Tyr **Cys** Met Ala Gln Leu Leu Asp Asn Ser

L: ... ... His Val **Gly** His Val Asp Val Thr Tyr
O: Asp Thr Ala Glu Pro Thr Lys Ala **Gly** Arg Gly ... Ala Ser Gln

L: Gly **Pro** Asp ... **Thr** Ser Gly Lys **Asp** Val **Phe** **Gln** Lys **Lys** Lys
O: Pro **Pro** Thr Pro **Thr** Pro Ala Ser **Asp** Ala **Phe** **Gln** Arg **Lys** Leu

L: Leu **Gly** **Cys** Gln Leu **Leu** Gly Lys **Tyr** Lys Gln Ile Ile Ala Val
O: Glu **Gly** **Cys** Arg Phe **Leu** His Gly **Tyr** His Arg Phe Met His Ser

L: Leu Ala Gln Ala **Phe** ***
O: Val Gly Arg Val **Phe** Ser Lys Trp Gly Glu Ser Pro Asn Arg Ser

O: **Arg** **Arg** His Ser Pro His Gln Ala Leu Arg Lys Gly Val Arg Arg
O: Thr Arg Pro Ser Arg Lys Gly Lys Arg Leu Met Thr Arg Gly Gln

O: Leu Pro Arg ***

gp130. Although this was an attractive model, the low affinity of the cloned LIF receptor was a puzzle. Transfection of the LIF receptor cDNA into nonresponsive cells was attempted to test whether the low-affinity LIF receptor could participate in signal transduction. Transfection of the LIF receptor into B9 mouse plasmacytoma cells was tried first. B9 cells have IL-6 receptors and proliferate in response to IL-6 but do not respond to, nor bind, LIF. B9 cells transfected with the LIF receptor cDNA (B9/hLIFR cells) bound LIF with both low and high affinity, suggesting the presence in B9 cells of a non-LIF-binding, affinity-converting subunit for the LIF receptor (Gearing and Bruce, 1992). Interestingly, although mouse LIF is incapable of binding the low-affinity human LIF receptors on transfected COS or B9/hLIFR cells, the mixed-species high-affinity LIF receptors on B9/hLIFR cells do bind mouse LIF (Gearing *et al.*, 1992a). Response to LIF in the B9/hLIFR cells was not measurable because the population became factor independent by a mechanism unrelated to the LIF receptor. Using a different hematopoietic cell line, BAF-B03, transfection of the LIF receptor resulted in the display of low-affinity LIF receptors. These cells did not become factor independent, but did not respond to LIF (D. Gearing and S. Ziegler, unpublished work, 1992).

In collaboration with the laboratory of Heinz Baumann (Roswell Park Cancer Center) our group has recently demonstrated the ability of the transfected LIF receptor to confer LIF responsiveness on the human hepatoma cell line Hep3B (H. Baumann and D. Gearing, unpublished results). Transfected Hep3B cells again displayed low- and high-affinity LIF receptors.

A. Oncostatin M Binds to the High-Affinity Leukemia Inhibitory Factor Receptor

The molecular basis for the affinity conversion of the low-affinity LIF receptor was facilitated by the observations of Rose and Bruce (1991) that LIF is related in structure to another cytokine, oncostatin

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FIG. 1. Comparison of the amino acid sequences of the precursors of human leukemia inhibitory factor (L) and oncostatin M (O). Gaps have been included (···) to optimize the alignment (based on Rose and Bruce, 1991). Identities are boldface, similarities are underlined. For comparison, residues in the LIF sequence conserved between human, mouse, rat, sheep, and pig LIF sequences (Willson *et al.*, 1992) are overlined. The mature amino termini are in bold italics and are preceded by an asterisk; the predicted site of cleavage of the oncostatin M proprotein (Linsley *et al.*, 1989) is in bold italics.

M (OSM). LIF and OSM are linked by virtue of two shared disulfide bonds among weak overall homology, the similar intron–exon boundary structure of their genes, and the demonstration that, as for the LIF gene, the OSM gene was on human chromosome 22 (Rose and Bruce, 1991) (Fig. 1). Indeed, the LIF and OSM genes have been structurally linked to within 22 kbp of each other (Jeffery *et al.*, 1993). OSM and LIF cause M1 leukemic cell differentiation at similar concentrations. Subsequent receptor binding experiments demonstrated that OSM and LIF could bind to the same receptor on M1 cells (Gearing and Bruce, 1992). There are also separate high- and low-affinity OSM-specific receptors on some solid tumor cells, and the LIF receptors on the macrophage cell line NS1.1 do not bind OSM. These differences were resolved by making use of the cDNA-directed LIF receptors expressed on transfected COS cells, B9 cells, and the LIF receptor in isolation as a soluble extracellular domain. OSM could bind the high-affinity component, but not the low-affinity component, of the LIF receptors on B9/hLIFR cells (like LIF, OSM did not appear to bind to untransfected B9 cells) and could not bind the low-affinity LIF receptor component on transfected COS cells or the soluble LIF receptor (Gearing and Bruce, 1992). The cross-competition of OSM and LIF binding on M1 cells was to a high-affinity LIF receptor, whereas the LIF binding to the NS1.1 cells was of low affinity. In summary, it appears that OSM could bind to the high-affinity LIF receptor, but not the low-affinity LIF receptor, and that a separate OSM-specific receptor also exists. As for the LIF binding, we deduced that because B9 cells did not bind OSM directly, the endogenous affinity-converting subunit also did not bind OSM. These observations led to the conclusion that OSM would make the ideal reagent to identify the affinity-converting subunit of the high-affinity LIF receptor complex.

By transfecting the LIF receptor cDNA together with pools of cDNAs from a placental expression library and screening for OSM binding, we reasoned that we would either clone a cDNA encoding the affinity convertor, or we would clone an OSM-binding subunit in its own right. Fortuitously, we cloned a cDNA that appeared to have both properties (Gearing *et al.*, 1992b). COS cells transfected with this novel cDNA bound OSM with low affinity (10 n*M*) and when cotransfected with the LIF receptor, cDNA bound both OSM and LIF with higher affinity (~0.3 n*M*). However, the affinity of binding was 2- to 30-fold lower than to the high-affinity LIF receptors on responsive cells (10–200 p*M*), suggesting the presence of other subunits in the high-affinity LIF receptor complex . Sequence analysis of

the cDNA revealed it to be identical to the affinity convertor of the IL-6 receptor complex, also known as gp130 (Taga *et al.*, 1989; Hibi *et al.*, 1990). Thus, gp130 is an OSM receptor in its own right and contributes to the binding of at least three ligands, IL-6, LIF, and OSM. The high-affinity LIF-, OSM-, and IL-6-binding sites on B9/hLIFR cells, but not on B9 parental cells, were cross-competitive with each of the other ligands in turn, suggesting that mouse gp130 was involved in the formation of the high-affinity LIF/OSM receptor displayed on B9/hLIFR cells (Gearing *et al.*, 1992b). Confirmatory studies revealed that gp130 is also a component of the OSM-specific receptor (Liu *et al.*, 1992). The sharing of a common subunit to form multiple high-affinity complexes has been observed elsewhere in the hematopoietin receptor family. The high-affinity GM-CSF, IL-3, and IL-5 receptor complexes share an affinity-converting and signaling component, called KH97 or AIC2B, that does not bind either ligand directly (Kitamura *et al.*, 1991; Devos *et al.*, 1991; Takaki *et al.*, 1991).

The relationship of affinity conversion to signal transduction has been tested in our laboratory using the BAF-B03 system. We observed that only when the LIF receptor and gp130 were expressed together would BAF-B03 cells respond to LIF and OSM (D. Gearing and S. Ziegler, unpublished work, 1992). This result suggests that affinity conversion is required for signaling and that despite binding OSM directly, gp130 cannot signal an OSM proliferative response directly.

B. Ciliary Neurotrophic Factor and the Leukemia Inhibitory Factor Receptor

Another role for the cloned LIF receptor subunit has been suggested by studies of signal transduction in a transformed neural cell line, MAH (Ip *et al.*, 1992). MAH cells respond to LIF and yet another cytokine, the ciliary neurotrophic factor (CNTF), in a similar manner, and following LIF or CNTF stimulation the same set of cytoplasmic proteins appears to be phosphorylated. Stimulation by LIF and CNTF was blocked by antibodies to gp130 and one of the induced phosphoproteins was similar in size to the LIF receptor. These results suggest that the CNTF receptor complex is made up in part by the same components as the high-affinity LIF receptor, namely the cloned LIF receptor and gp130. CNTF binds to a specific hematopoietin family receptor component that is attached to the membrane by a glycosylphosphatidylinositol linkage (Davis *et al.*, 1991). This receptor component is expressed by MAH cells and thus the high-affinity CNTF receptor has been suggested to be a triplex structure of CNTF receptor, LIF receptor, and gp130 (Ip *et al.*, 1992).

Because the CNTF receptor is not expressed on M1 cells (Ip *et al.*, 1992), the high-affinity LIF receptor complex cannot be identical to this high-affinity CNTF receptor complex. High- and low-affinity LIF, OSM, and CNTF receptors are shown in Fig. 2.

C. LIF, OSM, CNTF, IL-6, AND IL-11: REDUNDANCY, PLEIOTROPYE, OR SHADES OF GREY?

Many of the examples of LIF biology and receptor function discussed above have included reference to the related cytokines, OSM, CNTF, and IL-6. Recently, participants at a CIBA foundation conference explored the relationship between LIF and IL-6 (Bock *et al.*, 1992). In this context it is worth considering the broader implications of the relationship between all four cytokines and to IL-11, a cytokine closely related to IL-6.

Like LIF, IL-6 is active in stimulating hepatocytes to produce acute-phase proteins (Gauldie *et al.*, 1987); IL-6 also influences bone remodeling (Ishimi *et al.*, 1990), neuronal differentiation (Satoh *et al.*, 1988), adipogenesis (Jablons *et al.*, 1990), and myeloid leukemic cell differentiation (Miyaura *et al.*, 1988), it synergizes with IL-3 to stimulate hematopoietic stem cell (Ikebuchi *et al.*, 1987) and megakaryocyte progenitor proliferation (Ishibashi *et al.*, 1989), and may also have a role in the early embryo (Murray *et al.*, 1990). As with LIF-overproducing mice (Metcalf and Gearing 1989a,b), IL-6-producing tumor cells cause hypercalcemia, cachexia, and thrombocytosis when injected into nude mice (Black *et al.*, 1991). In contrast to LIF, IL-6 induces the proliferation of renal mesangial cells and is strongly implicated in mesangial proliferative glomerulonephritis (Tomida *et al.*, 1990; Horii *et al.*, 1989). A possible correlation between LIF and kidney graft rejection has, however, been observed (Blancho *et al.*, 1992). IL-6 also stimulates T cell proliferation and differentiation, myeloma, plasmacytoma, and hybridoma growth and B cell differentiation (Hirano *et al.*, 1990).

The relationship of the LIF receptor cDNA to the IL-6 signal transducer, gp130, initially suggested a mechanism by which LIF and IL-6 might exert their similar effects (Gearing *et al.*, 1991). The involvement of gp130 in the high-affinity LIF receptor complex (Gearing *et al.*, 1992b) simplified the explanation because signaling through gp130 would provide a more likely route. Yet, despite LIF and IL-6 sharing common signaling pathways in certain cell types, such as M1 leukemic cells (Lord *et al.*, 1991), they do not always provide exactly the same signal. The best example is the H-35 rat hepatoma cell line in which IL-6 stimulation of thiostatin gene ex-

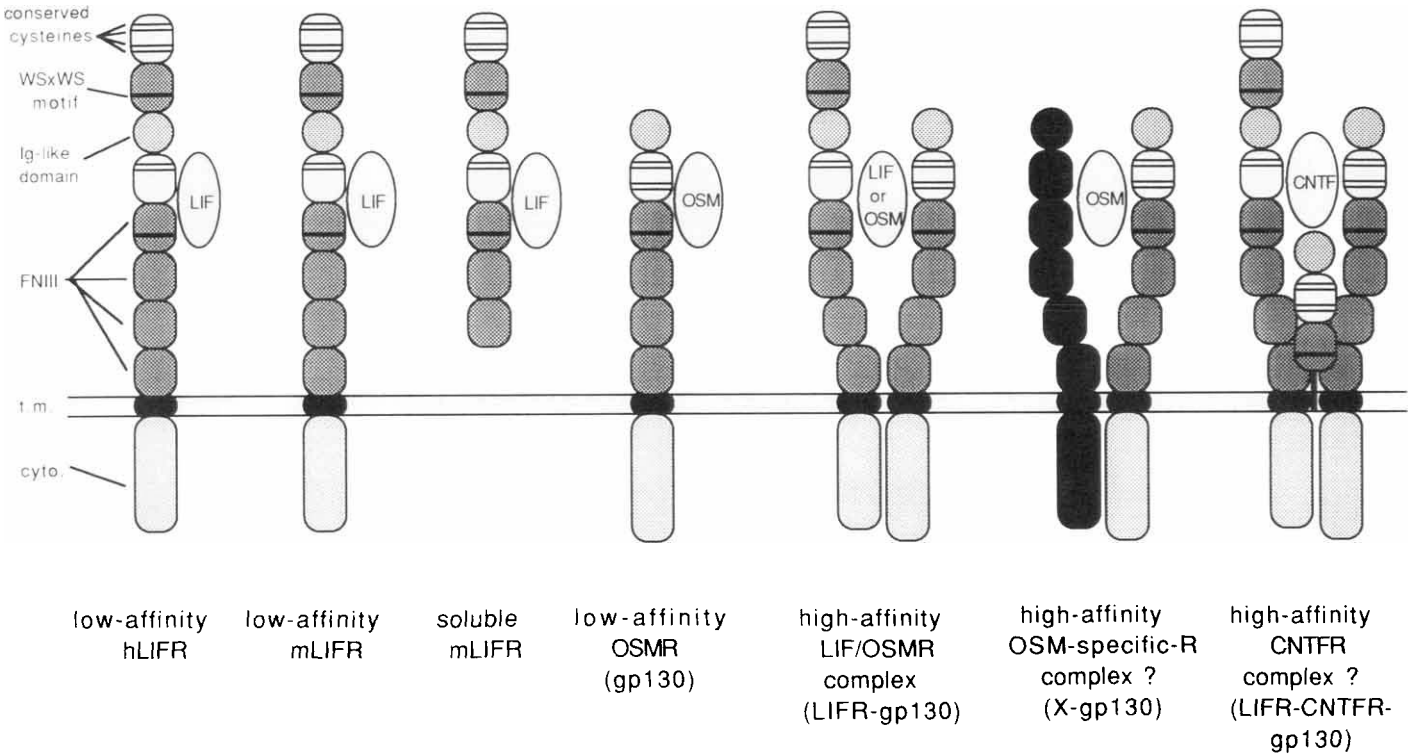

FIG. 2. High- and low-affinity LIF, OSM, and CNTF receptors. The arrangements of the receptor subunits involved in high-affinity complexes are based on data from Gearing *et al.* (1992b) and Ip *et al.* (1992). The domain structure common to the hematopoietin family of receptors is indicated. Thin horizontal lines correspond to cysteine residues conserved in the family and thick lines correspond to the WS × WS sequence motif. Domains with homologies to the fibronectin type III domain are heavily shaded; t.m., transmembrane; cyto, cytoplasm.

pression is strongly enhanced by dexamethasone; however, LIF stimulation of the same gene is inhibited by dexamethasone (Baumann and Gauldie, 1990; Baumann *et al.*, 1992).

Because OSM can bind the high-affinity LIF receptor in addition to an OSM-specific receptor, OSM would likely have the same range of effects as LIF (Gearing and Bruce, 1992) and then more. Shared activities between OSM and LIF have included induction of differentiation of M1 leukemic cells (Rose and Bruce, 1991), growth inhibition of aortic endothelial cells (Brown and Shoyab, 1991) inhibition of ES cell diferentiation (Gearing and Bruce, 1992), and induction of acute-phase proteins by hepatocytes (Richards *et al.*, 1992). On the other hand there are other actions of OSM not shared with LIF, by definition, and these are mediated by the OSM-specific receptor. These actions include the inhibition of growth of several tumor and nontumor lines, including A375 melanoma cells (Zarling *et al.*, 1986), stimulation of plasminogen activator activity in cultured bovine aortic endothelial cells (Brown *et al.*, 1990), induction of IL-6 production by umbilical vein endothelial cells (Brown *et al.*, 1991), and stimulation of AIDS-related Kaposi's sarcoma cells (Miles *et al.*, 1992). The stimulation of acute-phase protein production by hepatocytes is stronger with OSM than with LIF, suggesting the presence of both types of receptor (Richards *et al.*, 1992). Similarly, aortic endothelial cell growth is inhibited by both cytokines, but only OSM stimulates the plasminogen activity of these cells (Ferrara *et al.*, 1992; Brown *et al.*, 1991a,b). Thus, the OSM-specific receptor and the LIF/OSM receptor appear to couple with different signaling pathways. The molecular structure of the high-affinity OSM-specific receptor (Linsley *et al.*, 1989) has not been completely elucidated, but one component is probably gp130, which binds OSM with low affinity (Gearing *et al.*, 1992b; Liu *et al.*, 1992).

The proposed structure of the high-affinity CNTF receptor complex, based on a similar bioactivity of CNTF and LIF and phosphoprotein analyses of MAH cells, was a combination of CNTF receptor, gp130, and LIF receptor (Ip *et al.*, 1992). Functionally, LIF and CNTF promote the noradrenergic-to-cholinergic switch in cultured sympathetic neurons and affect the survival or differentiation of motor and sensory neurons (Yamamori *et al.*, 1989; Murphy *et al.*, 1991; Sendtner *et al.*, 1990; Hall and Rao, 1992). The absence of CNTF receptor mRNA in high-affinity LIF receptor-positive M1 myeloid leukemic cells (Ip *et al.*, 1992) predicts that CNTF does not interact with the LIF receptor. Thus, wherever there is a CNTF response

there would be a LIF response, but not vice versa; CNTF promotes the survival of ciliary neurons and affects glial differentiation, but LIF does not (Rao *et al.*, 1990, 1992). Furthermore, LIF alters neuropeptide levels in sensory neurons whereas CNTF does not (Nawa *et al.*, 1990; Rao *et al.*, 1992). CNTF has also been observed to induce acute-phase protein production (H. Baumann and D. Gearing, unpublished work, 1992) and to prevent ES cell differentiation (C. Stewart, personal communication). Like LIF, the CNTF receptor is expressed in muscle tissue and may also have a role in myoblast proliferation (Davis *et al.*, 1991). Finally, when injected *in vivo*, CNTF is, like LIF, toxic to mice (Sendtner *et al.*, 1992). It would be worth testing whether CNTF affects calcium and fat metabolism, the primary systems responsible for sickness in the LIF-overproducing mice (Metcalf and Gearing, 1989a,b). The CNTF receptor can be shed from the surface of cells and in this form might act like the soluble IL-6 receptor or p40 subunit of IL-12, both of which have been suggested to act as agonists of function (Davis *et al.*, 1991; Taga *et al.*, 1989; Gearing and Cosman, 1991).

Another cytokine worthy of mention is IL-11 because it also induces acute-phase protein production by hepatocytes (Baumann and Schendel, 1991), causes hematopoietic progenitor proliferation and megakaryocyte precursor proliferation in the presence of IL-3 (Bruno *et al.*, 1991; Musashi *et al.*, 1991), inhibits adipogenesis (Kawashima *et al.*, 1991), and stimulates the growth of lymphoid cells (Paul *et al.*, 1990). IL-11 binds to a distinct receptor that does not cross-compete with IL-6 (Yin *et al.*, 1992).

Why are so many cytokines performing so many tasks and why are so many tasks shared? These question are central to the work on any of the cytokines mentioned. In the case of LIF, why does OSM do everything that LIF does, and then more? Is it that the two cytokines are differentially regulated in space and time? Apparently not. Both LIF and OSM are produced by activated T cells and macrophages and a variety of mesenchymal cell types (Gearing and Bruce, 1992). A mouse version of OSM has not been identified, apparently due to the lack of cross-reactivity to the human cDNA sequence. It will be important to determine if OSM exists in the mouse. Similarly, what is the purpose of having two similar neuroactive cytokines, LIF and CNTF? Perhaps each has different thresholds of activity: when one signal reaches a maximum, the second cytokine might then put the system into overdrive. Alternatively, multiple cytokines might act synergistically, allowing function at doses too low to cause toxicity by either alone.

VI. Life without LIF: The LIF Knockout Mouse

Functional redundancy can best be determined by deletion of the gene using homologous recombination. In the case of LIF and its receptor, this would seem to be a particularly enlightened route of investigation, because, according to current models, OSM might be able to replace LIF, and LIF knockout mice would have no phenotype. If the LIF receptor is involved in the signaling by several cytokines, its knockout would be expected to be detrimental. The LIF gene was recently deleted from the mouse genome by homologous recombination in ES cells (Stewart *et al.*, 1992). Breeding chimeras transmitting the mutant gene led to viable homozygous animals lacking functional LIF protein. The animals, although smaller, were essentially normal, with no obvious gross phenotype. However, although adult males were fertile, adult females were infertile and implantation of embryos was shown to be defective. Significantly, the development of the embryo itself was normal, as shown by the ability of homozygous mutant embryos to implant and develop normally when transplanted into a normal uterus, whereas normal embryos failed to develop in mutant mothers.

Implications from this important work are numerous. The essentially normal development of the mutant mice suggests that if LIF is an important molecule the redundancy of action with OSM, assuming a homologue exists in mice, might enable the mice to survive. The fact that Stewart and colleagues found any phenotype at all is especially important, suggesting that LIF and OSM may be differentially expressed in either a tissue-specific or time-dependent manner, LIF being uniquely capable of supporting implantation. Perhaps LIF is essential for the normal development of the uterine wall. Maintenance of the ES cell phenotype by LIF *in vitro* would suggest that LIF is available in the developing blastocyst (above), yet homozygous mutant embryos develop normally. Therefore, other support mechanisms must exist in the embryo, e.g., production of OSM, for the stem cells to survive. The production of LIF in the developing blastocyst indicates that LIF still contributes to the blastocyst environment (Conquet and Brulet, 1990; Murray *et al.*, 1990).

The smaller size of the LIF knockout mice suggests that other more subtle and non-life-threatening disorders occur. For example, the role of LIF as an alarm molecule would not have been tested in the sterile mouse-house environment used to maintain the mice. It will be interesting to determine their response to infection and in-

flammatory attack. Given the number of other systems affected by LIF this analysis could be extensive.

VII. Conclusions

It is now understood that most cytokines are pleiotropic and redundant in action. The mechanism that underlies both features appears to lie in the nature of the receptors rather than in the cytokines themselves. Although structural homology between functionally related ligands is rare, a common receptor framework exists and the high-affinity-converting, signal-transducing receptor subunits are often shared. The shared receptor components most easily account for the issue of redundancy, although, as noted above, gp130 is common to LIF, OSM, IL-6, and CNTF receptors, yet different signals can result. Because receptor subunits are common to different tissues, the molecular basis of cytokine pleiotropy appears to be due to tissue-specific expression of different cytoplasmic signal-transducing components, e.g., protein tyrosine kinases, rather than to variants of the receptor components per se.

Why does the body make so many cytokines, all doing much the same job? It is hard to imagine they are maintained as a fail-safe mechanism. Yet that is essentially the conclusion to be obtained from the LIF knockout mice, assuming that systems other than the uterus are not affected. Even if a cytokine is active on a particular cell type *in vitro*, does that mean it is active *in vivo*? In the case of LIF, most of its effects *in vitro* have been observed *in vivo*. The likely answer is that LIF is made close to its site of action, and only small amounts would be required under such a scenario. Local action also provides a means for the disasterous effects of systemic LIF exposure to be avoided, and the high levels of circulating LIF receptor in serum might provide a sink for escaped LIF, preventing such side effects. Similar binding proteins would be expected for the LIF-related cytokines, OSM and CNTF. However, the CNTF receptor, by homology to the IL-6 receptor and IL-12 (Taga *et al.*, 1989; Gearing and Cosman, 1991), is likely to be an agonist of CNTF function. These issues are clearly difficult to interpret and will require much more experimentation to clarify.

Acknowledgments

I thank Steve Dower, John Sims, and Anne Bannister for their critical comments on this manuscript and my colleagues at Immunex for their contributions to the work described herein. Dedicated to Sarah on the occasion of her birth.

References

Abdollahi, A., Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1991). Interferon regulatory factor 1 is a myeloid differentiation primary response gene induced by interleukin-6 and leukemia inhibitory factor: Role in growth inhibition. *Cell Growth Differ.* **2,** 401–407.

Abe, E., Tanaka, H., Ishimi, Y., Miyaura, C., Hayashi, T., Nagasawa, H., Tomida, M., Yamaguchi, Y., Hozumi, M., and Suda, T. (1986). Differentiation-inducing factor purified from conditioned medium of mitogen-treated spleen cell cultures stimulates bone resorption. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 5958–5962.

Abe, T., Murakami, M., Sato, T., Kajiki, M., Ohno, M., and Kodaira, R. (1989). Macrophage differentiation inducing factor from human monocytic cells is equivalent to murine leukemia inhibitory factor. *J. Biol. Chem.* **264,** 8941–8945.

Allan, E. H., Hilton, D. J., Brown, M. A., Evely, R. S., Yumita, S., Metcalf, D., Gough, N. M., Ng, K. W., Nicola, N. A., and Martin, T. J. (1990). Osteoblasts display receptors for and responses to leukemia-inhibitory factor. *J. Cell. Physiol.* **145,** 110–119.

Austin, L., and Burgess, A. W. (1991). Stimulation of myoblast proliferation in culture by leukemia inhibitory factor and other cytokines. *J. Neurol. Sci.* **10,** 193–197.

Bard, J. B., and Ross, A. S. (1991). LIF, the ES-cell inhibition factor, reversibly blocks nephrogenesis in cultured mouse kidney rudiments. *Development* **113,** 193–198.

Baumann, H., and Gauldie, J. (1990). Regulation of hepatic acute phase plasma protein genes by hepatocyte-stimulating factors and other mediators of inflammation. *Mol. Biol. Med.* **7,** 147–159.

Baumann, H., and Schendel, P. (1991). Interleukin-11 regulates the hepatic expression of the same plasma protein genes as interleukin-6. *J. Biol. Chem.* **266,** 20424–20427.

Baumann, H., and Wong, G. G. (1989). Hepatocyte-stimulating factor III shares structural and functional identity with leukemia-inhibitory factor. *J. Immunol.* **143,** 1163–1167.

Baumann, H., Won, K. A., and Jahreis, G. P. (1989). Human hepatocyte-stimulating factor-III and interleukin-6 are structurally and immunologically distinct but regulate the production of the same acute phase plasma proteins. *J. Biol. Chem.* **264,** 8046–8051.

Baumann, H., Marinkovic-Pajovic, S., Won, K. A., Jones, V. E., Campos, S. P., Jahreis, G. P., and Morella, K. K. (1992). The action of interleukin-6 and leukemia inhibitory factor in liver cells. *In* "Polyfunctional Cytokines: IL-6 and LIF" (G. R. Bock, J. Marsh, and K. Widdows, eds.), pp. 80–99. Wiley, Chichester (*CIBA Found. Symp. No. 167*).

Bazan, J. F. (1990). Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 6934–6938.

Bhatt, H., Brunet, L. J., and Stewart, C. L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 11408–11412.

Black, K., Garrett, I. R., and Mundy, G. R. (1991). Chinese hamster ovarian cells transfected with the murine interleukin-6 gene cause hypercalcemia as well as cachexia, leukocytosis and thrombocytosis in tumor-bearing nude mice. *Endocrinology* **128,** 2657–2659.

Blancho, G., Moreau, J. F., Anegon, I., and Soulillou, J. P. (1992). HILDA/LIF is present in the urine of rejecting kidney graft recipients. *Transplant. Int.* **5,** 57–58.

Bock, G. R., Marsh J., and Widdows, K. (eds.) (1992). "Polyfunctional Cytokines: IL-6 and LIF." Wiley, Chichester (*CIBA Found. Symp. No. 167*).

Brown, T. J., Rowe, J. M., Shoyab, M., and Gladstone, P. (1990). Oncostatin M: A novel

regulator of endothelial cell properties. *In* "Molecular Biology of the Cardiovascular System" (R. Roberts and M. D. Schneider, eds.), Vol. 131, p. 195. *UCLA Symp. Mol. Cell. Biol.* Wiley-Liss, New York.

Brown T. J., Rowe, J. M., and Shoyab, M. (1991a). Differential effects of leukemia inhibitory factor and oncostatin M on endothelial cell physiology. *J. Cell. Biochem. (Supp.)* **15F,** CF405.

Brown, T. J., Rowe, J. M., and Shoyab, M. (1991b). Regulation of interleukin-6 expression by oncostatin M. *J. Immunol.* **147,** 2175–2180.

Bruno, E., Briddell, R. A., Cooper, R. J., and Hoffman, R. (1991). Effects of recombinant interleukin-11 on human megakaryocyte progenitor cells. *Exp. Hematol.* **19,** 378–381.

Budarf, M., Emanuel, B. S., Mohandas, T., Goedell, D. V., and Lowe, D. G. (1989). Human differentiation stimulating factor (leukemia inhibitory, human interleukin DA) gene maps distal to the Ewing's sarcoma breakpoint on 22q. *Leukemia* **3,** 9–13.

Capecchi, M. R. (1989). Altering the genome by homologous recombination. *Science* **244,** 1288–1292.

Conquet, F., and Brulet, P. (1990). Developmental expression of myeloid leukemia inhibitory factor gene in preimplantation blastocysts and in extraembryonic tissue of mouse embryos. *Mol. Cell. Biol.* **10,** 3801–3805.

Cory, S., Maekawa, T., McNeall, J., and Metcalf, D. (1991). Murine erythroid cell lines derived with myc retroviruses respond to leukemia inhibitory factor, erythropoietin and interleukin-3. *Cell Growth Differ.* **2,** 165–172.

Cosman, D., Lyman, S. D., Idzerda, R. L., Beckmann, M. P., Park, L. S., Goodwin, R. G., and March, C. J. (1990). A new cytokine receptor superfamily. *Trends Biochem. Sci.* **15,** 265–270.

Davis, S., Aldrich, T. H., and Valenzuela, D. M. (1991). The receptor for ciliary neurotrophic factor. *Science* **253,** 59–63.

De Felici, M., and Dolci, S. (1991). Leukemia inhibitory factor sustains the survival of mouse primordial germ cells cultured on TM4 feeder layers. *Dev. Biol.* **147,** 281–284.

Devos, R., Plaetinck, G., Van der Heyden, J., Cornelis, S., Vandekerckhove, J., Fierce, W., and Tavernier, J. (1991). Molecular basis of a high affinity murine IL-5 receptor. *EMBO J.* **10,** 2133–2137.

Evans, M. J., and Kaufmann, M. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature (London)* **292,** 154–156.

Ferrara, N., Winer, J., and Henzel, W. J. (1992). Pituitary follicular cells secrete an inhibitor of aortic endothelial cell growth: Identification as leukemia inhibitory factor. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 698–702.

Fletcher, F. A., Williams, D. E., Maliszewski, C., Anderson, D., Rives, M., and Belmont, J. W. (1990). Murine leukemia inhibitory factor enhances retroviral-vector infection efficiency of hematopoietic progenitors. *Blood* **76,** 1098–1103.

Fletcher, F. A., Moore, K. A., and Ashlenazi, M. (1991). Leukemia inhibitory factor improves the survival of retoviral vector-infected hematopoietic stem cells *in vitro*, allowing efficient long-term expression of vector-encoded human adenosine deaminase *in vivo*. *J. Exp. Med.* **174,** 837–845.

Fukada, K. (1985). Purification and partial characterization of a cholinergic neuronal differentiation factor. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8795–8799.

Gascan, H., Godard, A., Ferenz, C., Naulet, J., Praloran, V., Peyrat, M. A., Hewick, R., Jacques, Y., Moreau, J. F., and Soulillou, J. P. (1989). Characterization and NH_2-terminal amino acid sequence of natural human interleukin for DA cells: Leukemia

inhibitory factor. Differentiation inhibitory activity secreted by a T lymphoma cell line. *J. Biol. Chem.* **264,** 21509–21515.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., and Baumann, H. (1987). Interferon beta 2/B-cell stimulating factor type 2 shares identity with monocyte-derived hepatocyte stimulating factor and regulates the major acute phase protein response in liver cells. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7251–7255.

Gearing, D. P., and Bruce, A. G. (1992). Oncostatin M binds to the high-affinity leukemia inhibitory factor receptor. *New Biol.* **4,** 61–65.

Gearing, D. P., and Cosman, D. (1991). Homology of the p40 subunit of natural killer cell stimulatory factor (NKSF) with the extracellular domain of the interleukin-6 receptor. *Cell (Cambridge, Mass.)* **66,** 9–10.

Gearing, D. P., and Ziegler, S. D. (1993). The hematopoietic growth factor receptor family. *Curr. Opin. Hematol.* (in press).

Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A., and Metcalf, D. (1987). Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *EMBO J.* **6,** 3995–4002.

Gearing, D. P., King, J. A., and Gough, N. M. (1988). Complete sequence of murine myeloid leukaemia inhibitory factor (LIF). *Nucleic Acids Res.* **16,** 9857.

Gearing, D. P., Nicola, N. A., Metcalf, D., Foote, S., Willson, T. A., Gough, N. M., and Williams, R. L. (1989a). Production of leukemia inhibitory factor in *Escherichia coli* by a novel procedure and its use in maintaining embryonic stem cells in culture. *Biotechnology* **7,** 1157–1161.

Gearing, D. P., King, J. A., Gough, N. M. and Nicola, N. A. (1989b). Expression cloning of a receptor for human granulocyte-macrophage colony stimulating factor. *EMBO J.* **8,** 3667–3676.

Gearing, D. P., Thut, C. J., VandenBos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D., and Beckmann, M. P. (1991). Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. *EMBO J.* **10,** 2839–2848.

Gearing, D. P., VandenBos, T., Beckmann, M. P., Thut, C. J., Comeau, M. R., Mosley, B., and Ziegler, S. F. (1992a). Reconstitution of high affinity leukemia inhibitory factor (LIF) receptors in hematopoietic cells transfected with the cloned human LIF receptor. *In* "Polyfunctional Cytokines: IL-6 and LIF" (G. R. Bock, J. Marsh, and K. Widdows, eds.), pp. 245–255. Wiley, Chichester (*CIBA Found. Symp. No. 167*).

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F., and Cosman, D. (1992b). The IL-6 signal transducer, gp130: An oncostatin M receptor and affinity convertor for the LIF receptor. *Science* **255,** 1434–1437.

Godard, A., Gascan, H., Naulet, J., Peyrat, M. A., Jacques, Y., Soulillou, J. P., and Moreau, J. F. (1988). Biochemical characterization and purification of HILDA, a human lymphokine active on eosinophils and bone marrow cells. *Blood* **71,** 1618–1623.

Godard, A., Heymann, D., Raher, S., Anegon, I., Peyrat, M. A., Le Mauff, B., Mouray, E., Gregoire, M., Virdee, K., Soulillou, J. P., Moreau, J. F., and Jacques, Y. (1992). High and low affinity receptors for human interleukin for DA cells/leukemia inhibitory factor on human cells. *J. Biol. Chem.* **267,** 3214–3222.

Godin, I., Deed, R., Cooke, J., Zsebo, K., Dexter, M., and Wylie, C. C. (1991). Effects of the steel gene product on mouse primordial germ cells in culture. *Nature (London)* **352,** 807–809.

Gough, N. M., Gearing, D. P., King, J. A., Willson, T. A., Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988). Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2623–2627.

Hall. A. K., and Rao, M. S. (1992). Cytokines and neurokines: Related ligands and related receptors. *Trends Neurosci.* **15,** 35–37.

Heath, J. (1992). *In* "Polyfunctional Cytokines: IL-6 and LIF" (G. R. Bock, J. Marsh, and K. Widdows, eds.), p 40. Wiley, Chichester (*CIBA Found. Symp. No. 167*).

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kishimoto, T. (1990). Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell (Cambridge, Mass.)* **63,** 1149–1157.

Hilton, D. J., and Nicola, N. A. (1992). Kinetic analyses of the binding of leukemia inhibitory factor to receptor on cells and membranes and in detergent solution. *J. Biol. Chem.* **267,** 10238–10247.

Hilton, D. J., Nicola, N. A., Gough, N. M., and Metcalf, D. (1988a). Resolution and purification of three distinct factors produced by Krebs ascites cells which have differentiation-inducing activity on murine myeloid leukemic cell lines. *J. Biol. Chem.* **263,** 9238–9243.

Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988b). Purification of a murine leukemia inhibitory factor from Krebs ascites cells. *Anal. Biochem.* **173,** 359–367.

Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988c). Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 5971–5975.

Hilton, D. J., Nicola, N. A., and Metcalf, D. (1991). Distribution and comparison of receptors for leukemia inhibitory factor on murine hemopoietic and hepatic cells. *J. Cell Physiol.* **146,** 207–215.

Hirano, T., Akira, S., Taga, T., and Kishimoto, T. (1990). Biological and clinical aspects of interleukin 6. *Immunol. Today* **11,** 443–449.

Horii, Y., Muraguchi, A., Iwano, M., Matsuda, T., Hirayama, T., Yamada, H., Fujii, Y., Dohi, K., Ishikawa, H., Ohmoto, Y., Yoshizaki, K., Hirano, T., and Kishimoto, T. (1989). Involvement of IL-6 in mesangial glomerulonephritis. *J. Immunol.* **143,** 3949–3955.

Ikebuchi, K., Wong, G. G., Clark, S. C., Ihle, J. N., Hirai, Y., and Ogawa, M. (1987). Interleukin-6 enhancement of interleukin-3 dependent proliferation of multipotential hemopoietic progentiors. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9035–9039.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1992). CNTF and LIF act on neuronal cells via shared signalling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell (Cambridge, Mass.)* **69,** 1121–1132.

Ishibashi, T., Kimura, H., Shikama, Y., Uchida, T., Kariyone, S., Hirano, T., Kishimoto, T., Takatsuki, F., and Akiyama, Y. (1989). Interleukin-6 is a potent thrombopoietic factor *in vivo* in mice. *Blood* **74,** 1241–1244.

Ishimi, Y., Miyaura, C., Jin, C. H., Akatsu, T., Abe, E., Nakamura, Y., Yamaguchi, A., Yoshiki, S., Matsuda, T., Hirano, T., Kishimoto, T., and Suda, T. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J. Immunol.* **145,** 3297–3303.

Jablons, D., Nordan, R. P., and McIntosh, J. (1990). Interleukin-6 inhibits adipocyte lipoprotein lipase activity *in vivo* and *in vitro*. *FASEB J.* **4,** A1713.

Jeffery, E., Price, V., and Gearing, D. (1993). Close proximity of the genes for leukemia inhibitory factor and oncostatin M. *Cytokine* (in press).

Kawashima, I., Ohsumi, J., Mita-Honjo, K., Shimoda-Takano, K., Ishikawa, H., Sakakibara, S., Miyadai, K., and Takiguchi, Y. (1991). Molecular cloning of cDNA encod-

ing adipogenesis inhibitory factor and identity with interleukin-11. *FEBS Lett.* **283,** 199–202.

Kitamura, T., Stao, N., Arai, K., and Miyajima, A. (1991). Expression cloning of the human IL-3 receptor cDNA reveals a shared beta subunit for the human IL-3 and GM-CSF receptors. *Cell (Cambridge, Mass.)* **66,** 1165–1174.

Kola, I., Davey, A., and Gough, N. M. (1990). Localization of the murine leukemia inhibitory factor gene near the centromere on chromosome 11. *Growth Factors* **2,** 235–240.

Layton, M. J., Cross, B. A., Metcalf, D., Ward, L. D., Simpson, R. J., and Nicola, N. A. (1992). A major binding protein for leukemia inhibitory factor in normnal mouse serum: Identification as a soluble form of the cellular receptor. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 8616–8620.

Le, P. T., Lazorick, S., and Whichard, L. P. (1990). Human thymic epithelial cells produce IL-6, granulocyte-monocyte CSF and leukemia inhibitory factor. *J. Immunol.* **145,** 3310–3315.

Leary, A. G., Wong, G. G., Clark, S. C., Smith, A. G., and Ogawa, M. (1990). Leukemia inhibitory factor differentiation-inhibiting activity/human interleukin for DA cells augments proliferation of human hematopoietic stem cells. *Blood* **75,** 1960–1964.

Linsley, P. S., Bolton-Hanson, M., Horn, D., Malik, N., Kallestad, J. C., Ochs, V., Zarling, J. M., and Shoyab, M. (1989). Identification and characterization of cellular receptors for the growth regulator, oncostatin M. *J. Biol. Chem.* **264,** 4282–4289.

Liu, J., Modrell, B., Aruffo, A., Marken, J. S., Taga, T., Yasukawa, K., Murakami, M., Kishimoto, T., and Shoyab, M. (1992). Interleukin-6 signal transducer gp130 mediates oncostatin M binding. *J. Biol. Chem.* **267,** 16763–16766.

Lord, K. A., Abdollahi, A., Thomas, S. M., De, M. M., Brugge, J. S., Hoffman, L. B., and Liebermann, D. A. (1991). Leukemia inhibitory factor and interleukin-6 trigger the same immediate early response, including tyrosine phosphorylation, upon induction of myeloid leukemia differentiation. *Mol. Cell. Biol.* **11,** 4371–4379.

Lorenzo, J. A., Sousa, S. L., and Leahy, C. L. (1990). Leukemia inhibitory factor (LIF) inhibits basal bone resorption in fetal rat long bone cultures. *Cytokine* **2,** 266–271.

Lowe, D. G., Nunes, W., Bombara, M., McCabe, S., Ranges, G. E., Henzel, W., Tomida, M., Yamamoto, Y. Y., Hozumi, M., and Goeddel, D. V. (1989). Genomic cloning and heterologous expression of human differentiation-stimulating factor. *DNA* **8,** 351–359.

Lowe, C., Cornish, J., Martin, T. J., and Reid, I. R. (1991). Effects of leukemia inhibitory factor on bone resorption and DNA synthesis in neonatal mouse calvaria. *Calcif. Tissue Int.* **49,** 394–397.

Maekawa, T., and Metcalf, D. (1989). Clonal suppression of HL60 and U937 cells by recombinant human leukemia inhibitory factor in combination with GM-CSF or G-CSF. *Leukemia* **3,** 270–276.

Maekawa, T., Metcalf, D., and Gearing, D. P. (1990). Enhanced suppression of human myeloid leukemic cell lines by combinations of IL-6, LIF, GM-CSF and G-CSF. *Int. J. Cancer* **45,** 353–358.

Martin, G. R. (1981). Isolation of a pluripotential cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7634–7638.

Martin, T. J., Allan, E. H., Evely, R. S., and Reid, I. R. (1992). Leukemia inhibitory factor and bone cells function. *In* "Polyfunctional Cytokines: IL-6 and LIF" (G. R. Bock, J. Marsh, and K. Widdows, eds.), pp. 141–155. Wiley, Chichester (*CIBA Found. Symp. No. 167*).

Matsui, Y., Toksoz, D., Nishikawa, S., Nishikawa, S., Williams, D., Zsebo, K., and

Hogan, B. L. (1991). Effect of Steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. *Nature (London)* **353,** 750–752.

Matsui, Y., Zsebo, K., and Hogan, B. L. M. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell (Cambridge, Mass.)* **70,** 841–847.

Metcalf, D. (1988). Suppression of myeloid leukemic cells by normal hematopoietic regulators. *In* "Mochida Memorial Symposium. Recent Progress in Cytokine Research," pp. 119–134.

Metcalf, D., and Gearing, D. P. (1989a). Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 5948–5952.

Metcalf, D., and Gearing, D. P. (1989b). A myelosclerotic syndrome in mice engrafted with cells producing high levels of leukemia inhibitory factor (LIF). *Leukemia* **3,** 847–852.

Metcalf, D., Hilton, D. J., and Nicola, N. A. (1988). Clonal analysis of the actions of the murine leukemia inhibitory factor on leukemic and normal murine hemopoietic cells. *Leukemia* **2,** 216–221.

Metcalf, D., Nicola, N. A., and Gearing, D. P. (1990). Effects of injected leukemia inhibitory factor on hematopoietic and other tissues in mice. *Blood* **76,** 50–56.

Metcalf, D., Hilton, D. J., and Nicola, N. A. (1991). Leukemia inhibitory factor can potentiate murine megakaryocyte production *in vitro*. *Blood* **76,** 50–56.

Metcalf, D., Waring, P., and Nicola, N. A. (1992). Actions of leukemia inhibitory factor on megakaryocyte and platelet formation. *In* "Polyfunctional Cytokines: IL-6 and LIF" (G. R. Bock, J. Marsh, and K. Widdows, eds.), pp. 174–182. Wiley, Chichester (*CIBA Found. Symp. No. 167*).

Michishita, M., Satoh, M., Yamaguchi, M., Hirayoshi, K., Okuma, M., and Nagata, K. (1991). Phosphorylation of the stress protein hsp27 is an early event in murine myelomonocytic leukemic cell differentiation induced by leukemia inhibitory factor/D-factor. *Biochem. Biophys. Res. Commun.* **176,** 979–984.

Miles, S. A., Martinez-Maza, O., Rezai, A., Magpantay, L., Salahuddin, S. Z., Nakamura, S., Radka, S. F., and Linsley, P. S. (1992). Oncostatin M as a potent mitogen for AIDS-Kaposi's sarcoma-derived cells. *Science* **255,** 1432–1434.

Miyaura, C., Onozaki, K., Akiyama, Y., Taniyama, T., Hirano, T., Kishimoto, T., and Suda, T. (1988). Recombinant human interleukin-6 (B-cell stimulatory factor 2) is a potent inducer of differentiation of mouse myeloid leukemia cells (M1). *FEBS Lett.* **234,** 17–21.

Moreau, J. F., Donaldson, D. D., Bennett, F., Witek, G. J., Clark, S. C., and Wong, G. G. (1988). Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells. *Nature (London)* **336,** 690–692.

Mori, M., Yamaguchi, K., and Abe, K. (1989). Purification of a lipoprotein lipase-inhibiting protein produced by a melanoma cell line associated with cancer cachexia. *Biochem. Biophys. Res. Commun.* **160,** 1085–1092.

Murphy, M., Reid, K., Hilton, D. J., and Bartlett, P. F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 3498–3501.

Murray, R., Lee, F., and Chiu, C. P. (1990). The genes for leukemia inhibitory factor and interleukin-6 are expressed in mouse blastocysts prior to the onset of hemopoiesis. *Mol. Cell. Biol.* **10,** 4953–4956.

Musashi, M., Yang, Y. C., Paul, S. R., Clark, S. C., Sudo, T., and Ogawa, M. (1991). Direct and synergistic effects of interleukin-11 on murine hemopoiesis in culture. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 765–769.

Nawa, H., Yamamori, T., and Patterson, P. H. (1990). Generation of neuronal diversity: Analogies and homolgies with hematopoiesis. *Cold Spring Harbor Symp. Quant. Biol.* **55**, 247–253.

Nichols, J., Evans, E. P., and Smith, A. G. (1990). Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development* **110**, 1341–1348.

Noda, T., Hasson, D. M., Vogel, R. L., Nicola, N. A., and Rodan, G. A. (1990). Leukemia inhibitory factor suppresses proliferation, alkaline phosphatase activity and type I collagen mRNA level and enhances osteopontin mRNA levels in murine osteoblast-like (MC3T3-E1) cells. *Endocrinology* **127**, 185–190.

Oliff, A., Defea-Jones, D., Boyer, M., Martinez, D., Kiefer, D., Vuocolo, G., Wolfe, A., and Socher, S. H. (1987). Tumors secreting human TNF-cachectin induce cachexia in mice. *Cell (Cambridge, Mass.)* **50**, 555–583.

Patterson, P. H., and Fann, M. J. (1992). Further studies of the distribution of CDF/LIF mRNA. *In* "Polyfunctional Cytokines: IL-6 and LIF" (G. R. Bock, J. Marsh, and K. Widdows, eds.), pp. 125–135. Wiley, Chichester (*CIBA Found. Symp. No. 167*).

Paul, S. R., Bennett, F., Calvetti, J. A., Kelleher, K., Wood, C. R., O'Hara, R. M., Leary, A. C., Sibley, B., Clark, S. C., Williams, D. A., and Yang, Y. C. (1990). Molecular cloning of a cDNA encoding interleukin-11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7512–7516.

Pease, S., Braghetta, P., Gearing, D., Grail, D., and Williams, R. L. (1990). Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). *Dev. Biol.* **141**, 344–352.

Rao, M. S., Landis, S. C., and Patterson, P. H. (1990). The cholinergic neuronal differentiation factor from heart cell conditioned medium is different from the cholinergic factors in sciatic nerve and spinal cord. *Dev. Biol.* **139**, 65–74.

Rao, M. S., Tyrrell, S., Landis, S. C., and Patterson, P. H. (1992). Effects of ciliary neurotrophic factor (CNTF) and depolarization on neuropeptide expression in cultured sympathetic neurons. *Dev. Biol.* **150**, 281–293.

Rathjen, P. D., Nichols, J., Toth, S., Edwards, D. R., Heath, J. K., and Smith, A. G. (1990a). Developmentally programmed induction of differentiation inhibiting activity and the control of stem cell populations. *Genes Dev.* **4**, 2308–2318.

Rathjen, P. D., Toth, S., Willis, A., Heath, J. K., and Smith, A. G. (1990b). Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. *Cell (Cambridge, Mass.)* **62**, 1105–1114.

Reid, I. R., Lowe, C., Cornish, J., Skinner, S. J. M., Hilton, D. J., Willson, T. A., Gearing, D. P., and Martin, T. J. (1990). Leukemia inhibitory factor: A novel bone-active cytokine. *Endocrinology* **126**, 1416–1420.

Richards, C. D., Brown, T. J., Shoyab, M., Baumann, H., and Gauldie, J. (1992). Recombinant oncostatin M stimulates the production of acute phase proteins in HepG2 cells and rat primary hepatocytes *in vitro*. *J. Immunol.* **148**, 1731–1736.

Rodan, S. B., Wesolowski, G., Hilton, D. J., Nicola, N. A., and Rodan, G. A. (1990). Leukemia inhibitory factor binds with high affinity to preosteoblastic RCT-1 cells and potentiates the retinoic acid induction of alkaline phosphatase. *Endocrinology* **127**, 1602–1608.

Rose, T. M., and Bruce, A. G. (1991). Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8641–8645.

Sakata, T., Iwagami, S., Tsuruta, Y., Yamaguchi, M., Michishita, M., Nagata, K., Takai, Y., Ogata, M., Teraoka, H., and Fujiwara, H. (1992). Constitutive expression of leukemia inhibitory factor (LIF) mRNA and production of LIF by a cloned murine

thymic stromal cell line. *Thymus* **19,** 89–95.

Satoh, T., Makamura, S., and Taga, T. (1988). Induction of neural differentiation in PC12 cells by B cell stimulatory factor/interleukin-6. *Mol. Cell. Biol.* **8,** 3546–3549.

Sendtner, M., Kreutzberg, G. W., and Thoenen, H. (1990). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature (London)* **345,** 440–441.

Sendtner, M., Schmalbruch, H., Stoeckli, K. A., Carroll, P., Kreutzberg, G. W., and Thoenen, H. (1992). Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. *Nature (London)* **358,** 502–504.

Simpson, R. J., Hilton, D. J., Nice, E. C., Rubira, M. R., Metcalf, D., Gearing, D. P., Gough, N. M., and Nicola, N. A. (1988). Structural characterization of a murine myeloid leukaemia inhibitory factor. *Eur. J. Biochem.* **175,** 541–547.

Sims, J. E., March, C. J., Cosman, D., Widmer, M. B., MacDonald, H. R., McMahan, C. J., Grubin, C. E., Wignall, J. M., Jackson, J. L., Call, S. M., Friend, D., Alpert, A., Gillis, S., Urdal, D. L., and Dower, S. K. (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science* **241,** 585–589.

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature (London)* **336,** 688–690.

Smith, A. G., Nichols, J., Robertson, M., and Rathjen, P. D. (1992). Differentiation inhibiting activity (DIA/LIF) and mouse development. *Dev. Biol.* **151,** 339–351.

Stahl, J., Gearing, D. P., Willson, T. A., Brown, M. A., King, J. A., and Gough, N. M. (1990). Structural organization of the genes for murine and human leukemia inhibitory factor. Evolutionary conservation of coding and noncoding regions. *J. Biol. Chem.* **265,** 8833–8841.

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Koentgen, F., and Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature (London)* **359,** 76–79.

Sutherland, G. R., Baker, E., Hyland, V. J., Callen, D. F., Stahl, J., and Gough, N. M. (1989). The gene for human leukemia inhibitory factor (LIF) maps to 22q12. *Leukemia* **3,** 9–13.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., and Kishimoto, T. (1989). Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell (Cambridge, Mass.)* **58,** 573–581.

Takaki, S., Ita, S., Kitamura, T., Yonehara, S., Yamaguchi, N., and Tominaga, A. (1991). Identification of the second subunit of the murine interleukin-5 receptor: Interleukin-3 receptor-like protein, AIC2B is a component of the high affinity interleukin-5 receptor. *EMBO J.* **10,** 2833–2838.

Tomida, M., Yamamoto-Yamaguchi, Y., and Hozumi, M. (1984). Purification of a factor inducing differentiation of mouse myeloid leukemic M1 cells from conditioned medium of mouse fibroblast L929 cells. *J. Biol. Chem.* **259,** 10978–10982.

Tomida, M., Yamamoto-Yamaguchi, Y., Hozumi, M., Holmes, W., Lowe, D. G., and Goeddel, D. V. (1990). Inhibition of development of Na(+)-dependent hexose transport in renal epithelial LLC-PK1 cells by differentiation-stimulating factor for myeloid leukemic cells/leukemia inhibitory factor. *FEBS Lett.* **268,** 261–264.

Tsan, M. F., White, J. E., and Wong, G. H. W. (1992). D-factor and growth hormone enhance tumor necrosis factor-induced increase of Mn superoxide dismutase mRNA and oxygen tolerance. *Cytokine* **4,** 101–105.

Verfaillie, C., and McGlave, P. (1991). Leukemia inhibitory factor/human interleukin for DA cells: A growth factor that stimulates the *in vitro* development of multipoten-

tial human hematopoietic progenitors. *Blood* **77,** 263–270.

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature (London)* **336,** 684–687.

Willson, T. A., Metcalf, D., and Gough, N. M. (1992). Cross species comparison of the sequence of the leukemia inhibitory factor gene and its protein. *Eur. J. Biochem.* **204,** 21–30.

Yamamori, T. (1991). CDF/LIF selectively increases c-fos and jun-B transcripts in sympathetic neurons. *Neuroreport* **2,** 173–176.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M. J., and Patterson, P. H. (1989). The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* **246,** 1412–1416.

Yamamoto-Yamaguchi, Y., Tomida, M., and Hozumi, M. (1986). Specific binding of a factor inducing differentiation to mouse myeloid leukemic M1 cells. *Exp. Cell. Res.* **164,** 97–102.

Yin, T., Miyazawa, K., and Yang, Y. C. (1992). Characterization of interleukin-11 receptor and protein tyrosine phosphorylation induced by interleukin-11 in mouse 3T3-L1 cells. *J. Biol. Chem.* **267,** 8347–8351.

Zarling, J. M., Shoyab, M., Marquardt, H., Hanson, M. B., Lioubin, M. N., and Todaro, G. J. (1986). Oncostatin M: A growth regulator produced by differentiated histiocytic lymphoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 9739–9743.

This article was accepted for publication on 8 October 1992.

ADVANCES IN IMMUNOLOGY, VOL. 53

Role of CD4 and CD8 in T Cell Activation and Differentiation

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I. Introduction

CD4 and CD8 are cell surface glycoproteins expressed on T lymphocytes that have specificity for class II or class I major histocompatibility complex (MHC) proteins, respectively (reviewed in Swain, 1983). T cells that express CD4 are generally, though not always, helper/inducer cells, while those that express CD8 are usually cytotoxic cells (reviewed in Parnes, 1989). These proteins have been shown to play major roles in both the activation of mature peripheral T cells and the thymic differentiation process that leads to the mature T cell repertoire and the expression of CD4 and CD8 on mutually exclusive T cell subsets. The molecular biology and function of CD4 and CD8 were reviewed by Parnes in 1989. Since that time there has been a wealth of new information on the mechanisms by which these molecules influence T cell differentiation and function. For example, the crystal structures of immunoglobulin-like domains of CD4 (Wang *et al.*, 1990; Ryu *et al.*, 1990) and CD8 (Leahy *et al.*, 1992) have been determined, binding sites for CD8 on class I MHC proteins (Norment *et al.*, 1988; Potter *et al.*, 1989; Salter *et al.*, 1989, 1990) and CD4 on class II MHC proteins (Cammarota *et al.*, 1992; König *et al.*, 1992) have been mapped, and both proteins have been shown to be associated via their cytoplasmic tails with the relatively T cell-specific, *src*-family tyrosine kinase $p56^{lck}$ (Rudd *et al.*, 1988; Veillette *et al.*, 1988). This review summarizes the role of CD4 and CD8 in both T cell activation and differentiation from the perspective of such new findings.

II. Function of CD4 and CD8 in T Cell Activation

A. CD4 and CD8 Contribute to TCR-Mediated Recognition and Activation

Early studies demonstrated that antisera or monoclonal antibodies (mAbs) directed against CD4 and CD8 cell surface antigens interfere with the activation and differentiation of antigen-induced T cell re-

sponses. Antibodies directed against CD4 generally interfere with MHC class II-restricted responses and thus most T "helper" function, whereas antibodies against CD8 affect MHC class I-restricted responses and thus "killer" function (reviewed in Parnes, 1989). Since the ability of anti-CD4 and anti-CD8 to block T cell function is quite variable, affecting clones with different specificities and affinities for antigen/MHC to different extents, the role of CD4 and CD8 cannot be considered essential for all T cell activation and recognition. Thus, these molecules have been termed "accessory molecules." However, there is an increasing body of evidence to suggest that CD4 and CD8 are of primary importance in the activation, differentiation, and effector functions of most T cells.

Over the past few years many experiments have been directed at understanding the mechanism by which CD4 and CD8 contribute to antigen-specific recognition, activation, and differentiation. It was suggested that CD4 and CD8 may be adhesion molecules that increase the overall avidity of a T cell and its cognate partner by binding to their respective MHC class II and class I ligands, thus facilitating specific TCR/antigen–MHC interactions. Expression of high levels of CD4 (Doyle and Strominger, 1987) or CD8 (Norment *et al.*, 1988; Salter *et al.*, 1989, 1990; Rosenstein *et al.*, 1989) in transfected cells or membrane vesicles increased the ability of those cells or vesicles to bind to cells or membranes expressing the appropriate MHC class II or class I ligand, demonstrating that CD4/class II or CD8/class I interactions can influence the avidity of one cell for another. Expression of CD4 in a class-I restricted hybridoma demonstrated that CD4 contributed to antigen-specific IL-2 production only when class II was present on the antigen-presenting cell (APC) (Gay *et al.*, 1987). Similarly, expression of CD8 in a hybridoma in which the TCR was restricted to class II influenced IL-2 production only when class I was present on the APC (Ratnofsky *et al.*, 1987). These data are consistent with CD4 and CD8 acting as adhesion molecules and were interpreted to support this notion. By individually examining the effects of various antibodies on the "adhesive" and "lytic" phases of CTL activity, it was demonstrated that mAbs directed against CD4 and CD8 could sometimes block the adhesive phase. However, the relative adhesive effects of CD4 or CD8 proved to be minor compared with those of CD2 and LFA-1. Interestingly, in these studies, blocking with anti-CD4 or anti-CD8 significantly inhibited activity when present only during the lytic phase, indicating an additional role for these molecules in activation (De Vries *et al.*, 1989). However, the validity of an adhesive phase that is entirely

separate from and precedes the lytic phase must be questioned in light of recent data by O'Rourke, Mescher, and co-workers (1990, 1992) and Mazerolles *et al.* (1990, 1991), which will be discussed later.

The first indication that CD4 and CD8 may play a more active role in TCR-mediated cell signaling came from studies demonstrating that anti-CD4 and anti-CD8 mAbs inhibited mitogen-induced activation in the absence of relevant MHC ligands (Bank and Chess, 1985; Bekoff *et al.*, 1985; Wassmer *et al.*, 1985; Tite *et al.*, 1986; Rosoff *et al.*, 1987). Similarly, anti-CD3/TCR-directed cytotoxicity against target cells that lacked MHC class I expression could be inhibited by anti-CD8 mAbs (Van Seventer *et al.*, 1986) and anti-TCR-induced activation could be blocked with mAbs directed against CD4 (Rojo *et al.*, 1980; Dianzani *et al.*, 1992a). This ability of antibodies directed against CD4 or CD8 to inhibit TCR- or mitogen-induced T cell activation was termed "negative signaling." However, there remains no clear evidence for the existence of a signal delivered on CD4 or CD8 ligation that actively downregulates a TCR-mediated signal. Rather, a lack of signaling that normally accompanies TCR activation has generally been reported. An alternate explanation for these results suggests that the presence of anti-CD4 or anti-CD8 mAbs may interfere with the formation of functional complexes between the TCR and CD4 or CD8 and/or their associated $p56^{lck}$. This idea, in part, gave rise to the "coreceptor" hypothesis of CD4 or CD8 function (see Section II, D).

B. Protein Tyrosine Kinase $p56^{lck}$ Is Physically Associated with the Cytoplasmic Tails of CD4 and CD8

The idea that CD4 and CD8 have signal transduction as well as ligand-binding capabilities gained support with recent findings that the intracellular *src*-like tyrosine kinase $p56^{lck}$ binds to the cytoplasmic tails of both CD4 and CD8α (Rudd *et al.*, 1988; Veillette *et al.*, 1988; Zamoyska *et al.*, 1989; Shaw *et al.*, 1989; Turner *et al.*, 1990); that crosslinking CD4 or CD8 can contribute to increases in *in vivo* tyrosine phosphorylation of protein substrates (Veillette *et al.*, 1988; 1989a, 1989c; Luo and Sefton, 1990); and that tyrosine phosphorylation is the earliest known signaling event associated with TCR-mediated recognition and activation (Samelson *et al.*, 1986; Hsi *et al.*, 1989; June *et al.*, 1990a). It has been demonstrated that crosslinking CD4 with anti-CD4 mAb results in an increase in the *in vitro* kinase activity of $p56^{lck}$ associated with CD4 (Veillette *et al.*, 1989a, 1991c; Luo and Sefton, 1990). It has been more difficult to demonstrate an upregulation of CD8-associated *in vitro* kinase activity on CD8

crosslinking with mAb and secondary antibodies (Veillette *et al.*, 1989c; Luo and Sefton, 1990). However, an increased tyrosine phosphorylation of *in vivo* substrates has been demonstrated to accompany CD8 crosslinking (Veillette *et al.*, 1989c; Luo and Sefton, 1990).

Quantitative analysis showed that 50 and 20% of cellular $p56^{lck}$ are bound to CD4 and CD8, respectively (Veillette *et al.*, 1988, Veillette *et al.*, 1989c; Luo and Sefton, 1990). This association is noncovalent and is disrupted by ionic detergents or agents capable of modifying free sulfhydryl groups on cysteine residues (Veillette *et al.*, 1991; Shaw *et al.*, 1990). These characteristics led to the suggestion that the interaction between the CD4 or CD8 cytoplasmic tail and $p56^{lck}$ may involve cysteines participating in a metal ion coordination complex. In keeping with this idea, deletion and site-specific mutagenesis studies have demonstrated that sequences localized between amino acids 394 and 421 of CD4, 194 and 203 of CD8α, and 2 and 34 of $p56^{lck}$ containing crucial cysteines are sufficient for complex formation (mouse numbering used in each case) (Shaw *et al.*, 1989; Turner *et al.*, 1990). A cysteine-X-cysteine motif shared by CD4 and CD8α cytoplasmic tails and a related cysteine-X-X-cysteine motif present in the N-terminal portion of $p56^{lck}$ have been demonstrated to be particularly important, since mutation of any of these cysteines significantly diminishes or eliminates CD4/$p56^{lck}$ or CD8α/$p56^{lck}$ binding when these molecules are expressed in nonlymphoid cells or T cell hybridomas (Rudd *et al.*, 1988; Veillette *et al.*, 1988; Shaw *et al.*, 1989; Turner *et al.*, 1990; Zamoyska *et al.*, 1989). Furthermore, it has been demonstrated that the cytoplasmic tail of the CD8α chain rather than the CD8β chain is entirely responsible for CD8α/$p56^{lck}$ association (Zamoyska *et al.*, 1989). A complementarity of charge likely also facilitates interactions between CD4 or CD8 cytoplasmic tails and $p56^{lck}$. Mouse CD4 and CD8α cytoplasmic tails have net positive charges while mouse $p56^{lck}$ is largely negatively charged (particularly within the amino-terminal 32 amino acids). Peptides reproducing 13 CD4 cytoplasmic residues can compete with native CD4 for binding to $p56^{lck}$ *in vitro* (Vega *et al.*, 1990). Furthermore, interactions between peptides representing CD4 or CD8α cytoplasmic portions and $p56^{lck}$ up-regulate the enzymatic activity and affect substrate specificity of $p56^{lck}$ *in vitro*. Polycations can mimic CD4 cytoplasmic fragments in some of these respects (Bramson *et al.*, 1991).

$p56^{lck}$ is a member of the *src* gene family of intracellular protein tyrosine kinases (PTKs) and, as such, shares a number of salient features with other members of the *src* family (Fig. 1) (reviewed in

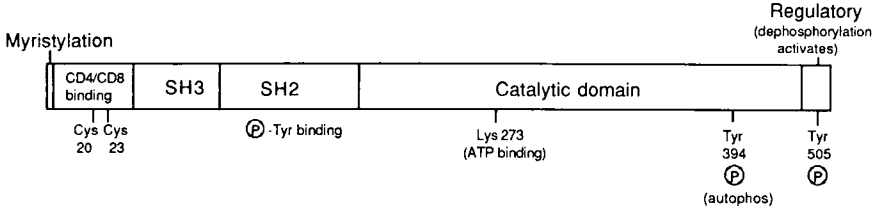


FIG. 1. Schematic representation of the intracellular tyrosine kinase $p56^{lck}$. The N-terminal glycine is known to be myristylated (N. Abraham and Veillette, 1990). The domain involved in CD4/CD8 association and the cysteines demonstrated to be crucial for the association (Shaw *et al.*, 1989; Turner *et al.*, 1990) are shown. The SH3 and SH2 domains are thought to be involved in substrate interaction. This most likely is partially a result of the ability of the SH2 domain to bind phosphotyrosine (Koch *et al.*, 1991; Overduin *et al.*, 1992). The catalytic domain contains an ATP-binding site crucial to enzymatic function, as well as a site of autophophorylation (Cooper, 1990). Tyrosine phosphorylation at this site is thought to contribute to $p56^{lck}$ activity *in vivo* (Caron *et al.*, 1992). Phosphorylation at position Tyr-505 is thought to inhibit enzymatic activity of $p56^{lck}$ (Marth *et al.*, 1988; Amrein and Sefton, 1988; N. Abraham and Veillette, 1990).

Cooper, 1990). The N-terminal glycine (glycine 2 in $p56^{lck}$) is myristylated, allowing for association with the membrane, presumably (by analogy to $p60^{src}$) by binding to an internal membrane myristyl-kinase receptor (Resh and Ling, 1990). The amino-terminal domain is the least conserved among family members, is approximately 60 amino acids long, and for $p56^{lck}$ contains the sequences known to be involved in CD4/CD8 binding (Shaw *et al.*, 1989). Myristylation of $p56^{lck}$ at glycine 2 is not required for association with CD4, though it appears to facilitate the interaction, most likely by localizing $p56^{lck}$ to the cytoplasmic face of the plasma membrane, where it is ideally positioned for binding CD4 or CD8 cytoplasmic tails (Shaw *et al.*, 1990; Caron *et al.*, 1992). The *src* homology 3 (SH3) and 2 (SH2) domains span amino acid residues (66–122 and 123–234 (mouse Lck numbering) and are thought to be involved in cytoskeletal association and substrate specificity, respectively (Koch *et al.*, 1991). Isolated SH2 domains have been shown to bind directly phosphotyrosine and phosphotyrosine-containing proteins, and SH2 domains from different proteins bind to phosphotyrosine-containing peptides or phosphoproteins with a particular specificity (Fantl *et al.*, 1992; Kashishian *et al.*, 1992). Activation-induced tyrosine phosphorylation of proteins within or associated with transmembrane receptor complexes is thought to contribute to the recruitment and binding of SH2-containing signaling proteins that modify and/or effect signal

transduction (reviewed in Koch *et al.*, 1991). Though little has been published to date on the binding properties of the $p56^{lck}$ SH2 domain, it has recently been reported that a bacterial fusion protein containing the $p56^{lck}$ SH2 domain binds to several phosphotyrosine-containing proteins from stimulated T cells (Caron *et al.*, 1992).

Amino acid residues 235–494 encompass the kinase domain, which contains an ATP-binding site centered around lysine 273, as well as an autophosphorylation site at tyrosine 394 (Casnelli *et al.*, 1982; N. Abraham and Veillette, 1990; Luo and Sefton, 1990). This domain has a high degree of sequence identity with kinase domains of nonreceptor tyrosine kinases (i.e., *src* family members) as well as receptor tyrosine kinases, such as the platelet-derived growth factor (PDGF) receptor (reviewed in Cooper, 1990). Phosphorylation of tyrosine 394 most likely results from trans-phosphorylation between $p56^{lck}$ molecules on kinase dimerization accompanying activation, and is thought to play an upregulatory role (see below). Myristylation at glycine 2 is required for *in vivo* phosphorylation at this site (Caron *et al.*, 1992).

The carboxy-terminal domain of all *src*-like kinases (spanning amino acids 495–509 in $p56^{lck}$) contains a regulatory tyrosine (Y505 in $p56^{lck}$) (Cooper, 1990). In $p56^{lck}$ this is the major site of *in vivo* phosphorylation (Veillette *et al.*, 1988; Luo and Sefton, 1990). A number of findings suggest that $p56^{lck}$ enzymatic function is likely to be negatively regulated by tyrosine 505 phosphorylation (see below). It has been shown that $p56^{lck}$ molecules with defective ATP-binding sites are not enzymatically active, but are still phosphorylated at position 505 *in vivo* (Veillette *et al.*, 1991). Therefore, tyrosine 505 is most likely not a site of autophosphorylation, but rather is phosphorylated by another PTK that has yet to be identified. A PTK named csk (c-*src* kinase) specifically phosphorylates $p60^{src}$ at the analogous regulatory site (Y527) and has recently been cloned and characterized. While csk transcripts were detected in all tissues examined, a cross-hybridizing transcript of a different size was only detectable in spleen and thymus (Nada *et al.*, 1991). Given its restricted tissue distribution, the protein encoded by this cross-hybridizing message represents a potential candidate for the $p56^{lck}$ Y505 kinase (Nada *et al.*, 1991). Finally, the tyrosine phosphatase CD45 may dephosphorylate $p56^{lck}$ at position Y505, thus upregulating $p56^{lck}$ kinase activity and T cell activation (Ostergaard *et al.*, 1989; Ostergaard and Trowbridge, 1990).

C. GTP-Binding Protein Associates with CD4 and CD8

Telfer and Rudd (1991) recently reported that a 32-kDa phosphoprotein coprecipitates with CD4–$p56^{lck}$ and CD8–$p56^{lck}$ complexes

from the human T cell tumor line HPB-ALL as well as from normal human thymocytes and peripheral blood lymphocytes. This protein is recognized by antiserum to a peptide representing a consensus GTP-binding region found in G proteins [Gly-$(X)_4$-Gly-Lys], and the peptide used to generate the antiserum blocked recognition of the 32-kDa protein. Immunoprecipitated CD4 and CD8 complexes bound to GTP and hydrolyzed it to GDP. Additionally, this 32-kDa protein was covalently linked to [α-^{32}P]GTP by ultraviolet photoaffinity labeling. These data demonstrate that a functional GTP-binding protein is associated with CD4 and CD8. How this G protein might be involved in T cell activation remains unknown, though activation of GTP-binding proteins in T cells by GTP analogs that cannot be hydrolyzed has been shown to induce phosphoinositol turnover (Harnett and Klaus, 1988). Furthermore, G proteins have been demonstrated to affect the enzymatic activity of phospholipase C in other systems (Cockcroft and Gomperts, 1985; Gilman, 1987). As PLC_γ is known to be involved in T cell activation, this is a potential mechanism through which this G protein could influence T cell signals. Studies in which [α-^{32}P]GTP was crosslinked to T cell proteins by ultraviolet light revealed as many as 10 GTP-binding proteins in T cells (including one at 32 kDa) (Pessa-Morikawa *et al.*, 1990), thus one cannot yet directly link this particular 32-kDa protein with a function in T cells.

D. The Coreceptor Model

The "coreceptor" model holds that CD4 or CD8 forms a complex with the TCR, binding to the same antigen-bearing MHC molecule to function as a coreceptor for recognition and activation (reviewed in Janeway, 1992). This model is particularly appealing, because it could help explain the association between MHC class II recognition and CD4 expression and between MHC class I recognition and CD8 expression. Thus, during thymic selection, when a $CD4^+CD8^+$ thymocyte encounters antigen presented by class I or class II MHC, only the CD4 or CD8 coreceptor that can bind to that same MHC molecule as the TCR is called into play to contribute to or modify a differentiation/activation signal. Such a signal may result in the maintenance of expression of the appropriate CD4 or CD8 coreceptor molecule, the downregulation of expression of the other coreceptor, and possibly the differentiation program involved in maturation into functional helper or killer T cells. The role for CD4 and CD8 "coreceptors" in differentiation will be discussed in depth later in this article (see Section III). Similarly, this hypothesis predicts that maximal activation of mature T cell function, such as proliferation, lym-

phokine production, or cytotoxic granule release, occurs when the TCR and CD4 or CD8 bind to the same MHC ligand and interact as coreceptors for MHC/antigen recognition and signal transduction. Data to support the coreceptor model will be discussed here.

E. Association between the TCR and Coreceptor Molecules

A physical association between CD4 or CD8 and the TCR has been demonstrated using a number of different approaches, including coimmunoprecipitation (Gallagher *et al.*, 1989; Beyers *et al.*, 1992), cocapping (Saizawa *et al.*, 1987; Kupfer *et al.*, 1988; Rojo *et al.*, 1989), and comodulation (Takada and Engleman, 1987; Weyand *et al.*, 1987; Anderson *et al.*, 1988) of these molecules, and by fluorescence resonance energy transfer between these molecules (Mittler *et al.*, 1989; Collins *et al.*, 1992). While this association is barely detectable before T cell activation, TCR triggering induces a greater degree of association. When maximally associated, it appears that two CD4 molecules associate with each TCR (Saizawa *et al.*, 1987). Fluorescence microscopy studies initially demonstrated that cell surface CD4 or CD8 and the TCR colocalized to the site of direct interaction between a T cell and an APC only when the appropriate antigen/MHC combination was present on the APC (Kupfer *et al.*, 1987). Similarly, a number of groups demonstrated that CD4 or CD8 comodulates with the TCR complex off the cell surface following T cell activation (Weyand *et al.*, 1987; Anderson *et al.*, 1988). More convincingly, CD4, CD8, and $p56^{lck}$ have been coprecipitated with the TCR on T cell tumors and in peripheral blood cells when mild detergents were used for cell lysis (i.e., Triton X-100 and Brij 96, respectively) (Gallagher *et al.*, 1989; Beyers *et al.*, 1992). In one report, the sensitivity of the *in vitro* kinase assay allowed for detection of the association between CD4/$p56^{lck}$ and CD3/TCR components on a T cell tumor even when NP40 was used for cell lysis (Burgess *et al.*, 1991).

Over the past few years, Janeway's group has generated a considerable body of evidence to suggest that the TCR undergoes a conformational change on TCR activation, resulting in a greater association between CD4 and the TCR and more efficient activation (Dianzani *et al.*, 1992a; Saizawa *et al.*, 1987; Rojo *et al.*, 1989; Janeway, 1992). Working with the class II-restricted T cell clone D10 and a set of mAbs directed against different epitopes of the variable region of its TCR complex, they demonstrated that some mAbs could induce cocapping of CD4 with the TCR, whereas others could not (Rojo *et al.*, 1989). There was a direct correlation between the ability of a mAb to

induce cocapping and its ability to stimulate D10 to proliferate (Rojo *et al.*, 1989; Janeway, 1992). Those mAbs capable of inducing cocapping of the TCR and CD4 required 30- to 300-fold fewer TCR ligands per cell for maximal activation. It has been demonstrated that these results do not simply reflect the affinity of the mAb for the TCR. Rather, they have been interpreted to suggest that the mAbs that are maximally stimulatory bind the TCR in such a way that they induce a conformational change that induces association of the TCR with CD4, and thus more efficient signaling. An extension of these studies substantiated this idea by demonstrating that only mAbs that induced efficient CD4/TCR interactions could induce efficient tyrosine phosphorylation of the TCR ζ chain, which is known to accompany TCR-mediated activation (Dianzani *et al.*, 1992a).

A potential site of interaction between CD4 and the TCR has been identified by epitope interference studies with the D10 T cell and a panel of anti-TCR and anti-CD4 mAbs (Janeway, 1991; Dianzani *et al.*, 1992a). mAbs directed against CD4 inhibited TCR-mediated activation by anti-TCR variable region mAbs directed against a particular epitope of the D10 TCR (Janeway, 1991; Dianzani *et al.*, 1992a). Stimulation of D10 by anti-TCR Vβ mAbs was unaffected by anti-CD4 mAbs, demonstrating the fine specificity of the epitope interference and presumably the interaction between CD4 and the TCR. Much of what was previously referred to as "negative signaling" by anti-CD4 mAbs might now be understood in terms of steric interference with CD4/TCR interactions required for effective TCR-mediated signaling. Appropriate interpretation of these data might be complicated by the finding that the TCR can signal through at least two distinct signaling modules (centered around CD3ϵ and the associated ζ chain) (Irving and Weiss, 1991; Wegener *et al.*, 1992). It is possible that each module may be differentially stimulated by various anti-TCR mAbs and that signaling through one of these modules may be affected to a greater degree by CD4/p56^{lck} or p56^{lck}/TCR interaction. How CD4 and p56^{lck} might influence TCR-mediated signaling will be readdressed later (see Section II,L).

A number of groups have deliberately forced an interaction between the TCR and either CD4 or CD8 by artificially crosslinking the TCR and coreceptor utilizing the following means: heterospecific mAbs containing binding sites for both CD4 or CD8 and the TCR (Ledbetter *et al.*, 1988, 1990), secondary crosslinking antibodies that bind both anti-CD4 or anti-CD8 and anti-TCR mAbs (Emmrich *et al.*, 1986; Owens *et al.*, 1987; Haughn *et al.*, 1992), biotin conjugation of anti-CD4 and anti-TCR followed by avidin crosslinking (Emmrich *et*

al., 1987; Ledbetter *et al.*, 1988; Kanner *et al.*, 1992), or adherence of anti-CD4 or anti-CD8 and anti-TCR to beads or plates (Emmrich *et al.*, 1986, 1987; Anderson *et al.*, 1987). In each case conditions leading to coligation of CD4 or CD8 with the TCR dramatically increased TCR-mediated signaling when suboptimal concentrations of anti-TCR antibodies were used. Signals that were measured include protein tyrosine phosphorylation of $PLC_{\gamma}1$ (Kanner *et al.*, 1992; Deans *et al.*, 1992), CD3-associated ζ (Dianzani *et al.*, 1992a), the proto-oncogene *vav* (Bustelo *et al.*, 1992; Margolis *et al.*, 1992), and a number of as yet undefined proteins (Veillette *et al.*, 1989c; Luo and Sefton, 1990; Ledbetter *et al.*, 1990; Kanner *et al.*, 1992); inositol triphosphate production (Ledbetter *et al.*, 1988); Ca^{2+} flux (Ledbetter *et al.*, 1988); proliferation (Emmrich *et al.*, 1987; Anderson *et al.*, 1987; Haughn *et al.*, 1992); and lymphokine production (Owens *et al.*, 1987). Crosslinking the TCR alone could result in all of these signals; however, the intensity of the signal was not as great as when the TCR was crosslinked to a coreceptor molecule. Crosslinking CD4 alone has in some instances been reported to result in small increases in production of inositol phosphates (Ledbetter *et al.*, 1988); in Ca^{2+} flux (Kanner *et al.*, 1992); in tyrosine phosphorylation of the ζ chain (Veillette et al., 1989b), $p56^{lck}$ (Veillette *et al.*, 1989b), and some other unidentified substrates (Rudd *et al.*, 1988, Ledbetter *et al.*, 1990); and in an increase in the kinase activity of the associated $p56^{lck}$ (Veillette *et al.*, 1989a, 1991; Luo and Sefton, 1990), but these signals have not generally been correlated with functional consequences of T cell activation such as proliferation or lymphokine production. It has been reported that in some instances crosslinking CD4 in the absence of TCR signaling can lead to decreased TCR expression or decreased T cell adhesiveness. These data will be discussed later (see Sections II,I and II,J, respectively).

F. Coreceptor/MHC Binding Sites

In the coreceptor model simultaneous binding of the TCR and the appropriate CD4 or CD8 coreceptor to the same MHC molecule during antigen recognition is required for maximal TCR-mediated activation. It was therefore imperative to establish that coreceptor/MHC binding sites and TCR/MHC binding sites did not overlap and that simultaneous binding was, in fact, physically possible. Interpretation of the crystal structures of MHC class I molecules allowed for the identification of residues within the $\alpha 1$ and $\alpha 2$ domains thought to contribute to binding and interaction with the TCR (Bjorkman *et al.*,

1987). Characterization of mutant mouse and human class I molecules substituted at positions 227 and 245, respectively, implicated the $\alpha 3$ domain as the potential contact site for CD8α (Norment *et al.*, 1988; Potter *et al.*, 1989). Further mutagenesis of the $\alpha 3$ domain of class I MHC molecules demonstrated that three clusters of $\alpha 3$ domain residues (including amino acids 220–232, 233–235, and 245–247) contribute to the adherence of cells expressing normal or mutated human class I MHC molecules to human CD8α-transfected CHO cells (Norment *et al.*, 1988; Salter *et al.*, 1989, 1990). Furthermore, in both mouse and human systems, cells expressing class I MHC molecules mutated at key residues within the $\alpha 3$ domain were demonstrated to be less functionally competent at activating T cell responses restricted by those mutant class I molecules, despite the surface expression of class I molecules with intact $\alpha 3$ domains, to which CD8 could bind independently of the TCR (Connolly *et al.*, 1988, 1990; Potter *et al.*, 1989; Salter *et al.*, 1990). Taken together, these data demonstrated that simultaneous binding of CD8 and the TCR to the same MHC molecule was not only possible, but likely occurs physiologically during T cell activation. The role of the CD8β chain in class I MHC binding remains an open question.

Recent mutagenesis studies aimed at establishing the CD4 binding site on class II MHC proteins have implicated a region between residues 137–143 (mouse numbering) as a primary determinant for class II MHC/CD4 interaction (König *et al.*, 1992). These data are consistent with those obtained using a human class II HLA-DR peptide binding assay (Cammarota *et al.*, 1992). This region of the class II β chain $\beta 2$ domain is structurally analogous to the CD8 binding region within the $\alpha 3$ domain of MHC class I molecules, and binding to this site would be consistent with simultaneous binding of CD4 and the TCR to the same MHC molecule.

The crystal structures of extracellular portions of CD4 (Ryu *et al.*, 1990; Wang *et al.*, 1990) and CD8α (Leahy *et al.*, 1992) have also recently been solved. Coupled with studies in which CD4 and CD8α have been mutagenized and assayed for binding and function in antigen-specific T cells (Clayton *et al.*, 1989; Fleury *et al.*, 1991), these data have allowed for a better understanding of how CD8/class I MHC and CD4/class II MHC interaction may occur. Mutation of extracellular portions of CD4 has implicated regions within the immunoglobulinlike domains 1 and 2 of CD4 as playing a primary role in the interaction with class II MHC. In particular, mutations within the CDR1- and CDR3-like loops of CD4 greatly affected class II interaction (Clayton *et al.*, 1980; Fleury *et al.*, 1991). Whereas all

reports agreed that mutations within the CDR2-like loop affected CD4/HIV gp120 binding, there is a discrepancy as to whether or not the CDR2-like loop is involved in CD4/MHC class II interaction (Clayton *et al.*, 1989; Fleury *et al.*, 1991). Since the CDR1 and CDR3 loops are on the opposite face of the CD4 molecule from the CDR2 loop (Ryu *et al.*, 1990; Wang *et al.*, 1990), these data may imply that CD4 does not contact class II MHC molecules along the surface encompassing the CDR-like loops, but rather along a surface including only one side of the CDR1-like and CDR3-like loops. Mutagenesis of the CD8α immunoglobulin-like variable domain demonstrated that the CDR1-like and CDR2-like loops of CD8 are important in MHC class I binding (Sanders *et al.*, 1991). As the surface of CD8 bearing the CDR-like loops is primarily positively charged and the CD8-binding residues identified within the α3 domain of class I MHC are highly negatively charged, a complementarity of charge likely contributes to the CD8/class I MHC interaction (Leahy *et al.*, 1992).

If the interaction between CD8 and class I MHC is confined to the CDR1 and CDR2 loops, the crystal structure indicates that it is possible for the simultaneous binding of two HLA class I molecules to one CD8α homodimer (Leahy *et al.*, 1992). Such an interaction might contribute to crosslinking the TCR complex (including the CD8 coreceptor) *in vivo*. The CD4 crystal structure indicates that each CD4 molecule can only bind one class II MHC protein (Ryu *et al.*, 1990; Wang *et al.*, 1990). However, as mentioned earlier, it is thought that two CD4 molecules associate with one TCR complex (Saizawa *et al.*, 1987). This may contribute to TCR complex (including the CD4 coreceptor) crosslinking *in vivo*. Taken together, these data may help explain why crosslinking the TCR *in vitro* is required for activation.

G. Understanding the Mechanisms of Coreceptor Function Using Gene Transfer

A number of groups have used gene transfer coupled with mutagenesis to understand the mechanisms by which CD4, CD8, and $p56^{lck}$ potentiate T cell activation. T cell hybridomas often lose surface expression of CD4 or CD8 in culture, thus providing the investigator with an antigen-specific $CD4^-CD8^-$ T cell into which wild-type or mutant forms of CD4, CD8α, and CD8β can be transfected, so that their relative abilities to potentiate T cell responses can be compared. Initial gene transfer studies demonstrated that expression of CD8 or CD4 in either class I- or class II-restricted hybridomas could augment antigen-induced IL-2 production relative to the parental hybridoma (Dembić *et al.*, 1987; Gabert *et al.*, 1987; Gay *et al.*, 1987;

Ratnofsky *et al.*, 1987; Sleckman *et al.*, 1987; Ballhausen *et al.*, 1988). We have recently used this technique to assess the relative potentiating effects of CD4 or CD8 expression under circumstances wherein they either can or cannot bind to the same MHC molecule as the TCR, and thus can or cannot act as a coreceptor. Furthermore, we have expressed truncated forms of CD4 and CD8α in order to determine whether the cytoplasmic tail and/or the associated p56^{lck} are required for potentiation under each of these circumstances (Zamoyska *et al.*, 1989; Parnes *et al.*, 1989; Miceli *et al.*, 1991; Miceli and Parnes, 1991).

DC27.10 is a mouse T cell hybridoma that requires CD8α surface expression for IL-2 production in response to the alloantigen K^b. It is a subclone of a CD4$^+$ helper hybridoma D0-11.10 (specific for I-A^d + ovalbumin) that was transfected with the TCRα and TCRβ chain genes from a CD8$^+$, K^b-specific CTL clone (Gabert *et al.*, 1987). DC27.10 was shown to produce IL-2 in response to the K^b alloantigen only when CD8α was transfected into and expressed by this cell. CD8β expression was not required. CD8α', a naturally occurring truncated form of CD8α, lacks all but a few amino acids of its cytoplasmic tail and thus cannot associate with p56^{lck} (Liaw *et al.*, 1986; Zamoyska *et al.*, 1989). Expression of CD8α' in this class I-restricted hybridoma potentiated IL-2 production to a much lesser extent than did CD8α, demonstrating that the cytoplasmic tail and/or its associated p56^{lck} are involved in enhancing class I-restricted responses when the CD8α molecule can act as a coreceptor by binding to the same MHC molecule as the TCR (Fig. 2). Expression of hybrid CD8/CD4 molecules consisting of the extracellular domains of CD8α and intracellular domains of CD4 or vice versa (i.e., $CD4_{out}/CD8_{in}$) in DC27.10 showed that the ability to augment antigen-induced IL-2 production in this hybridoma is primarily dictated by the extracellular portion of the coreceptor. Hybrid molecules containing extracellular portions of CD8α potentiated IL-2 production significantly, regardless of whether internal portions were CD8α or CD4, presumably because they could bind to the same MHC molecule as the TCR. In contrast, hybrid molecules containing external CD4 and internal CD8α or CD4 did not enhance IL-2 production (Zamoyska *et al.*, 1989; Parnes *et al.*, 1989). Similarly, Letourneur *et al.* (1990) have demonstrated that CD8α' is less efficient than CD8α at potentiating antigen-induced IL-2 production of a class I-restricted hybridoma when alloantigen is limiting. Chalupny *et al.* (1991) have extended the analysis of the dependency of class I responses on CD8α coreceptor function by assessing the ability of a panel of CD8α cytoplasmic tail mutants to potentiate TCR-mediated increases in intra-

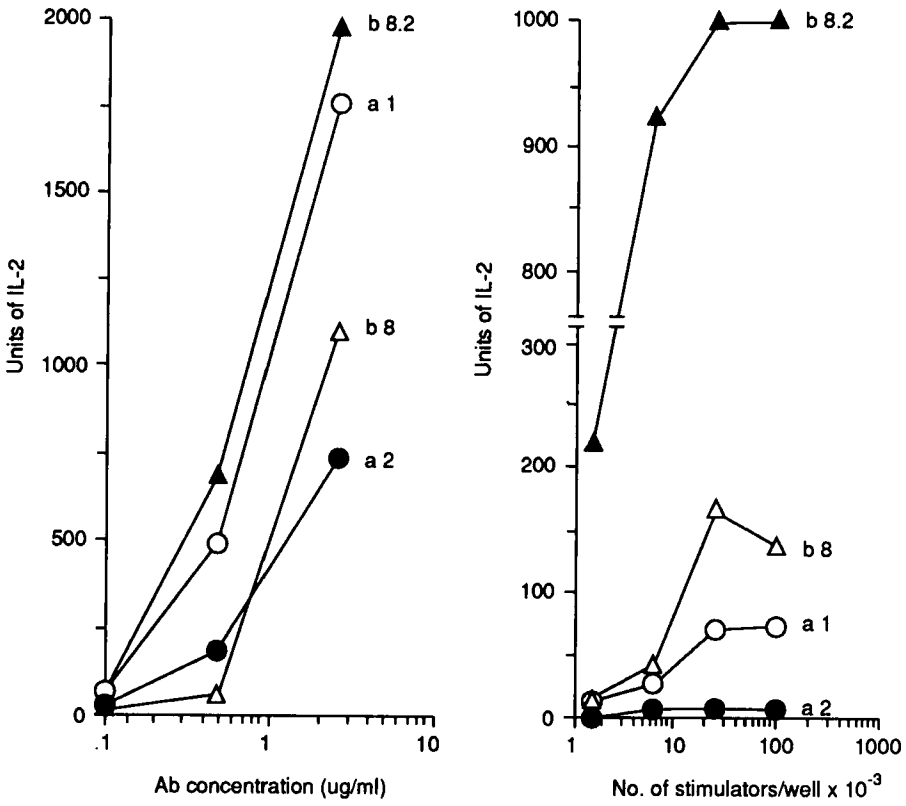


FIG. 2. Expression of CD8α enhances IL-2 production to a greater degree than expression of CD8α' in the DC27.10 hybridoma. Transfectants of the DC27.10 hybridoma expressing either CD8α homodimers (b8 and b8.2) or CD8α' (a1 and a2) were assayed for IL-2 production when stimulated with anti-TCR clonotypic mAb Desiré-1 (left panel) or with the MHC class I alloantigen for which the TCR is specific (K^b on transfected L cell stimulators) (right panel). These results demonstrate a role for the CD8α cytoplasmic tail in coreceptor function. Reprinted with permission from *Nature* (Zamoyska *et al.*). Copyright (1989) MacMillan Magazines Ltd.

cellular Ca^{2+} and protein tyrosine phosphorylation. These studies demonstrated that a functional $p56^{lck}$ binding site on the cytoplasmic tail of CD8α is required for early signaling events transduced by the CD8α cytoplasmic tail.

In order to better understand the mechanisms operating when CD4 or CD8 are expressed in a class II-restricted T cell hybridoma, we have been working with the beef insulin-specific, I-A$\alpha^b\beta^k$-restricted, $CD4^-CD8^-$ T cell hybridoma BI-141 (Reske-Kuna and Rüde, 1985). This cell has been shown to produce IL-2 at high antigen concentra-

tions in the absence of CD4 or CD8 expression (Ballhausen *et al.*, 1988). Expression of either CD4 or CD8α enhanced the ability of BI-141 to respond to beef insulin when suboptimal concentrations of antigen were used for stimulation. However, expression of CD4 potentiated antigen-induced IL-2 production to a much greater extent (10- to 30-fold) than did CD8α (Miceli *et al.*, 1991). Furthermore, expression of CD4 but not CD8α allowed for the detection of IL-2 production in response to pork insulin. We believe that the difference in the degree to which CD4 and CD8α could potentiate IL-2 production reflects the ability of CD4 but not CD8 to act as a coreceptor in this class II-restricted cell by binding to the same MHC molecule as the TCR. In support of this interpretation, truncation of the cytoplasmic tail only affected the ability of CD4 and not CD8α to facilitate antigen-induced IL-2 production. Truncated CD4, in which the cytoplasmic tail had been reduced to three amino acids, augmented IL-2 production by BI-141 only to the same extent as either CD8α or CD8α' (Miceli *et al.*, 1991) (Fig. 2). Since the CD8α cytoplasmic tail was shown to be important for full enhancement of IL-2 production in class I-restricted responses (Zamoyska *et al.*, 1989; Letourneur *et al.*, 1990; Chalupny *et al.*, 1991), these data suggest that the cytoplasmic tail and/or the associated p56^{lck} are primarily important in coreceptor function. That CD8α, CD8α', and truncated CD4 did potentiate antigen-induced IL-2 production by BI-141 to some limited extent illustrates that minor contributions can be made by coreceptor molecules through a mechanism (or mechanisms, probably including adhesion) distinct from the cytoplasmic tail and signaling through p56^{lck} (Miceli *et al.*, 1991).

A role for the cytoplasmic tail of CD4 and the associated p56^{lck} in mediating coreceptor signaling has also been demonstrated in other studies (Sleckman *et al.*, 1988; Glaichenhaus *et al.*, 1991). By expressing a panel of CD4 cytoplasmic tail mutants in a CD4-dependent, class II-restricted hybridoma, Glaichenhaus *et al.* (1991) demonstrated that the ability of CD4 to function as a coreceptor was almost entirely compromised by mutating the p56^{lck}-binding site. Interestingly, there is some suggestion that the cytoplasmic tail may also contribute to coreceptor activity through a mechanism independent of p56^{lck}, since a mutant expressing only the first four amino acids of the CD4 cytoplasmic tail weakly potentiated antigen-induced IL-2 production despite its inability to bind p56^{lck}, while CD8α did not. Such a coreceptor function was not evident in the aforementioned BI-141 system, where the truncated CD4 potentiated antigen responsiveness to the same extent as either CD8α or CD8α'. This may be the result of a higher baseline activity (i.e., in the ab-

sence of CD4 or CD8) in BI-141, not allowing for enough sensitivity to detect this minor coreceptor contribution as a difference in functional capacity between tailless CD4 and CD8α or CD8α'. In contrast, the class II-restricted T cell hybridoma used by Glaichenhaus *et al.* (1991) was entirely dependent on CD4 for IL-2 production, and CD8α could not stimulate IL-2 production. These results are akin to those described above for DC27.10, which is dependent on CD8α for IL-2 release and in which CD8α' is much less effective but CD4 does not lead to a detectable response. Collins *et al.* (1992) have recently demonstrated that an intact p56^{lck}-binding site on the CD4 cytoplasmic tail is also required for CD4/TCR association accompanying activation, as measured by fluorescence resonance energy transfer, as well as for IL-2 production. These data suggest that p56^{lck} may be directly responsible for the physical association between the TCR and CD4 that accompanies activation.

In order to assess more directly the role of p56^{lck} in TCR-mediated signaling, we, in collaboration with Veillette's group, have overexpressed wild-type, nonfunctional "kinase killed" (in which the ATP binding site K273 as well as the regulatory Y505 have been mutated) or constitutively activated (in which the regulatory Y505 has been mutated to F505) forms of p56^{lck} in the class II-restricted BI-141 T cell hybridoma discussed above (N. Abraham *et al.*, 1991). Overexpression of constitutively activated p56^{lckF505} (which cannot be phosphorylated at Y505) enhanced TCR-mediated signaling even in the absence of CD4 or CD8 expression. Yet, p56^{lck} activity *in vivo* was still controlled by TCR ligation, since increased tyrosine phosphorylation (Fig. 3) and IL-2 production (Fig. 4) were only apparent on specific TCR stimulation by mAb or antigen/MHC, respectively. While all of the same protein substrates detectable in total cell lysates were tyrosine phosphorylated in response to TCR ligation in both the parental and p56^{lckF505}-overexpressing hybridomas (see Fig. 5), there was a disproportional enhancement of some of these bands on TCR ligation in p56^{lckF505}-expressing cells. Similar enhancement of TCR-mediated tyrosine phosphorylation was seen whether F23.1 (anti-Vβ8), 2C11 (anti-CD3ϵ), or H57-597 (anti-$\alpha\beta$ TCR) was used to stimulate the TCR. Overexpression of either kinase-killed or wild-type p56^{lck} did not lead to an increase in TCR-mediated tyrosine phosphorylation or IL-2 production. These data directly demonstrated that p56^{lck} can positively contribute to TCR-mediated signaling even in the absence of CD4 or CD8 expression (N. Abraham *et al.*, 1991).

This system has been further manipulated to delineate the portions

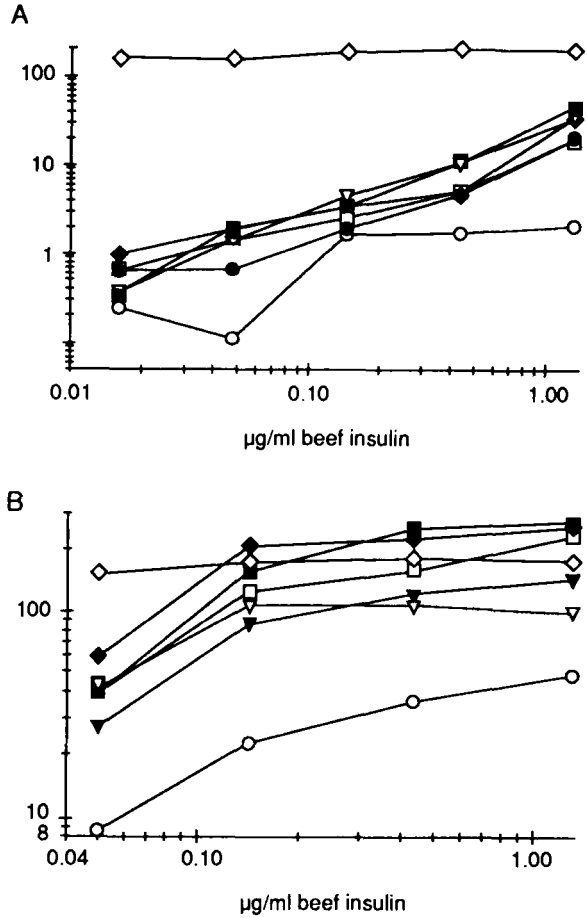


FIG. 3. Ability of tailless CD4 to stimulate IL-2 production by BI-141. A genetically engineered truncated CD4 containing only three cytoplasmic amino acids augments antigen-induced IL-2 release by BI-141 only as well as CD8α or CD8α′, indicating a coreceptor function associated with the CD4 cytoplasmic tail and/or the associated $p56^{lck}$ in this class II-restricted hybridoma. The contribution of the cytoplasmic tail of CD4 was assessed by comparing the abilities of transfectants expressing a truncated CD4 (CD4cyt$^-$), truncated CD8 (CD8α′), full-length CD8α, or full-length CD4 to release IL-2 in response to antigen (beef insulin)-pulsed antigen-presenting cells. A and B represent two independent experiments. The following transfectants were used: (A) BI-141, ○; mCD4.C5, ◇; mCD8α′.C3, □; mCD8α.B3, ▽; mCD4cyt−.2B4, ●; mCD4cyt−.25B4, ◆; mCD4cyt−.25D1, ■; (B) BI-141, ○; mCD4.5b, ◇; mCD8α.B3.a, ◁; mCD8α.B3.b, □; mCD4cyt−.25B1, ◆; mCD4cyt−.2B4, ■; mCD4cyt−.25B4, ◆. IL-2 was measured as counts per minute of [^{3}H]thymidine incorporated by the IL-2-dependent HT-2 cell line (Miceli *et al.*, 1991).

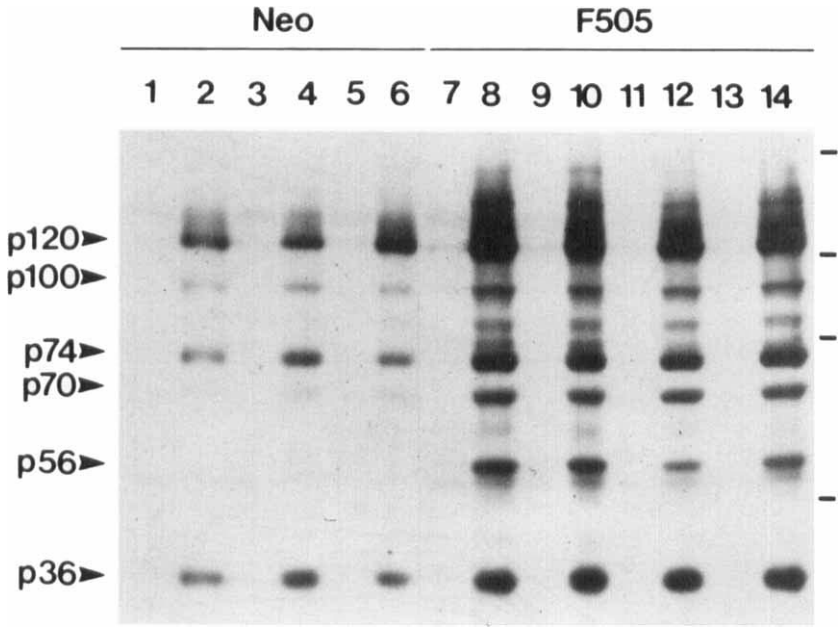


FIG. 4. Expression of p56^{lck505} in BI-141 enhances TCR/CD3-induced tyrosine protein phosphorylation. Crosslinking on BI-141 cells transfected with p56^{lck505} (lanes 7–14) or with the neomycin resistance vector alone (lanes 1–6) was performed with mAb 2C11 directed against CD3ε and rabbit antihamster (RAH) IgG for 1 min at 37°C (lanes 2, 4, 6, 8, 10, 12, and 14) or with RAH IgG alone (lanes 1, 3, 5, 7, 9, 11, and 13). Total cell lysates were immunoblotted and probed with antiphosphotyrosine antiserum and subsequently with ^{125}I-labeled protein A. Lanes 1 and 2, Neo.1; lanes 3 and 4, Neo.2; lanes 5 and 6, Neo.3; lanes 7 and 8, F505.7; lanes 9 and 10, F505.9; lanes 11 and 12, F505.10; lanes 13 and 14, F505.12. Reprinted with permission from *Nature* (N. Abraham *et al.*). Copyright (1991) MacMillan Magazines Ltd.

of p56$^{lck\text{F}505}$ that are required for TCR-mediated potentiation of protein tyrosine phosphorylation and antigen-induced IL-2 production. When overexpressed in BI-141 cells, a form of constitutively activated p56$^{lck\text{F}505}$ in which the entire SH2 domain was deleted was shown to be dramatically compromised in its ability to stimulate anti-TCR-mediated tyrosine phosphorylation of proteins, including PLC$_\gamma$1, and antigen-induced IL-2 production (Caron *et al.*, 1992). Similarly, mutation of either G2 or the Y394 autophosphorylation site hampered the ability of p56$^{lck\text{F}505}$ to stimulate both tyrosine phosphorylation and IL-2 production. Interestingly, deletion of the SH3 domain did not affect the ability of p56$^{lck\text{F}505}$ to contribute to early

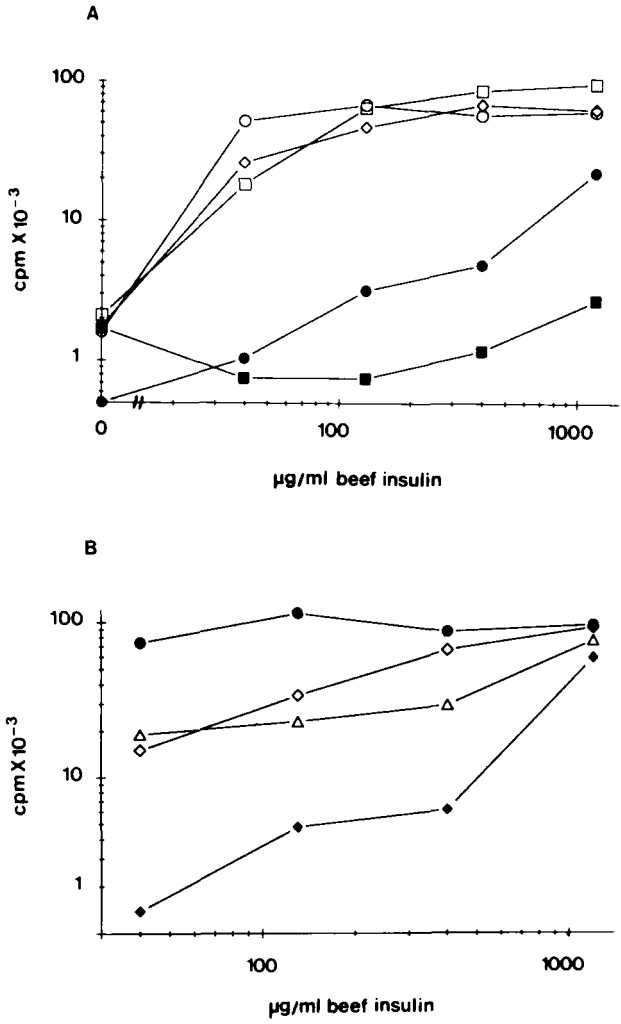


FIG. 5. Expression of $p56^{lck505}$ in BI-141 enhances antigen-induced IL-2 production. IL-2 production in response to various concentrations of beef insulin in association with I-Aα^b Aβ^k (class II MHC) was tested in $p56^{lck505}$-expressing BI-141 transfectants (F505), transfectants expressing the neomycin resistance vector alone (neo), a CD4 transfectant (CD4.C5), or untransfected BI-141. IL-2 was measured as counts per minute of [^{3}H]thymidine incorporation by the IL-2-dependent HT-2 cell line. A and B represent separate experiments. The following transfectants were used: (A) Neo.1, ■; Neo.2, ●; F505.3, □; F505.7, ○; F505.9, ◇; spontaneous IL-2 production is shown at the zero point on the abscissa; (B) BI-141, ◆; F505.9, ◇; F505.12, △; CD4.C5, ●. Reprinted with permission from *Nature* (N. Abraham *et al.*). Copyright (1991) MacMillan Magazines Ltd.

tyrosine phoshorylation events detectable by antiphosphotyrosine Western blotting of total cell lysates. However, deletion of the SH3 domain did compromise the ability of $p56^{lckF505}$ to potentiate antigen-induced IL-2 production (Caron *et al.*, 1992). Since SH3 domains have previously been demonstrated to play a role in cytoskeletal interactions (Koch *et al.*, 1991), it is possible that the inability to produce IL-2 in response to antigen reflects a deficiency in cytoskeletal association necessary for translating tyrosine phosphorylation into IL-2 production. Alternatively, tyrosine phosphorylation of a key substrate not abundant enough to be detected by antiphosphotyrosine Western blots of total cell lysates is affected by the absence of the SH3 domain.

H. Additional Evidence Substantiating a Role for $p56^{lck}$ in T Cell Activation

Thymocyte development was shown to be severely defective in mice overexpressing a mutant transgenic $p56^{lckF505}$. Furthermore, these mice developed thymic tumors (K. M. Abraham *et al.*, 1991a,b). These effects were dose dependent, becoming more severe with increasing transgene dosage. Interestingly, overexpression of wild-type $p56^{lck}$ by transgenic techniques similarly led to defective thymocyte maturation and could also lead to the emergence of tumors. However, the phenotype observed in animals overexpressing wild-type $p56^{lck}$ approximated that seen with sevenfold lower levels of $p56^{lckF505}$. These results further exemplify the relatively increased *in vivo* kinase activity of $p56^{lckF505}$ over wild-type $p56^{lck}$. However, unlike the studies mentioned above (see Section II,G), where overexpression of wild-type $p56^{lck}$ in a $CD4^-CD8^-$ T hybridoma had no apparent effect (N. Abraham *et al.*, 1991), as little as a twofold increase in $p56^{lck}$ levels substantially disrupted the appearance of functional thymocytes. This difference could be a reflection of differential sensitivity to $p56^{lck}$ kinase activity at various stages of differentiation. Indeed, characterization of thymic tumors developing from these mice revealed that they did not express CD3, and in those tumors developing in mice expressing the $p56^{lckF505}$ transgene neither CD4 nor CD8 was expressed (K. M. Abraham *et al.*, 1991a,b). These data may suggest that $p56^{lck}$ plays a role in T lymphocyte development independent of CD4, CD8, or TCR signaling.

Analysis of a mutant Jurkat T cell line, JCaM1, identified by its inability to show induction of inositol phosphates and intracellular Ca^{2+} following stimulation of the TCR, has further demonstrated that $p56^{lck}$ plays a major role in TCR-mediated activation. JCaM1 has re-

cently been shown to be defective in functional $p56^{lck}$ expression and TCR-mediated tyrosine phosphorylation. Transfection of *lck* cDNA into JCaM1 led to the expression of functional $p56^{lck}$ and to rescue of TCR-mediated signaling. JCaM1 does not express CD8 and expresses only very low levels of CD4 (Straus and Weiss, 1992). It is therefore possible that $p56^{lck}$ is acting to rescue T cell signaling by contributing to TCR-mediated signaling independently of CD4 or CD8. This would not be surprising, since, as mentioned above, it has been previously demonstrated that a constitutively activated form of $p56^{lck}$ ($p56^{lck505}$) can affect TCR-mediated signaling in the absence of CD4 or CD8 expression (N. Abraham *et al.*, 1991). Until expression of a mutant $p56^{lck}$ that lacks a functional CD4 interaction site is shown to be capable of rescuing the JCaM1 signaling defect, it will remain unclear whether the low levels of CD4 expressed by JCaM1 are participating in TCR-mediated signaling after $p56^{lck}$ transfection.

While $p56^{lck}$ may normally augment TCR-mediated signals, the phenotype of mice deficient in $p56^{lck}$ gene expression demonstrated that $p56^{lck}$ is not absolutely required for the TCR to mediate a signal (Molina *et al.*, 1992). These mice demonstrated a profound role for $p56^{lck}$ in development, since most T cells never matured or exited the thymus (the role in development will be discussed in depth later (see Section III,E). However, a very few T cells were present in the periphery. This minor subset of cells that apparently did not go through conventional thymic maturation and education could still proliferate (albeit at reduced levels) in response anti-TCR$\alpha\beta$, anti-CD3ϵ, or mitogens. These results should not be misinterpreted to suggest that $p56^{lck}$ does not normally play a role in TCR-mediated signaling. Rather, they may demonstrate the adaptability of some cells to the lack of $p56^{lck}$ and may point to some redundancy within *src* family members or other signaling proteins.

Recent studies by Haughn *et al.* (1992) substantiate the hypothesis that the phenomenon of "negative signaling" by CD4 (and, by inference, CD8) reflects interference with molecular associations normally associated with TCR signaling, and that $p56^{lck}$ plays an up-regulatory role in T cell activation. This group investigated TCR-mediated activation of $CD4^+$ and $CD4^-$ variants of a class II-specific T cell clone. In this clone CD4 does not provide a prerequisite signal, as both the $CD4^+$ and $CD4^-$ variants responded equally well to a broad range of antigen concentrations presented by APCs. Similar independence of CD4 function has been previously reported and is generally associated with clones bearing high-affinity TCRs (reviewed in Parnes, 1989). Despite its apparent lack of a require-

ment for CD4 to function, mAbs against CD4 blocked TCR-mediated activation of the $CD4^+$ clone by antigen presented by antigen-presenting cells expressing the appropriate class II molecule. Furthermore, when either plate-bound H57.597 (anti-TCR$\alpha\beta$) or an anti-Vβ4 mAb was used to stimulate the variants, the clone expressing CD4 proliferated less well than the one not expressing CD4, even in the absence of anti-CD4 mAbs (Haughn *et al.*, 1992). This implies that expression of CD4 resulted in inefficient TCR-mediated signaling when these anti-TCR mAbs were used for stimulation. When CD4 was expressed in the $CD4^-$ variant, its ability to respond to either anti-Vβ4 or H57.597 was similarly diminished. An intact $p56^{lck}$ binding site on the CD4 cytoplasmic tail was required for inhibition of TCR-mediated signaling, since a mutant CD4 lacking the crucial cysteines required for CD4/$p56^{lck}$ interaction did not affect signaling in response to anti-Vβ4 or H57.597 when expressed in the $CD4^-$ variant. However, signaling in response to anti-CD3ϵ antibody 2C11 was unaffected by CD4 expression. As predicted by the coreceptor hypothesis, when CD4 and the TCR were artificially crosslinked with secondary antibody, a synergy between CD4 and the TCR resulted in the $CD4^+$ variant signaling better than either variant did when stimulated with anti-Vβ4 alone (Haughn *et al.*, 1992).

The interpretation of these results by the authors relies on the supposition that $p56^{lck}$ is involved in TCR-mediated signaling regardless of whether or not CD4 is present. This is not an unreasonable suggestion, since experiments mentioned above by N. Abraham *et al.* (1991) and Straus and Weiss (1992) have implicated $p56^{lck}$ in the augmentation of TCR-mediated signals in the absence of CD4 or CD8 expression. Thus, when the TCR is stimulated by anti-Vβ4 or H57.597, CD4 is not included in the TCR complex and sequesters $p56^{lck}$, keeping it from participating in and augmenting TCR-mediated signaling. This is in keeping with Janeway's suggestion that stimulating with anti-Vβ antibodies does not induce coassociation between CD4 and the TCR (Rojo *et al.*, 1989; Janeway, 1992). On the other hand, when CD4 and the TCR are colocalized deliberately by crosslinking antibodies, CD4-associated $p56^{lck}$ is efficiently localized to the TCR complex, where it can participate in signaling (Haughn *et al.*, 1992). Similarly, "negative signaling" by mAb-induced aggregation of CD4 can be attributed to $p56^{lck}$ sequestration. These results are consistent with the notion that $p56^{lck}$ is required to couple functionally the $\alpha\beta$ TCR to signal transduction. Furthermore, they may imply that $p56^{lck}$ is not involved in regulating signals by CD3ϵ, or that its involvement is under different functional or physi-

cal constraints, since stimulation by 2C11 is unaffected by CD4 expression. The latter suggestion may be more reasonable considering that anti-CD3ϵ signaling is enhanced by overexpression of constitutively activated $p56^{lck}$ (N. Abraham *et al.*, 1991), as mentioned above (see Section II,G).

This interpretation may also help explain earlier results described by this same group, and may have interesting implications regarding how $p56^{lck}$ influences TCR-mediated signals. Newell *et al.* (1990) demonstrated that mAb H57.597, directed against TCR$\alpha\beta$, stimulated DNA synthesis by $CD4^+$ splenic T cells. Crosslinking of CD4 prior to stimulation with H57.597 inhibited the TCR-mediated induction of DNA synthesis. More interestingly, pretreatment by crosslinking CD4 with specific mAb and secondary antibody increased a TCR-mediated signal for induction of programmed cell death (apoptosis) as measured by DNA fragmentation. Up to 10 times the level of DNA fragmentation was observed when CD4 was crosslinked prior to stimulation of the $\alpha\beta$ TCR relative to when the $\alpha\beta$ TCR and CD4 were stimulated simultaneously. 2C11 mAb directed against CD3ϵ also induced DNA synthesis in this $CD4^+$ population, though it was resistant to pretreatment by CD4 crosslinking, and no increase in TCR-mediated apoptsosis was observed (Newell *et al.*, 1990). The requirement for CD4 crosslinking with a secondary antibody may reflect a requirement for efficient activation of the associated $p56^{lck}$. Alternatively, it may imply that this phenomenon results from sequestration of $p56^{lck}$ (and/or CD4) from participating in TCR-mediated signaling. The latter seems more likely given the requirement for pretreatment with anti-CD4, as $p56^{lck}$ would also be activated (albeit possibly differently) when CD4 and the TCR are stimulated simultaneously. If this is the correct interpretation, these data may suggest that inclusion of CD4 and/or $p56^{lck}$ may contribute to TCR-mediated signals that lead to proliferation, whereas exclusion allows for the generation of a more efficient apoptosis signal. It seems unlikely that CD4 interaction with class II *in vivo* would result in a similar phenomenon in peripheral T cells, as it would then be difficult to explain why most T cells are not primed for death rather than immune response. After all, it seems quite likely that any given T cell might encounter a class II molecule loaded with a peptide that does not trigger its TCR prior to encountering one that does. This mechanism may have physiological significance, however, in the thymus, where there has been some suggestion (discussed in Section II,H) that signaling through CD4 binding in the absence of TCR costimulation may have relevant effects (Nakayama *et al.*, 1990a,b). This is particu-

larly intriguing, as programmed cell death may be the desired outcome of TCR engagement at particular stages of thymic development.

I. An Additional Signaling Role for CD4 in Thymocytes

Most immature $CD4^+CD8^+$ thymocytes express a low level of TCR on their surface and are only marginally capable of signaling in response to TCR stimulation. Nakayama *et al.* (1990a,b) have suggested that CD4-dependent interactions are responsible for maintaining low TCR levels and ζ chain tyrosine phosphorylation on these cells, which therefore have minimal signaling capacity. Specifically, they have reported that TCR ζ chains from $CD4^+CD8^+$ thymocytes are constitutively phosphorylated *in vivo*, that dephosphorylation of ζ occurred on removal of $CD4^+CD8^+$ thymocytes from the thymic microenvironment by culturing at 37°C, and that rephosphorylation of the ζ chain was induced *in vitro* by stimulation with mAbs against CD4, CD8, or the $\alpha\beta$ TCR (i.e., with mAb H57.597) (Nakayama *et al.*, 1990b). Similarly, low TCR expression was demonstrated in intact thymus and was induced to high levels on the removal of thymocytes from the thymic microenvironment and culture at 37°C (Nakayama *et al.*, 1990a,b). Furthermore, $CD4^+CD8^+$ cells that had been induced to higher TCR levels demonstrated an increased ability to generate a Ca^{2+} flux in response to mAb specific for the $\alpha\beta$ TCR (H57.597). Crosslinking with anti-CD4, but not anti-CD8, specifically inhibited the induction of higher levels of TCR (Nakayama *et al.*, 1990a). These data are consistent with studies reporting that the *in vivo* administration of mAb specific for CD4, but not CD8, resulted in a polyclonal increase in the level of TCR expression on $CD4^+CD8^+$ thymocytes (McCarthy *et al.*, 1988). This presumably results from anti-CD4 mAb interfering with the thymic CD4/ligand interaction responsible for maintaining low TCR expression. However, since *in vivo* administration of mAbs specific for class-II MHC proteins did not result in increased TCR levels (McCarthy *et al.*, 1988), the ligand interacting with CD4 in the thymus appears not to be class II MHC. The authors suggest that a novel ligand for CD4 is present in the thymus and is responsible for interacting with CD4 to send the signal necessary for ζ phosphorylation and maintenance of low $\alpha\beta$ TCR expression. No ligand for CD4 other than class II has yet been found in the thymus; however, it is possible that interaction with such a ligand may "prime" T cells for apoptosis, as discussed above (see Section II,H).

J. Coreceptor Molecules May Modulate Increased Adhesiveness That Accompanies TCR Triggering

Recently, O'Rourke *et al.* (1990) and O'Rourke and Mescher (1992) have shown that CD8 binding to class I MHC is increased on activation of the TCR with fluid-phase anti-TCR mAb. Stimulation of cytotoxic T lymphocytes (CTLs) with soluble anti-TCR mAb F23.1 (directed against the Vβ element used by this cell) increased the ability of these cells to subsequently bind to immobilized class I molecules in a CD8-dependent manner. Such an interaction may have a role in stabilizing T cell interactions with target or antigen-presenting cells after specific TCR/antigen/MHC recognition. Furthermore, this increased binding was also demonstrated to contribute to signaling required for the release of cytotoxic granules associated with T cell function. While fluid-phase anti-TCR mAb F23.1 did not stimulate poly(phosphoinositide) hydrolysis, Ca^{2+} flux, or cytotoxic granule release, subsequent exposure of CTLs that had been "primed" with soluble F23.1 to immobilized class I molecules stimulated the CTLs to signal in these ways. There was no requirement for a particular allotype of class I MHC, implying that in this system there was no requirement that CD8 and the TCR bind to the same MHC molecule (Goldstein and Mescher, 1988; O'Rourke *et al.*, 1990). However, as this system required priming with soluble anti-TCR mAb followed by interaction with plate-bound class I molecules, it is possible that some TCR complexes were trapped at the site of CD8 crosslinking and were in this way colocalized with CD8 and its associated $p56^{lck}$. Under normal physiological situations this would occur when CD8 and the TCR bind to the same MHC molecule. TCR-mediated activation of CD8 adhesiveness was shown to be dependent on a tyrosine kinase pathway, as it could be blocked by the tyrosine kinase inhibitors herbimycin A and genistein (O'Rourke and Mescher, 1992). No similar up-regulation of CD4 binding to class II MHC in response to TCR triggering has been reported as yet.

Mazerolles *et al.* (1990, 1991) have demonstrated that CD4/MHC class II interaction may play a role in downregulating the low-affinity binding of resting $CD4^+$ T cells to B cells when the TCR is not engaged during the interaction. While $CD4^+$ cells showed similar maximal binding to class II-bearing B cells and to variant B cells lacking class II expression, disassociation from the class II-positive cells occurred significantly more rapidly (Mazerolles *et al.*, 1990, 1991). It has been suggested that such a mechanism may be operational in order to avoid chance interactions, which could result in the

breakdown of specificity of the immune response. Additional studies by this same group have suggested that CD4/MHC interactions also affect the high-affinity binding of $CD4^+$ T cells to class II MHC-bearing cells induced by anti-CD3 crosslinking (Mazerolles et al., 1991). They and others have demonstrated that the increased adhesivness of a T cell for target cells that occurs on TCR-mediated triggering is primarily due to the increased adhesiveness of LFA-1 for its ligand, ICAM-1. Furthermore, their data suggests that interaction of CD4 with class II MHC without simultaneous TCR triggering may regulate adhesion by influencing LFA-1-mediated adhesion (Mazerolles et al., 1991).

K. Role for CD8β in T Cell Recognition and Activation

Most of the studies aimed at dissecting CD8 function have focused primarily on CD8α in the homodimeric form. As described above, CD8α homodimers have been demonstrated to be functionally competent to restore or augment TCR-mediated responses such as IL-2 production when expressed in cells lacking CD8 (Dembić *et al.*, 1987; Gabert *et al.*, 1987; Zamoyska *et al.*, 1989; Letourneur *et al.*, 1990; Chalupny *et al.*, 1991). On most mouse thymocytes and T cells CD8 exists primarily as $\alpha\beta$ heterodimers (Ledbetter *et al.*, 1981; Walker *et al.*, 1984), although some $\alpha\alpha$ homodimers have been found on human $\alpha\beta$ TCR T cells (Terry *et al.*, 1990; Moebius *et al.*, 1991). Until recently very little was known regarding the function of the CD8β chain, primarily because CD8β requires CD8α for cell surface expression (Gorman *et al.*, 1988).

The most convincing data indicating an immunological role for CD8β comes from experiments done in our laboratory by Wheeler *et al.* (1992) By directly comparing class I alloantigen-induced IL-2 production by T cell hybridoma transfectants expressing either the CD8α chain alone or both the CD8α and β chains, we have demonstrated that CD8$\alpha\beta$ heterodimers are more functionally competent than CD8α homodimers. In these experiments we used an H-$2K^b$-specific, $CD4^-CD8^-$ mouse T cell hybridoma, HTB-157.7, which produces IL-2 in response to L cells stably transfected with H-$2K^b$, but not in response to untransfected L cells or L cells expressing H-2^{bm10} (others have reported a minimal response to H-2^{bm10}). HTB-157.7 transfectants expressing either CD8α homodimers or CD8 $\alpha\beta$ heterodimers demonstrated increased IL-2 production in response to H-$2K^b$ as well as a novel response to H-2^{bm10}. However, CD8$\alpha\beta$-bearing cells were significantly more potent than CD8α-bearing cells at producing IL-2 when limiting numbers of H-$2K^b$-transfected L

cells were included as antigen-presenting cells and even at higher densities of H-2^{bm10}-transfected L cells. Furthermore, a hybrid molecule consisting of the extracellular portions of CD8β and the transmembrane and cytoplasmic portions of CD8α was stably expressed on the surface of HTB.157 and demonstrated to be capable of augmenting antigen-specific IL-2 production (Wheeler *et al.*, 1992). These results suggest that the extracellular portion of CD8β can act independently of CD8α extracellular portions to potentiate T cell responses. In these cells CD8β may contribute to responses by directly binding to class I molecules, by contributing an increased adhesiveness to CD8, or by allowing for better coupling of CD8 with the TCR in coreceptor function. Recent data by Karaki *et al.* (1992) using the H-$2K^b$ allospecific mouse T cell hybridoma HTB176.10 similarly demonstrated that the expression of CD8$\alpha\beta$ could potentiate IL-2 production in response to mutated H-$2K^b$ molecules (K^bK^bB7 containing a human class I MHC α3 domain) above the level produced by CD8α homodimer-expressing clones. Furthermore, a CD8$\alpha\beta$ expressing clone responded to the mouse H-$2K^b$ molecule mutated at position 224 (to contain the human-type amino acid Q) within the α3 domain, whereas the parental CD8α-expressing clone did not. These data may imply that the CD8β chain acts, at least in part, by influencing the ability of the CD8$\alpha\beta$ heterodimer either directly or indirectly to bind portions of the α3 domain or that CD8β has an independent binding site. A slightly different α chain structure in CD8$\alpha\alpha$ homodimers and CD8$\alpha\beta$ heterodimers has been suggested by biochemical (Kirszbaum *et al.*, 1989) and crystallographic studies (Leahy *et al.*, 1992), demonstrating the presence in the immunoglobulin-like domain of one intrachain disulfide bridge in $\alpha\alpha$ homodimers and another in $\alpha\beta$ heterodimers. The disulfide loop in the homodimers is analogous to the disulfide loop in immunoglobulin variable domains, while that in the heterodimers is unique. How this structural difference relates to function remains unknown.

L. TCR-Mediated Signaling and Possible Contributions of CD4, CD8, and p56^{lck}

The TCR consists of at least seven polypeptide chains (α, β, CD3, γ, δ, ϵ, and ζ, or sometimes ζ and η) (reviewed in Frank *et al.*, 1990). It is well documented that TCR triggering results in rapid tyrosine phosphorylation of a number of proteins within seconds to minutes of receptor ligation (Hsi *et al.*, 1989; June *et al.*, 1990a), and this phosphorylation appears to be crucial, as its inhibition prevents T cell activation (June *et al.*, 1990b). Stimulation of the TCR is also known

to result in the activation of $PLC_\gamma1$ (Park *et al.*, 1991; Weiss *et al.*, 1991), which in turn leads to the accumulation of inositol phosphates and diacylglycerol, which are responsible for the release of intracellular Ca^{2+} stores and activation of protein kinase C, respectively (Berridge, 1984). Activation of $PLC_\gamma1$ activity is probably due to TCR-induced tyrosine phosphorylation of $PLC_\gamma1$ (Ullrich and Schlessinger, 1990). It is likely that $p56^{lck}$ either directly or indirectly contributes to the tyrosine phosphorylation of $PLC_\gamma1$, since both CD4 crosslinking to the TCR and overexpression of constitutively activated $p56^{lck}$ have been reported to upregulate the degree of tyrosine phosphorylation of $PLC_\gamma1$ that accompanies TCR activation (Caron *et al.*, 1992; Kanner *et al.*, 1992). Furthermore, Weber *et al.* (1992) have recently reported the induction of an association between $PLC_\gamma1$, $p56^{lck}$, and tyrosine-phosphorylated proteins of 36, 38, 58, and 63 kDa with stimulation of the TCR on Jurkat cells. An identical pattern of proteins was precipitated from TCR-activated Jurkat cells using TrpE fusion proteins containing SH2 domains from $PLC_\gamma1$, indicating that the SH2 domain of $PLC_\gamma1$ is responsible for the association (Weber *et al.*, 1992).

How TCR ligation is translated into tyrosine kinase activity and which tyrosine kinases are involved are still not well understood. Recent data suggest that the TCR may couple to signal transduction in more than one way (Irving and Weiss, 1991; Letourneur and Klausner, 1992; Wegener *et al.*, 1992). A number of groups have demonstrated that the cytoplasmic tail of ζ in isolation from other TCR/CD3 components (i.e., grafted onto CD8α or IL-2 receptor transmembrane and extracellular domains) (Irving and Weiss, 1991; Letourneur and Klausner, 1992) can couple to tyrosine kinase activity and lead to IL-2 production after mAb crosslinking of the external domains. Similarly, both the cytoplasmic portion CD3ϵ grafted onto transmembrane and extracellular portions of the IL-2 receptor (Letourneur and Klausner, 1992) and a deficient TCR/CD3 complex consisting of only functional α, β, γ, ϵ, and δ (Wegener *et al.*, 1992) chains have also been shown capable of signaling tyrosine phosphorylation and IL-2 production on stimulation with appropriate mAbs (anti-IL2 receptor and anti-CD3ϵ, respectively). Receptor coupling presumably is achieved through functional domains that contain a consensus sequence [YXXL$(X)_7$YXXL] that is common to the cytoplasmic portions of many of the polypeptides associated with signal transduction in the immune system (e.g., antigen and Fc receptors). Sequences including this putative consensus protein tyrosine kinase binding motif are contained in the ζ, ϵ, γ, and δ chains of the TCR/CD3 complex

(Reth, 1989). The signals generated through isolated CD3ε and ζ subunits are somewhat redundant, as they both signal IL-2 production and induce tyrosine phosphorylation of proteins with relative molecular masses similar to those phosphorylated on intact TCR signaling (Irving and Weiss, 1991; Letourneur and Klausner, 1992; Wegener *et al.*, 1992). However, there are some differences between the signal generated through ζ and that through γ, ε, and δ, in that the relative degree of tyrosine phosphorylation of various substrates differs. Furthermore, the γ, ε, and δ module appears to be incapable of substituting for the ζ cytoplasmic tail to sustain activation through Thy-1, Ly-6, or CD2 (Wegener *et al.*, 1992).

Currently, there are three candidate tyrosine kinases that may participate in coupling with the TCR, including ZAP-70, $p59^{fyn}$, and $p56^{lck}$. TCR ligation with mAb to the TCRβ chain has been reported to induce the association of the ζ chain with ZAP-70, a 70-kDa phosphoprotein. It has been suggested that ZAP-70 may be the primary tyrosine kinase that associates with ζ, since tyrosine kinase activity has been demonstrated to associate with immunoprecipitates of the ζ chain (Chan *et al.*, 1991). In keeping with this idea, Wange *et al.* (1992) have recently demonstrated that CD3 components and ζ associate with tyrosine kinase activity and a 70-kDa phosphoprotein (ZAP-70) on TCR stimulation with mAb directed against either TCRβ chain or CD3ε. Furthermore, this 70-kDa tyrosine phosphoprotein was demonstrated to be capable of binding to a photoaffinity analog of ATP (Wange *et al.*, 1992).

The recent cloning and expression of the cDNA encoding ZAP-70 has clearly demonstrated that ZAP-70 is indeed a protein tyrosine kinase (Chan *et al.*, 1992). While it bears hallmark features of a tyrosine kinase within sequences encoding the putative catalytic domain and encodes what appears to be two SH2 domains, ZAP-70 is not a member of the *src* family of tyrosine kinases. It does not have an N-terminal myrisylation site or a SH3 domain, nor is it regulated by phosphorylation of a C-terminal tyrosine. In studies where ZAP-70 and $p59^{fyn}$ or $p56^{lck}$ were co-expressed in COS cells stably expressing chimeric CD8-ζ molecules (in which the extracellular portion of CD8α has been grafted onto the cytoplasmic portion of the ζ chain), the ability of ZAP-70 to associate with the ζ chain cytoplasmic tail was demonstrated to be largely dependent on $p59^{fyn}$ or $p56^{lck}$ expression. Furthermore, ZAP-70 was demonstrated to only associate with homodimers of ζ that were themselves tyrosine phosphorylated (possibly by $p59^{fyn}$ or $p56^{lck}$). Finally, tyrosine phosphorylation of cellular substrates was also dramatically increased in cells co-

expressing $p59^{fyn}$ or $p56^{lck}$ and ZAP-70. However, in these COS cells neither expression or triggering of the ζ cytoplasmic tail was necessary for this increased activity (Chan *et al.*, 1992). These data suggest that $p59^{fyn}$, $p56^{lck}$, and ZAP-70 may interact to couple PTKs to the TCR and upregulate the TCR-associated tyrosine kinase activity known to accompany TCR triggering.

The intracellular tyrosine kinase $p59^{fyn}$ has also been demonstrated to coprecipitate with TCR components (Samuelson *et al.*, 1990) and to cocap with the TCR (Gassmann *et al.*, 1992), though it has been difficult to demonstrate either an increase in $p59^{fyn}$ tyrosine kinase activity or in the degree of $p59^{fyn}$ association accompanying TCR ligation. However, analysis of mutant mice lacking $p59^{fyn}$ (Appleby *et al.*, 1992; Stein *et al.*, 1992) or overexpressing a $p59^{fyn}$ (wild-type or mutant) (Cooke *et al.*, 1991) transgene have demonstrated that $p59^{fyn}$ plays a role in TCR-mediated signaling. Thymocytes from mice defective in $p59^{fyn}$ expression (as a result of gene targeting) demonstrated impaired TCR triggering. On the other hand, peripheral cells appeared to be relatively unaffected. This may indicate that $p59^{fyn}$ contributes to the TCR-mediated tyrosine kinase signal only in thymocytes, whereas another tyrosine kinase may serve this purpose in the periphery (Appleby *et al.*, 1992; Stein *et al.*, 1992).

Finally, $p56^{lck}$ might couple the TCR to tyrosine kinase activity. Indeed, as has been discussed previously (see Section II,E), CD4 and its associated $p56^{lck}$ have been coimmunoprecipitated with the TCR (Gallagher *et al.*, 1989; Beyers *et al.*, 1992; Burgess *et al.*, 1991). The association of CD4 and the TCR has been demonstrated to increase on TCR ligation (Rojo *et al.*, 1989; Janeway, 1992), and some data suggest that $p56^{lck}$ may be necessary for this association (Collins *et al.*, 1992). Constitutively activated mutant $p56^{lck}$, when expressed in an *in vitro* cell line, can contribute to TCR-mediated tyrosine phosphorylation and IL-2 production (N. Abraham *et al.*, 1991). Furthermore, Jurkat cells lacking $p56^{lck}$ are deficient in TCR-mediated tyrosine kinase activity on TCR stimulation (Straus and Weiss, 1992). Finally, a $CD45^{-}$ mutant of the HPB-ALL leukemic T cell line, which was demonstrated to be incapable of tyrosine phosphorylation or Ca^{2+} flux in response to anti-CD3 crosslinking, was activated to phosphorylate $PLC_{\gamma}1$ and $p56^{lck}$ on tyrosine and to flux Ca^{2+} when CD4 and CD3 were artificially crosslinked by biotinylated mAbs and avidin (Deans *et al.*, 1992).

Several pieces of data suggest that $p56^{lck}$ may not act by associating directly with the TCR, but may rather regulate the activity of another TCR-associated tyrosine kinase (e.g., $p59^{fyn}$ or ZAP-70). First, direct

analysis of ζ immunoprecipitates by blotting with anti-p56^{lck} antibodies has failed to reveal an association with p56^{lck}, though a tyrosine kinase activity is clearly present. This ζ-associated *in vitro* tyrosine kinase activity was demonstrated to be greatly reduced in the JCaM1 p56^{lck}-deficient mutant T cell tumor line, and its activity was rescued by transfection and expression of p56^{lck} in this cell. Similarly, cocapping experiments have failed to reveal an association between the TCR and p56^{lck} (Gassmann *et al.*, 1992). As mentioned before, the TCR may be capable of coupling to tyrosine kinase activity in more than one way and may rely on different couplings to different extents at various stages of differentiation and under different circumstances. This feature will undoubtably contribute to difficulty in defining precisely how TCR ligation results in tyrosine kinase activity and which tyrosine kinases are involved.

Finally, the hematopoietic cell-specific, transmembrane tyrosine phosphatase CD45 may affect tyrosine kinase signaling via the TCR (reviewed in Thomas, 1989; Janeway, 1992). Generation of CD45$^-$ mouse and human T cells has demonstrated that expression of CD45 is crucial for TCR-mediated activation (Hyman and Trowbridge, 1981; Hyman *et al.*, 1982; Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1992). Although its role in this process is still not entirely understood, there are data to suggest that CD45 may sometimes function by interacting with CD4 or CD8 and affecting the associated p56^{lck} tyrosine kinase activity. Indeed, experiments in which CD4 or CD8 were artificially crosslinked to CD45 demonstrated that coclustering of CD45 and coreceptor molecules could affect the phosphorylation state and enzymatic activity of the associated p56^{lck} (Ostergaard and Trowbridge 1990, 1991). Furthermore, in three sets of well-characterized pairs of parental CD45$^+$ and CD45$^-$ mutant cell lines it has been demonstrated that phosphorylation of p56^{lck} on tyrosine 505 is inversely correlated with CD45 expression (Ostergaard *et al.*, 1989). Similarly, purified CD45 has been demonstrated to dephosphorylate p56^{lck} at tyrosine 505 *in vivo* (Mustelin and Altman, 1990). As mentioned previously, Deans *et al.* (1992) recently demonstrated that a forced interaction between CD4 and CD3 could compensate for the defect in CD3/TCR coupling to tyrosine kinase activity and the PLC$_\gamma$1 signaling cascade in mutant CD45$^-$ HPB-ALL cells.

CD45 occurs in many isoforms, which are the result of alternative splicing of the CD45 transcript to include any combination of 0, 1, 2, or 3 of the exons A, B, and C that encode portions of the external domain of the CD45 cell surface protein (reviewed in Thomas, 1989;

Janeway, 1992). All isoforms have the same internal domain known to contain tyrosine phosphatase activity (Ostergaard *et al.*, 1989). Since inclusion or exclusion of exons A, B, or C can significantly change the structure of the external domain of the CD45 protein, it has been suggested that each isoform might associate with different cell surface molecules within the plane of the T cell membrane or bind to different ligands on the surface of other cells. Recent data indicate that, at least to some degree, both of these suggestions are true (Dianzani *et al.*, 1990, 1992b; Janeway, 1992; Stamenkovic *et al.*, 1991). Furthermore, the relative expression of particular CD45 isoforms has been reported to vary with the developmental stage of T cells (Dianzani *et al.*, 1990; Luqman *et al.*, 1991; Lee and Vitetta, 1992; Clement, 1992). For instance, in human T cells it has been suggested that "naive" T cells express CD45RA isoforms (those including determinants from exon A), whereas memory cells express the CD45RO isoform (not containing A, B, or C determinants) (reviewed in Clement, 1992). Similarly, using a mAb specific for mouse isoforms that utilize exon B, Dianzani *et al.* (1990) have demonstrated that different CD45 isoforms are expressed on naive and memory cells. Furthermore, they demonstrated that molecular associations between CD4, CD45, and CD3 also correlate with memory. On naive mouse T cells CD4, CD45, and CD3 behave as independent entities and thus do not cocap, whereas on memory mouse T cells CD4, CD45, and CD3 have been demonstrated to associate stably on the cell surface, as demonstrated by their ability to cocap with one another. Similarly, cloned T_{H1} and T_{H2} lines also differ in their expression of CD45 isoforms containing exon B determinants. On T_{H2} cells CD4 was shown to stably associate with CD45, however, in this instance the complex was not associated with CD3 (Dianzani *et al.*, 1990). The same group has demonstrated that on human peripheral T cells CD4 and CD8 associate with particular isoforms of CD45 identified by the mAb MCA.347, which probably recognizes CD45 isoforms containing exon C determinants (Dianzani *et al.*, 1992b). Furthermore, an increase in the association between CD45 and CD4 or CD8 has been reported to occur on stimulation with solid-phase anti-CD3 mAb or during a mixed lymphocyte reaction (MLR) (Mittler *et al.*, 1992).

Taken together, these data imply that at different stages of differentiation different isoforms of CD45 predominate. As particular isoforms contribute to different interactions among CD4, CD45, and the TCR/CD3 complex, and since CD45 phosphatase is known to be capable of modifying $p56^{lck}$ kinase activity, it has been suggested that CD45 isoform expression contributes to a slightly modified TCR-

mediated signal at different stages of T cell development and in different T cell subsets (Dianzani *et al.*, 1990, 1992a,b; Janeway, 1992). Furthermore, CD45RO (not containing determinants from A, B, or C exons) on human T cells has recently been demonstrated to be a ligand for CD22 expressed on B cells (Stamenkovic *et al.*, 1991). While ligands for other isoforms of CD45 on the cell surface of T cell cognate partners have yet to be described, this data raises the exciting possibility that the engagement of particular CD45 isoforms with ligands on cell surfaces with which T cells interact may contribute to determining the assembly of the TCR signaling machinary as well as controlling the quality of the signal generated.

M. Differential Signaling between CD4 and CD8

CD4 and CD8 have symmetrical functions such that one augments class II recognition and activation and the other augments class I recognition and activation. Both act as coreceptors by binding to the same MHC molecule as the TCR and augmenting signaling by a mechanism at least in part reliant on colocalizing the associated $p56^{lck}$ tyrosine kinase activity with the TCR signaling complex. Furthermore, the banding patterns on antiphosphotyrosine Western blots are similar when either CD4 or CD8 is coligated with the TCR (Ledbetter *et al.*, 1990), indicative of a large degree of overlap in the mechanisms by which CD4 and CD8 act to amplify TCR-mediated signals. Both contribute to increases in phosphoinositol (PI) turnover and Ca^{2+} flux and can contribute to IL-2 production in T hybridomas. However, if (as will be discussed later; see Section III) signaling through either CD4 or CD8 coreceptor on a thymocyte contributes to the development of that thymocyte into either a mature class II-restricted helper T cell or class I-restricted CTL, respectively, it is imperative that there be differences in the signals generated when either CD4 or CD8 functions as a coreceptor. Indeed, despite their many similarities, some differences have been found. How these differences relate to signaling unique differentiation pathways or functions remains entirely unknown, though a number of possible mechanisms will be discussed here.

First, unique signals could be sent by CD4 and CD8 simply due to their differential abilities to bind to and activate $p56^{lck}$. CD4 binds to more cellular $p56^{lck}$ than does CD8 (Veillette *et al.*, 1988, 1989c; Luo and Sefton, 1990). Furthermore, CD4 crosslinking has been demonstrated to activate the *in vitro* kinase activity of the associated $p56^{lck}$, whereas crosslinking CD8 may not (Veillette *et al.*, 1989a,c, 1991; Luo and Sefton, 1990). Similarly, it has been reported that the $p56^{lck}$-

catalyzed *in vitro* tyrosine phosphorylation of the peptide substrate angiotensin II was stimulated up to 20-fold by peptides representing the CD4 cytoplasmic tail, whereas peptides of similar charge and size representing portions of the cytoplasmic tail of CD8α only activated angiotensin II phosphorylation with a theoretical maximum of 4.7-fold (Bramson *et al.*, 1991). Unlike the studies demonstrating the requirement for crucial cysteines within the CD4 cytoplasmic tail for binding to $p56^{lck}$ (Shaw *et al.*, 1989; Turner *et al.*, 1990), stimulation of $p56^{lck}$ kinase activity by CD4 cytoplasmic peptides did not require the presence of these cysteines (Bramson *et al.*, 1991). This may indicate that other sequences within the cytoplasmic tails of CD4 and CD8 may be important in modulating $p56^{lck}$ kinase activity. Perhaps the differentiation signal required to induce development along a CD4 lineage requires a higher degree of tyrosine phosphorylation (and perhaps activation) of protein substrates than does the signal required for CD8 lineage development. Thus, during thymic development when CD4 is engaged as a coreceptor a more intense tyrosine phosphorylation signal is sent and development into the CD4 lineage ensues. Similarly, a lower intensity signal (albeit higher than that seen with no coreceptor) may be sent when CD8 is engaged in coreceptor activity, resulting in the development of CD8 lineage cells.

Second, interactions between the CD4 or CD8 cytoplasmic tails and $p56^{lck}$ may modulate $p56^{lck}$-catalyzed phophorylations in a substrate-dependent manner unique to either CD4 or CD8. Indeed, as mentioned in the preceding paragraph, an analysis of the kinetics of the phosphorylation of angiotensin II indicated that activation by the 31-residue peptide (mimicking the complete CD4 cytoplasmic tail) occurred through increasing the V_{max} 20-fold. (Again, a peptide representing the CD8α cytoplasmic tail only increased phosphorylation of the same substrate 4.7-fold.) This same CD4 cytoplasmic peptide stimulated $p56^{lck}$-catalyzed phophorylation of another *in vitro* substrate ("RR-SRC") by increasing the V_{max} only 3-fold (Bramson *et al.*, 1991). In both cases the apparent K_m was unaffected by activation with the peptide. Finally, $p56^{lck}$-catalyzed phosphorylation of a third peptide substrate described was unaffected by the CD4 cytoplasmic peptide (Bramson *et al.*, 1991). Clearly, these data demonstrate that $p56^{lck}$ kinase activity *in vitro* is altered in a substrate-dependent manner through interaction with CD4 cytoplasmic residues. Interaction of CD4/$p56^{lck}$ or CD8/$p56^{lck}$ with TCR components could further modulate kinase activity with regard to particular substrates. Indeed, as mentioned earlier, CD4 and CD8 coreceptors may contribute to TCR complex crosslinking through different mecha-

nisms (i.e., CD8 dimers may bind two MHC molecules, whereas CD4 only binds one, and two CD4 molecules may associate with one TCR complex) (Saizawa *et al.*, 1987; Ryu *et al.*, 1990; Wang *et al.*, 1990; Leahy *et al.*, 1992). These differences may result in a subtle difference in the juxtaposition and orientation of proteins responsible for coupling to and affecting tyrosine kinase activity that is initiated on TCR complex crosslinking. While no major differences in *in vivo* phosphoproteins detected by antiphosphotyrosine Western blotting of total cell lysates have been seen on activation by CD4/TCR or CD8/TCR crosslinking (Veillette *et al.*, 1989c; Ledbetter *et al.*, 1990), this does not mean that unique substrates are not phosphorylated by CD4/p56^{lck}/TCR interactions and CD8/p56^{lck}/TCR interactions *in vivo*. Rather, such unique substrates simply may not be abundant enough to be detected in total cell lysates. As proteins phosphorylated on tyrosine during T cell activation become better characterized and antibodies become available, a more complete survey of *in vivo* substrates of CD4/p56^{lck}/TCR and CD8/p56^{lck}/TCR activity may result in the discovery of specific CD4- or CD8-association-induced modulation of p56^{lck}-catalyzed phophorylation *in vivo*.

Additional differences between CD4 and CD8 have been demonstrated in their responses to phorbol myristate acetate (PMA), though their significance for signaling differentiation or activation is not yet obvious. It has been demonstrated that CD4 (Acres *et al.*, 1986, 1987) and CD8$\alpha\beta$, but not CD8$\alpha\alpha$ (Terry *et al.*, 1990), are induced to modulate off the T cell surface on treatment with PMA. Recent data demonstrated that while most $\alpha\beta$ TCR-bearing T cells express CD8$\alpha\beta$ heterodimers, some T cell subsets (e.g., $\gamma\delta$ TCR-bearing cells and NK cells) may only express CD8α homodimers, implying that CD8α and CD8β expression are not always coordinantly expressed, and in human $\alpha\beta$ TCR cells at least some cells may express CD8α homodimers as well as $\alpha\beta$ heterodimers (Terry *et al.*, 1990; Moebius *et al.*, 1991). Given the difference in the ability of CD4 (and CD8$\alpha\beta$ heterodimers) versus CD8α homodimers to modulate in response to PMA, different signals might be generated depending on whether CD4 or CD8 is the involved coreceptor. The PMA-responsive element of CD4 is contained within the cytoplasmic tail of CD4, as CD8/CD4 hybrid molecules containing the extracellular and transmembrane portions of CD8α and the cytoplasmic tail of CD4 respond to PMA by modulating off the cell surface when expressed in T hybridomas (R. Zamoyska and J. R. Parnes, unpublished data). Furthermore, it has been demonstrated that CD4/p56^{lck} interactions are interrupted

within 10 minutes after PMA treatment, whereas CD8/p56^{lck} interactions remain intact on PMA treatment (Hurley *et al.*, 1989). Disassociation between CD4 and p56^{lck} could attenuate a CD4-induced p56^{lck} signal by no longer localizing p56^{lck} near relevant substrates. Alternatively, disassociation may render p56^{lck} (perhaps now modified as a result of its interaction with CD4 or TCR components) free to interact with another substrate that is not accessible to p56^{lck} when it is bound to CD4 or CD8.

Finally, CD4 or CD8 may contribute to TCR-mediated signaling differently through mechanisms distinct from p56^{lck} activity. The cytoplasmic tails may be involved in transmitting signals other than p56^{lck} kinase activity (e.g., through the aforementioned associated GTP-binding protein), or the transmembrane or extracellular portions of CD4 or CD8 may contribute to coassociation with and/or signaling through additional signaling proteins. Indeed, in some circumstances, it has been reported that CD4 and CD8 may preferentially associate with different isoforms of CD45 (Thomas, 1989). In addition to influencing p56^{lck} activity, the CD45 tyrosine phosphatase most likely regulates other phophoproteins involved in signaling. A potential role in T cell differentiation for the transmembrane region of CD4 may be suggested by recent experiments. In these studies it was demonstrated that a signal for T cell differentiation to a CD4 cell lineage could be delivered to a class I-restricted T cell through a hybrid molecule containing CD8 external sequences and CD4 transmembrane and cytoplasmic domains (Seong *et al.*, 1992). While this result may be due to the CD4 cytoplasmic tail and the associated p56^{lck}, it cannot be ruled out that all or part of this phenomenon depends on contributions made by the transmembrane portion of CD4. These experiments will be discussed in greater detail in Section III,F).

III. Role of CD4 and CD8 in T Cell Differentiation

A. Thymocyte Development and Selection Processes

The development in the thymus of T cells bearing the $\alpha\beta$ TCR is a complex and only partially understood process during which precursor cells lacking both TCR and coreceptor molecules mature to yield functionally mature, antigen-reactive T cells expressing either CD4 or CD8. During this process thymocytes pass through a stage of expressing either CD8 (Ceredig *et al.*, 1983; Paterson and Williams, 1987) or CD4 (Hugo *et al.*, 1990) alone enroute to becoming "double-positive" thymocytes expressing both CD4 and CD8 (reviewed in

Fowlkes and Pardoll, 1989). Surface expression of the $\alpha\beta$ TCR complex appears, initially at low levels, during this double-positive stage. As these cells mature, TCR levels increase and expression of either CD4 or CD8 is silenced. Hence, the most mature thymocytes, like peripheral T cells, are "single positive" with respect to the CD4 and CD8 coreceptors. Studies in normal (Zúñiga-Pflücker et al., 1989b) and TCR transgenic (Teh *et al.*, 1988; Sha *et al.*, 1988b; Scott *et al.*, 1989; Berg *et al.* 1989b; Kaye *et al.*, 1989; Pircher *et al.* 1989) mice have confirmed the notion that it is the specificity of the TCR for class I or class II MHC proteins that determines the phenotype of the single-positive mature T cell with respect to CD8 or CD4: TCRs specific for class I select for CD8 cells, while TCRs specific for class II select for CD4 cells.

Thymocytes are exposed to at least two selective mechanisms during their development, and these shape the final TCR repertoire (reviewed in Fowlkes and Pardoll, 1989; Blackman *et al.*, 1990; von Boehmer and Kisielow, 1990; Nikolic-Zugic, 1991). Positive selection involves the selection for survival of only those maturing thymocytes that can recognize self-MHC, ensuring that mature peripheral T cells will recognize antigenic peptides bound to self (and not foreign) MHC proteins. Positive selection appears to be mainly dependent on the MHC proteins expressed on thymic epithelial cells (Lo and Sprent, 1986). It is highly likely that self-peptide is present in the antigen-binding groove of these MHC proteins and influences positive selection (Nikolic-Zugic and Bevan, 1990; Berg *et al.*, 1990; Sha *et al.*, 1990). Positive selection appears to occur in the thymic cortex at the double-positive stage (although the stage has not been definitively proved), resulting in a signal for cells to survive [perhaps by preventing programmed cell death (Sprent *et al.*, 1990]) and/or mature. Negative selection involves deletion in the thymus of developing thymocytes expressing TCRs that are potentially autoreactive, presumably because they bind with high affinity to self-MHC proteins, most likely with self-peptide in the binding groove. Negative selection is a major mechanism of tolerance induction in the T cell compartment. MHC proteins on bone marrow-derived hematopoietic cells in the thymus appear to be the major mediators of negative selection, although the epithelium may play some role under certain circumstances (reviewed in Fowlkes and Pardoll, 1989; Sprent *et al.*, 1990). Recent data from Pircher *et al.* (1991) have shown that a lower TCR avidity interaction is required for clonal deletion in the thymus than for activation of mature T cell function in the periphery. The timing of negative selection has been controver-

sial; some studies in normal (Fowlkes *et al.*, 1988; MacDonald *et al.*, 1988b) and transgenic (Kisielow *et al.*, 1988; Sha *et al.*, 1988) mice suggested that it occurs at the double-positive stage, while other studies in normal mice suggested that it occurs at the transition to single positives (Kappler *et al.*, 1987a, 1988; Marrack *et al.*, 1988). Comparing deletion in TCRβ chain versus $\alpha\beta$ transgenic mice, Berg *et al.* (1989a) concluded that TCR density and maturational state determine whether deletion occurs at the double-positive stage. A study in normal mice using cell transfers suggested that negative selection occurs after commitment to the single-positive CD4 or CD8 lineage and postulated that the target is a transitional thymocyte expressing medium levels of TCR and either $CD4^+CD8^{low}$ or $CD4^{low}CD8^+$ (Guidos *et al.*, 1990). Indeed, the timing (double positive or later) and location of negative selection have been shown to vary even with a single transgenic TCR with dual specificities depending on which specificity is involved in the negative selection; this may result from differences in affinity of the TCR for the deleting MHC/peptide or from differences in where the deleting elements are encountered in the thymus (Pircher *et al.*, 1989).

The existence of both positive and negative selective mechanisms that are dependent on binding to self-MHC plus self-peptide presents the obvious dilemma of how the two are controlled so that the T cells that are permitted to mature are capable of recognizing foreign peptide bound to self-MHC, while those that would be activated peripherally by self-peptide with self-MHC are eliminated. A number of potential mechanisms have been proposed. Perhaps the most likely is the affinity model, by which thymocytes that recognize self-MHC/self-peptide with low affinity are positively selected, while those that recognize self-MHC/self-peptide with high affinity are deleted (Sprent *et al.* 1988; Schwartz, 1989). This mechanism has received strong support from recent studies of Lee *et al.* (1992) and Robey *et al.* (1992) involving the influence of transgenic CD8 expression levels on positive versus negative selection (see Section III,D). A second potential mechanism suggests that a different set of self-peptides is presented by the cells mediating positive versus negative selection (Marrack and Kappler, 1987). According to this model positive selection would involve presentation by thymic epithelial cell MHC proteins of both a set of self-peptides unique to these cells and ubiquitously expressed self-peptides, while negative selection would involve presentation by bone marrow-derived hematopoietic cell MHC proteins of only ubiquitous self-peptides. This would result in elimination of thymocytes recognizing ubiquitous self-peptides and

survival of those recognizing thymic epithelial-specific self-peptides. It is also possible that both (or alternative) mechanisms may be involved. While the specificity and affinity of the TCR play a crucial role in both positive and negative selection, the CD4 and CD8 coreceptor molecules have also been shown to be intimately involved in these processes.

B. Positive Selection

1. Role of CD4 in Positive Selection

Two types of studies eliminating the ligand for CD4 (i.e., class II MHC proteins) during thymocyte development have resulted in a lack of development of mature, single-positive, class II-restricted $CD4^+$ T cells. The first involved treatment of mice from birth with mAb reactive with class II MHC proteins (Kruisbeek *et al.*, 1983, 1985), while the second involved genetic knockout of class II expression (Cosgrove *et al.*, 1991; Grusby *et al.*, 1991). In both cases development of normal CD8 single-positive cells proceeded unimpeded. However, these results do not necessarily imply a role for the CD4/class II interaction in positive selection, because the lack of class II also prevents interaction of class II-specific TCRs with their selecting ligand.

More direct evidence for a role of CD4 in positive selection came from studies in which pregnant mice were treated daily with intraperitoneal (i.p.) anti-CD4 mAb until the day of birth (Zúñiga-Pflücker *et al.*, 1989c). The neonatal mice born after such treatment lacked single-positive CD4 thymocytes yet had normal levels of double-positive and single-positive CD8 thymocytes. The presence of double-positive cells indicated that the mAb did not eliminate all cells bearing CD4. Similar results were obtained by treatment of fetal thymus organ cultures with anti-CD4 mAb. Whole antibody, $F(ab')_2$ (which was shown to be nondepleting of CD4 cells in adult mice) or Fab fragments were all effective in blocking development of single-positive CD4 cells. Similarly, Ramsdell and Fowlkes (1989) used a nondepleting anti-CD4 mAb to treat adult mice (either unirradiated or 9 days after irradiation and syngeneic bone marrow reconstitution) i.p. for 15–18 days. As in the study of Zúñiga-Pflücker *et al.* (1989c), this treatment resulted in a block in the development of CD4 single-positive cells in the thymus without affecting the development of CD8 single-positive cells, and the double-positive population was still present. Both studies demonstrated a role for CD4 in positive selection of CD4 single-positive cells and suggested that mAb treat-

ment interfered by blocking the interaction of CD4 with a ligand (i.e., class II) during this process.

The importance of CD4 in positive selection of mature, single-positive CD4 cells received further support from studies of homozygous mutant mice in which the CD4 gene was genetically disrupted and not expressed (Rahemtulla *et al.*, 1991). Of course, such mice lacked cells expressing CD4, but more importantly, with respect to positive selection, mature $CD4^+$ cells were not simply replaced by an equivalently sized population of mature $CD4^-$, $CD8^-$, $CD3^+$ cells that could represent normal, positively selected, CD4 single-positive cells simply lacking surface CD4. Furthermore, class II-restricted helper T cell function was markedly reduced in these mice. These findings reaffirm the importance of CD4 for positive selection of $CD4^+$, class II-restricted helper T cells. In contrast, CD8 cells that could function as class I-restricted CTLs developed normally, implying that a double-positive population is not required for development of the mature functionally competent CD8 subset. Of potential importance was the presence in such mice of a very small population of peripheral $TCR\alpha\beta^+$ $CD4^-$ $CD8^-$ T cells. The origin and pathway of development of these cells are not known, and its is possible that they are responsible for the small amount of residual helper function observed.

2. *Role of CD8 in Positive Selection*

In analogy to the CD4 studies cited above (see Section III,B,1), development of CD8 single-positive cells was blocked in studies of thymic selection in mice in which the CD8 ligand (i.e., class I MHC proteins) was unavailable for interaction, either because of mAb treatment (Marusic-Galesic *et al.*, 1988) or because of lack of cell surface expression resulting from genetic knockout of the β_2-microglobulin gene (Zijlstra *et al.*, 1990; Koller *et al.*, 1990). Again, these results do not allow one to conclude that the critical interaction is CD8/class I, because TCR/class I interactions were also absent in these mice.

The importance of CD8 for the positive selection of single-positive CD8 cells was demonstrated by the lack of development of this subset in the thymuses of adult mice (either unirradiated or 9 days postirradiation and syngeneic bone marrow reconstitution) treated i.p. for 15–18 days with a nondepleting anti-CD8 mAb (Ramsdell and Fowlkes, 1989). These mice maintained their double-positive thymocytes and had normal development of CD4 single-positive cells. Similarly, CD8 single-positive thymocytes did not develop in neonates

after i.p. anti-CD8 mAb treatment of the pregnant mothers and the resulting pups (Zúñiga-Pflücker *et al.*, 1990). Again, development of single-positive CD4 cells was not interrupted by this treatment, and double-positive thymocytes were still present (i.e., the treatment did not deplete all cells bearing CD8). Similar results were obtained using $F(ab')_2$ fragments as opposed to whole antibody. Furthermore, treatment of heterozygous mice bearing both CD8.1 and CD8.2 alleles with mAb specific for CD8.2 alone did not result in loss of the single-positive CD8 subset. These results imply that cell depletion cannot explain the lack of CD8 cells in the homozygous CD8.2 mice, but rather that CD8 must be available for a critical interaction with which the mAb interferes. In the CD8.1 × CD8.2 mice the CD8.1 allele would still be available for that purpose.

Mice homozygous for knockout of the CD8α gene by gene targeting also lack $CD8^+$ cells and cytotoxic T cell function (Fung-Leung *et al.*, 1991). As in the case of the CD4 knockouts, the important point regarding positive the role of CD8 in selection is that such knockout mice have normal CD4 single-positive cells and class II-restricted helper T cell function, but they do not have a sizeable population of $CD4^-$, $CD8^-$, $CD3^+$ T cells that could correspond to normal CD8 single-positives merely lacking CD8.

Strong evidence for the role of a CD8/class I interaction in positive selection of CD8 T cells has recently been provided in two studies in which this interaction has been disrupted. Aldrich *et al.* (1991) generated C3H transgenic mice expressing either the normal L^d class I protein or a mutant L^d in which the α3 domain had been replaced with that of the $Q7^b$ class I molecule. The α3 domain of the latter differs from that of L^d by five amino acids and also has a three-amino acid insertion. The mutant L^d does not interact with CD8-dependent CTLs, but it does bind to an L^d-restricted viral peptide (derived from the immediate early protein pp89 of mouse cytomegalovirus). In contrast to transgenics expressing native L^d, those expressing the mutant failed to generate CTLs reactive with the viral peptide presented on L^d stimulator cells. Similarly, no antipeptide CTL response was evident using mutant L^d stimulator cells. These results suggest that the inability of CD8 to bind to the mutant L^d blocked the positive selection of CD8 cells restricted by that class I MHC protein. They further imply a need for CD8 to bind to the same class I MHC protein as the TCR during positive selection, as there are other wild-type (i.e., capable of binding CD8) class I proteins on the selecting cells of the transgenics expressing the mutant L^d.

Killeen *et al.* (1992) reached a similar conclusion using transgenic

mice expressing a mutant class I MHC protein, D^b, in which the aspartic acid at residue 227 was substituted with lysine. This substitution abolishes binding to CD8. Such transgenics, on an *H-2*^{d} background, were mated to *H-2*^{d} mice expressing a transgenic $\alpha\beta$ TCR specific for the male antigen H-Y in association with D^b. Studies by von Boehmer's group had previously shown that positive selection of this class I-restricted TCR occurs only in mice expressing D^b, and in female mice (which do not express H-Y and, hence, do not delete cells expressing this TCR based on autoreactivity) this results in mature $CD8^+$ T cells expressing the transgenic TCR (Kisielow *et al.*, 1988; Teh *et al.*, 1988). However, in female *H-2*^{d} mice doubly transgenic for the H-Y + D^b-specific TCR and the mutant *H-2D*^{b}, positive selection of the the transgenic TCR did not occur, despite the fact that this mutant D^b could present the H-Y antigen to H-Y-specific CTLs (Killeen *et al.*, 1992). In contrast, female mice doubly transgenic for the H-Y + D^b-specific TCR and wild-type D^b did exhibit positive selection for the transgenic TCR on CD8 cells. These results point to a critical role for the interaction between CD8 and class I MHC in the positive selection of CD8 T cells. They further imply that CD8 must act as a coreceptor during positive selection in that it must bind to the same class I MHC protein as the TCR on the developing thymocyte.

The level of CD8 expression has been shown to influence positive selection of class I-restricted thymocytes (Robey *et al.*, 1991; Borgulya *et al.*, 1991; Seong *et al.*, 1992). In these studies increased cell surface CD8 expression in mice doubly transgenic for CD8 and the H-Y + D^b-specific TCR resulted in enhanced positive selection of $CD8^+$, transgenic TCR^+ cells.

In an attempt to study the mechanism of positive selection of $CD8^+$ cells, Carrera *et al.* (1992) recently found that CD8 and the TCR selectively associated in thymocytes of H-Y + D^b-specific TCR transgenic mice (on a SCID background) expressing the restricting class I MHC protein (D^b), but not in thymocytes of such mice expressing a nonrestricting MHC haplotype. This complex also contained activated forms of $p56^{lck}$ and $p59^{fyn}$. In contrast, no such complex was formed between CD4 and the TCR regardless of the presence of a selecting or nonselecting MHC. These findings suggest that the complex containing the TCR, CD8, and activated tyrosine kinases is formed on binding of CD8 and the TCR to the same MHC protein and that it is likely to be involved in transmitting the signals for positive selection.

C. Negative Selection

1. Role of CD4 in Negative Selection

The role of CD4 in negative selection has been established primarily by studies in which *in vivo* treatment of mice with anti-CD4 mAb blocked "superantigen"-mediated thymic deletion of T cells expressing particular Vβ segments that react with the selecting superantigen. For example, T cells bearing Vβ17a are normally negatively selected during thymocyte development in mice expressing I-E because of autoreactivity (Kappler *et al.*, 1987a,b, 1989). Vβ17a is expressed on functionally immature, double-positive, TCR^{low} thymocytes in such mice, but it is absent in both CD4 and CD8 single-positive cells. Fowlkes *et al.* (1988) performed syngeneic bone marrow transfers in irradiated C57BR mice ($I\text{-}E^+$, $V\beta17a^+$) and treated the reconstituting mice with anti-CD4 mAb. They found that deletion of CD8 single-positive T cells bearing Vβ17a was blocked by the anti-CD4 treatment. As in the studies on positive selection described above, this treatment eliminated the CD4 single-positive subset entirely, so Vβ17a expression could not be examined in those cells. These results suggested that anti-CD4 blocked negative selection of potentially autoreactive Vβ17a thymocytes at the double-positive stage, thus allowing the continued development of CD8 single-positive cells bearing Vβ17a. The latter cells were found not to be reactive with self-I-E, implying that the CD4 molecule is required for the anti-I-E autoreactivity of Vβ17a T cells. Importantly, the block in deletion observed in these mice implicated the CD4 molecule as a crucial player in the negative selection of Vβ17a T cells in these mice.

Similarly, MacDonald *et al.* (1988b) found that neonatal treatment of Mls^a mice with anti-CD4 but not anti-CD8 mAb blocked the clonal deletion of $V\beta6^+$ and Vβ8.1 T cells that is normally seen in Mls^a mice (MacDonald *et al.*, 1988a). As in the case of $V\beta17a^+$ cells in the $I\text{-}E^+$ mice described above, it could be shown that $V\beta6^+$ cells appeared in the CD8 single-positive population in the treated animals. Hence, these results also support a model in which anti-CD4 mAb treatment blocks negative selection of Mls^a-reactive thymocytes at the double-positive stage, thereby allowing continued development of the nonautoreactive CD8 single-positive cells bearing Vβ6.

Finally, Zúñiga-Pflücker *et al.* (1989a) demonstrated that anti-CD4 but not anti-CD8 mAb treatment could block the deletion of Vβ11-bearing T cells that is normally seen in $I\text{-}E^+$ mice, again leading to the expression of this TCRβ chain on CD8 single-positive T cells.

All of these studies point to a pivotal role of CD4 during negative selection of class II-reactive cells. Although all involve superantigen-mediated deletion, it is likely that the same requirement for CD4 holds for deletion of at least some if not all thymocytes expressing TCRs with specificity for conventional autoantigen/class II MHC. The mechanism by which anti-CD4 blocks negative selection has not been defined. Presumably it involves interference with CD4 co-receptor function as opposed to a simple block in adhesion, since anti-CD8 does not block deletion of class II-restricted T cells even though CD8 is present on the cells undergoing selection (at least in these superantigen systems). Possible modes of anti-CD4 action include (but are not limited to) blocking the ability of CD4 to interact with the same class II MHC proteins seen by the TCR, blocking interaction between CD4 and the TCR complex, or transmission through CD4 of signals that interfere with the selection process.

2. *Role of CD8 in Negative Selection*

A number of recent studies involving transgenic mice have implicated CD8/class I MHC interactions in the process of negative selection of class I-reactive T cells. Ingold *et al.* (1991) generated transgenic mice expressing chimeric class I MHC proteins consisting of the $\alpha 1$ and $\alpha 2$ domains of *H-2K*^{b} and the $\alpha 3$ and internal domains of either wild-type *H-2D*^{d} or a mutant *H-2D*^{d} in which the CD8 binding site had been ablated by substitution of lysine for glutamic acid at residue 227. After breeding to obtain the appropriate background MHC haplotype, these transgenics were examined for the generation of CTLs reactive with K^b. In contrast to the transgenics expressing the chimera with the wild-type D^d domain, those with the mutant D^d $\alpha 3$ domain failed to delete CD8-dependent CTLs reactive with either wild-type K^b or K^b with a D^d $\alpha 3$ domain. These results suggested that not only is a CD8/class I interaction required for the process of negative selection, but that CD8 must interact with the same MHC protein as the TCR, since the selecting cells would express wild-type endogenous class I proteins in addition to the selecting mutant chimera.

The same conclusion was reached by Aldrich *et al.* (1991) using the transgenics described above (see Section III,B,2) expressing either wild-type L^d or L^d with the $\alpha 3$ domain from $Q7^b$ (which does not bind CD8). They found that expression of the chimeric L^d did not result in the elimination of all L^d-reactive cells from the T cell repertoire as measured in a mixed lymphocyte culture. They therefore concluded that the inability of the chimeric L^d to interact with CD8 blocked the complete clonal deletion (negative selection) of cells reactive with L^d.

Also in agreement were Killeen *et al.* (1992), using the same system they used (as described in Section III, B, 2) to study CD8 function in positive selection, i.e., *H-2*^{d} background double transgenics for the H-Y + D^b-specific TCR and either wild-type or mutant *H-2D*^{b} that cannot bind CD8 but still reacts with H-Y-specific T cells). In male mice the presence of wild-type D^b led to deletion of the potentially autoreactive T cells expressing the transgenic TCR α and β chains. However, in male double transgenics expressing the mutant D^b, negative selection did not occur and mature CD8$^+$ T cells expressing the transgenic TCRα and TCRβ chains were present.

However, the importance of CD8 in negative selection may be TCR dependent. Studies of negative selection involving TCR transgenes in homozygous CD8α knockout mice that lack CD8 expression have shown that negative selection can proceed in the absence of CD8 for the 2C allo-class I-specific TCR (anti-L^d), but not for the H-Y + D^b-specific TCR or an LCMV-specific, class I-restricted TCR (Fung-Leung *et al.*, 1992). Just as differences in TCR affinity are thought to influence the level of dependency of mature T cells on CD8 for antigen-induced activation, it is likely that differences in TCR affinity also account for differences in CD8 dependency for negative selection. Hence, thymocytes expressing TCRs with extremely high affinity for self-MHC/self-peptide may delete even in the absence of a coreceptor (CD8 in this case) signal, while those of somewhat lower affinity would depend on a coreceptor signal for negative selection. This issue has not been directly addressed. However, as described in Section III,D, recent results from two groups do suggest that differences in the avidity of the TCR/coreceptor/MHC interaction influence whether positive or negative selection occurs.

D. Influence of Coreceptor Levels on Positive versus Negative Selection

Lee *et al.* (1992) generated a series of transgenic mice expressing different levels of transgenic CD8α, from twice up to 10 times normal levels. These mice were crossed to transgenics expressing the class I-restricted TCR 2C (Sha *et al.*, 1988), which is specific for L^d and positively selected by the K^b class I protein. Double transgenics (on an *H-2*^{b} background) were analyzed for their expression of thymocyte and splenic T cell subsets and 2C expression. As expected, those mice that expressed the transgenic TCR and moderately elevated levels of transgenic CD8α exhibited positive selection for the 2C TCR transgene. However, cells expressing the TCR transgene were deleted in mice expressing the highest levels of the CD8α transgene. Hence, altering

the level of CD8α expression can affect whether the developing thymocyte undergoes positive versus negative selection. These findings appear to be TCR dependent and not related to a more general alteration of T cell developmental pathways, since high levels of expression of a CD8 transgene (α or $\alpha\beta$) led to enhanced positive selection and not negative selection of the H-Y + D^b-specific TCR in female $H\text{-}2^b$ double transgenic mice (Robey *et al.*, 1991; Borgulya *et al.*, 1991; Seong *et al.*, 1992; Lee *et al.*, 1992). The most obvious interpretation of these data is that the increased level of CD8α on the developing thymocyte leads to greater binding to class I MHC proteins on the selecting cells, thereby increasing the avidity of the interaction between the developing thymocyte and the selecting cells. These results provide strong evidence for the affinity model of thymocyte selection (see Section III,A) and implicate CD8 (and presumably CD4) in contributing to the strength of the interactions determining whether positive or negative selection occurs.

These conclusion were further supported by the very similar findings of Robey *et al.* (1992) using CD8$\alpha\beta$ and 2C TCR double transgenics. Interestingly, these authors only crossed 2C TCR transgenics with a CD8$\alpha\beta$ transgenic expressing twice the normal level of CD8, yet they saw deletion in the double transgenics. It took much higher levels of transgenic CD8α expression to yield deletion of the 2C TCR-bearing thymocytes in the study of Lee *et al.* (1992). This apparent discrepancy may reflect an increased functional capacity of CD8$\alpha\beta$ heterodimers during the selection process as compared to CD8α homodimers. Indeed, we have demonstrated that CD8$\alpha\beta$ can enhance the activation of a mature T cell hybridoma more efficiently than CD8α homodimers (see Section II,K) (Wheeler *et al.*, 1992). This increased efficiency of the CD8$\alpha\beta$ heterodimers may result from higher affinity binding to class I MHC proteins and/or from enhanced interaction with the TCR complex or other associated proteins.

E. Mechanism of CD4 and CD8 Function during Thymocyte Selection

Although the above studies clearly indicate a critical role for CD4 and CD8 in the positive and negative selection of class II-restricted $CD4^+$ T cells and class I-restricted $CD8^+$ T cells, respectively, the mechanism(s) by which they function in the selection processes remain to be defined. For CD8 in both positive and negative selection the evidence summarized above (Sections III,B,2 and III,C,2) supports a requirement for binding to the same class I MHC protein as the TCR, as opposed to other class I proteins on the same cell, implying that

CD8 functions as a coreceptor in developmental processes as well as in activation. Furthermore, as in the case of mature T cell activation, the dependency on CD8 expression for negative selection may vary with the affinity of the TCR for self-MHC/self-peptide. Thus class I-restricted TCRs with very high affinity are likely to result in negative selection regardless of the expression of CD8. One would predict that both the importance of coreceptor function and the variation in requirement for the coreceptor depending on TCR affinity will also be shown to hold for CD4 during thymocyte development. Whether the molecular mechanisms involved in the function of CD8 and CD4 in T cell activation will also be shown to hold for their role in development is unclear. The importance of the protein tyrosine kinase $p56^{lck}$ in CD4 and CD8 coreceptor function in mature T cells was summarized above (see Sections II,B, II,E, II,G, II,H, II,L, and II,M). That this kinase is also critical for the normal development of thymocytes bearing the $\alpha\beta$ TCR has been amply demonstrated by the striking developmental block in mice in which the $p56^{lck}$ gene has been knocked out by homologous recombination (Molina *et al.*, 1992). Mice homozygous for the disrupted *lck* gene exhibited thymic atrophy, with only 3–10% of the number of thymocytes of littermate controls that expressed $p56^{lck}$. The number of double-negative thymocytes was similar to that in normal littermates, but there was a dramatic reduction in the number of double-positive thymocytes (from 1.7 to 8.9% of normal levels) and no detectable single-positive thymocytes. The double-positive population was missing $CD3^{low}$ cells, and had higher average levels of CD3 expression. The cell cycle profile of these double-positive thymocytes appeared similar to that of littermate controls. In the periphery there were only 5–10% T cells in spleen and lymph node, but these cells expressed the $\alpha\beta$ TCR and CD4 or CD8. Although these cells exhibited reduced proliferative responses to crosslinking of CD3 or the TCR, they did respond and this response could be increased by IL-2. These cells could also repond normally to either IL-2 alone or to PMA plus ionomycin. The implications of these findings with respect to activation were discussed in Section II,H. Regarding the role of $p56^{lck}$ in T cell differentiation, the markedly reduced (and abnormal) double-positive population in the thymuses of the $p56^{lck}$-deficient mice can be interpreted to imply that $p56^{lck}$ (also) plays a critical role in thymocyte development prior to the time when it transmits signals for selection processes through CD4 or CD8. This function could be manifest prior to surface expression of CD4 and/or CD8 and could involve transition from the double-negative stage. While further studies will be needed to elucidate the nature of the defect(s) in these mice, results of K. M.

Abraham *et al.* (1991a,b) using transgenic mice overexpressing wild-type or constitutively activated $p56^{lck}$ transgenes support the notion that $p56^{lck}$ can influence early thymic developmental stages. High levels of expression of either type of transgene induced thymic tumors of early stage thymocytes prior to TCR/CD3 surface expression.

Surprisingly, a recent study using an *in vitro* system of peptide antigen/class II MHC-induced clonal deletion showed that inhibition of tyrosine protein kinase activity with herbimycin A or genistein did not block the clonal deletion of developing thymocytes bearing a transgenic TCR specific for the presented peptide/class II MHC (Nakayama and Loh, 1992). Activity of $p56^{lck}$ was almost completely blocked at a concentration of herbimycin A that did not inhibit clonal deletion. The authors concluded that $p56^{lck}$ is not necessary for clonal deletion of thymocytes and that negative selection must occur via a distinct pathway. What is not clear at this time is whether these results extend to all class II-restricted TCRs, whether they can be extrapolated to the *in vivo* situation, or whether a small amount of residual tyrosine kinase activity of $p56^{lck}$ (or another tyrosine kinase that can substitute for it in negative selection) is sufficient to allow negative selection to proceed. It may be that $p56^{lck}$ is critical for negative selection with other TCRs with lower affinity for self-class II MHC/self-peptide. The fact that the dependency on CD8 for negative selection of class I-restricted thymocytes has already been shown to vary depending on the (affinity of the) TCR (see Section III,C,2) makes it likely that the dependency on $p56^{lck}$ will follow suit, and, as suggested above, one would predict the same for $CD4^+$ class II-restricted thymocytes.

Based on the apparently more strict requirement for CD4 or CD8 in positive selection of the respective T cells, one might guess that $p56^{lck}$ will be shown to be required for that process. However, that too is not certain. In a recent study reconstitution of $CD4^-$ mice with a truncated CD4 transgene lacking all but eight amino acids of the cytoplasmic tail resulted in apparently normal thymocyte and T cell development and functionally mature, class II-restricted $CD4^+$ (transgene), $CD8^-$, $\alpha\beta$ TCR^+ T cells (N. Killeen and D. R. Littman, personal communication). Does this mean that the CD4 cytoplasmic tail and $p56^{lck}$, which does not bind to the truncated CD4 transgene, are not necessary for positive selection of class II-restricted $CD4^+$ T cells? Perhaps, but probably not. There are a number of caveats regarding this experiment. First, the level of expression of the transgene, which was driven by the proximal $p56^{lck}$ promoter, was substantially higher [10- to 15-fold (Van Oers *et al.*, 1992)] than the normal level of expression of CD4. It is possible that this extremely high level of expression of the CD4 transgene

compensated for the lack of the full cytoplasmic tail and/or $p56^{lck}$ association by increasing adhesion and/or by increasing association with other relevant molecules, e.g., the TCR. Second, $p56^{lck}$ was still present in these cells, just not associated with the CD4 tail, and there may have been sufficient amounts of this kinase associated directly with the inner surface of the cell membrane to mediate the signals required for CD4 T cell development and activation in the setting of increased levels of (tailless) CD4. Although the CD4 T cell functions examined seemed normal, what is not known is how the TCR repertoire of these cells compared to normal; it is possible that the thymocytes that were positively selected under these circumstances had much higher affinity $\alpha\beta$ TCRs than those selected in the presence of full-length CD4. It will be critical to see whether the results of this study can be reproduced with a tailless CD4 that is expressed at normal levels and on only the correct cells.

Van Oers *et al.* (1991) have argued for a role of $p56^{lck}$ in both positive and negative selection based on their finding that 10- to 15-fold overexpression of a full-length CD4 transgene, but not the tailless construct described above (i.e., eight-amino acid cytoplasmic tail), interfered with positive and negative selection of the H-Y + D^b-specific TCR transgene. They found that only the full-length and not the tailless CD4 could compete with CD8 for binding of $p56^{lck}$, reducing the amount bound to CD8 to 3-fold less than in the presence of the tailless construct or no CD4 transgene. They concluded that the competition for $p56^{lck}$ was the likely cause of interference in the selective processes. However, the interpretation of these findings is not yet entirely clear, as the authors themselves point out. First, in the absence of the TCR transgene thymocyte development appeared normal in the presence of the full-length CD4 transgene, with a full (if not increased) complement of single-positive CD8 cells. Of course, the repertoire expressed by the latter cells is not known, and it is conceivable that they represent cells with relatively higher affinity TCRs that are less dependent on $p56^{lck}$ for positive selection. Second, the competition with $p56^{lck}$ correlated with the effects on selection but was not necessarily causal. The difference between the effects of full-length and truncated CD4 could relate to other potential interactions of CD4, such as with the TCR complex or other signaling molecules. Third, sequestration of $p56^{lck}$ by the overexpressed CD4 transgene might interfere with the development of TCR transgene-expressing cells at an earlier stage of maturation unrelated to positive or negative selection; as discussed above, deficiency of $p56^{lck}$ in homozygous knockout mice resulted in a drastic decrease in the number of double-positive thymo-

cytes (Molina *et al.*, 1992), and over-expression of a $p56^{lck}$ transgene (either wild type or constitutively activated) led to thymic tumors at a stage at which the $\alpha\beta$ TCR/CD3 complex was not yet expressed on the cell surface (K. M. Abraham *et al.*, 1991a,b).

F. Models for Development of CD4 versus CD8 T Cells

One of the remaining critical questions regarding the development of CD4 and CD8 T cells is how a double-positive thymocyte expressing an $\alpha\beta$ TCR that recognizes class II MHC proteins differentiates into a CD4 single-positive T cell, while one expressing an $\alpha\beta$ TCR that recognizes class I MHC proteins differentiates into a CD8 single-positive cell. Somehow the cell must "know" to maintain expression of the appropriate coreceptor and to ablate expression of the inappropriate one. A further question is how the program to become a helper T cell is linked to that to become a CD4 cell while the program to become a cytotoxic T cell is linked to that to become a CD8 cell. Two basic models have been suggested to explain the correlation between MHC recognition by the TCR and CD4 versus CD8 expression (von Boehmer, 1986; Janeway, 1988; Robey *et al.*, 1990). The instructive model postulates that a distinct signal is given to the developing thymocyte to become either CD4 or CD8 single-positive on binding of the TCR and presumably coreceptor molecule to the selecting class II or class I MHC protein, respectively. This model is consistent with a number of possibilities regarding the signals transduced. It is possible that CD4 and CD8 are both preprogrammed to be turned off and that the "instruction" signal is to maintain expression of the appropriate coreceptor. It is also possible that both are programmed to stay on unless a signal is transduced to turn off the inappropriate one. The selective or stochastic model postulates that double-positive thymocytes randomly lose expression of either CD4 or CD8, after which those cells that have the "proper" combination of $\alpha\beta$ TCR and coreceptor (i.e., class II-restricted TCR and CD4 or class I-restricted TCR and CD8) are selected and receive a signal to survive, presumably by TCR and coreceptor binding to the same selecting MHC molecule. In contrast, those developing thymocytes that have the "wrong" combination (i.e., class II-restricted TCR and CD8 or class I-restricted TCR and CD4) fail to receive a survival signal and die.

Evidence against a strict stochastic model has come from three groups, each of which examined the effects of inappropriate CD8$\alpha\beta$ or CD8α transgene expression on CD4 cells in (female) mice transgenic for the H-Y + D^b-specific TCR (Robey *et al.*, 1991; Borgulya *et al.*, 1991; Seong *et al.*, 1992). According to the stochastic model one would

predict that those thymocytes that had stochastically lost expression of CD8 and retained CD4 and that expressed the class I-restricted transgenic TCR would be "rescued" by expression of the transgenic CD8 and would therefore appear as mature T cells expressing the transgenic TCR and CD4 (and transgenic CD8) but not endogenous CD8. This was not observed in either the thymus or periphery of these doubly transgenic mice. These data are therefore incompatible with the stochastic model as described and consistent with, but not proof of, an instructive model for thymocyte development. In contrast, a recent study from the Littman lab has been interpreted as supportive of the stochastic model (C. Davis and D. R. Littman, personal communication). In that study a constitutively expressed mouse CD4 transgene was crossed onto a homozygous β_2-microglobulin-deficient knockout background. The β_2-microglobulin knockout mice had been shown previously to lack surface expression of (most) class I MHC proteins and to lack mature, CD8 single-positive T cells (Zijlstra *et al.*, 1990; Koller *et al.*, 1990). Expression of the CD4 transgene resulted in the reappearance of $CD8^+$ cells in the periphery of these mice, a finding that would be consistent with rescue by the CD4 transgene of thymocytes that expressed a class II-restricted TCR but had stochastically lost CD4 and retained CD8 expression during the developmental process. However, the β_2-microglobulin knockout mice are not truly class I MHC negative; they have been shown to express at least some cell surface D^b (Zijlstra *et al.*, 1990), a class I protein that does not absolutely require association with β_2-microglobulin for transport to the cell surface (Potter *et al.*, 1984; Allen *et al.*, 1987; Williams *et al.*, 1989). It may be that the low levels of surface class I (with abnormal conformation) in these mice, coupled with high levels of the inappropriate transgenic coreceptor (i.e., CD4), allowed for the development of some class I-restricted CD8 T cells. Perhaps additional study will reconcile these findings with those in the CD8 and TCR double-transgenic mice described above.

Our laboratory has further examined the mechanism by which thymocytes differentiate to the CD4 versus CD8 lineage by generating mice (DBA/2J) transgenic for a constitutively expressed chimeric CD8/CD4 protein consisting of the external portions of CD8α and the transmembrane region and cytoplasmic tail of CD4 (Seong *et al.*, 1992). These mice were mated to the H-Y + D^b-specific TCR transgenics (C57BL/6J) (Kisielow *et al.*, 1988; Teh *et al.*, 1988) to generate double transgenics. Bone marrow transplants were done from female double transgenics into C57BL/6J female mice to obtain a homozygous *H-2*^{b} background for positive selection of the transgenic TCR.

As controls, similar bone marrow transplants were done from double transgenics expressing the H-Y + D^b-specific TCR and constitutively expressed wild-type CD8α or from progeny of the transgenic TCR × transgenic chimeric CD8/CD4 cross expressing only the transgenic TCR and not the chimeric CD8/CD4. We found that expression of the chimeric CD8/CD4 but not the wild-type CD8 transgene led to the appearance of a large population (about 50%) of peripheral $CD4^+$ transgenic $\alpha\beta$ TCR-expressing cells (Fig. 6). That these cells resulted from alteration in the thymic developmental pathway was evidenced by the shifts in the ratio of $CD4^+$, endogenous $CD8^-$:$CD4^-$, endogenous $CD8^+$ thymocytes in the various bone marrow recipients, from 1:25 for the transgenic TCR alone to 1:40 for the transgenic TCR + wild-type CD8 (in which positive selection of the class I-restricted TCR is enhanced by the transgenic CD8), and 1:2 for the transgenic TCR + chimeric CD8/CD4 (Fig. 7a–c). These results imply that expression of the CD4 transmembrane region and/or cytoplasmic tail on the CD8/CD4 transgene leads to a specific signal for maintenance of CD4 expression on thymocytes (and their resultant T cells) expressing this class I-restricted TCR. Of the peripheral transgenic $\alpha\beta$ TCR-expressing cells expressing CD4, approximately 70% had turned off endogenous CD8 expression, as would be expected according to an instructive model (Fig. 7f). However, about 30% of these cells retained endogenous CD8 expression (as well as CD4) (Fig. 7f). It is possible that such cells might have received conflicting signals because of coexpression of endogenous wild-type and transgenic chimeric CD8/CD4, leading to continued expression of both CD4 and CD8. In any event, these results suggest that at least in the case of this transgenic TCR, a specific signal for differentiation of thymocytes to the CD4 or CD8 lineage is delivered by each of these coreceptor molecules through its transmembrane region and/or cytoplasmic tail. Although these findings are most consistent with an instructive model for thymocyte differentiation, additional approaches will be needed to address this issue more directly. Studies are currently underway to examine whether the $CD4^+$, endogenous $CD8^-$ T cells expressing the H-Y + D^b-specific TCR in our chimeric CD8/CD4 plus TCR transgenics have the phenotype of helper as opposed to cytotoxic T cells in order to determine whether this functional dichotomy results from the same signals that affect CD4 versus CD8 expression.

Another important remaining question is the mechanism by which the CD4 and CD8 transmembrane regions and/or cytoplasmic tails deliver different signals to differentiating thymocytes. An obvious can-

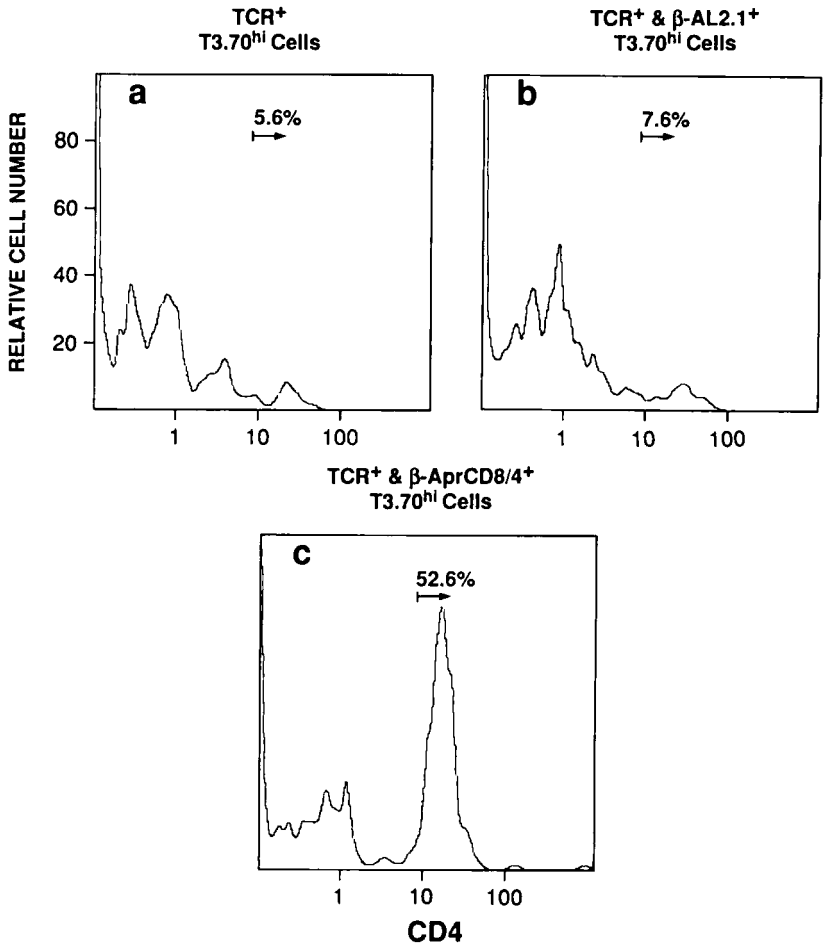
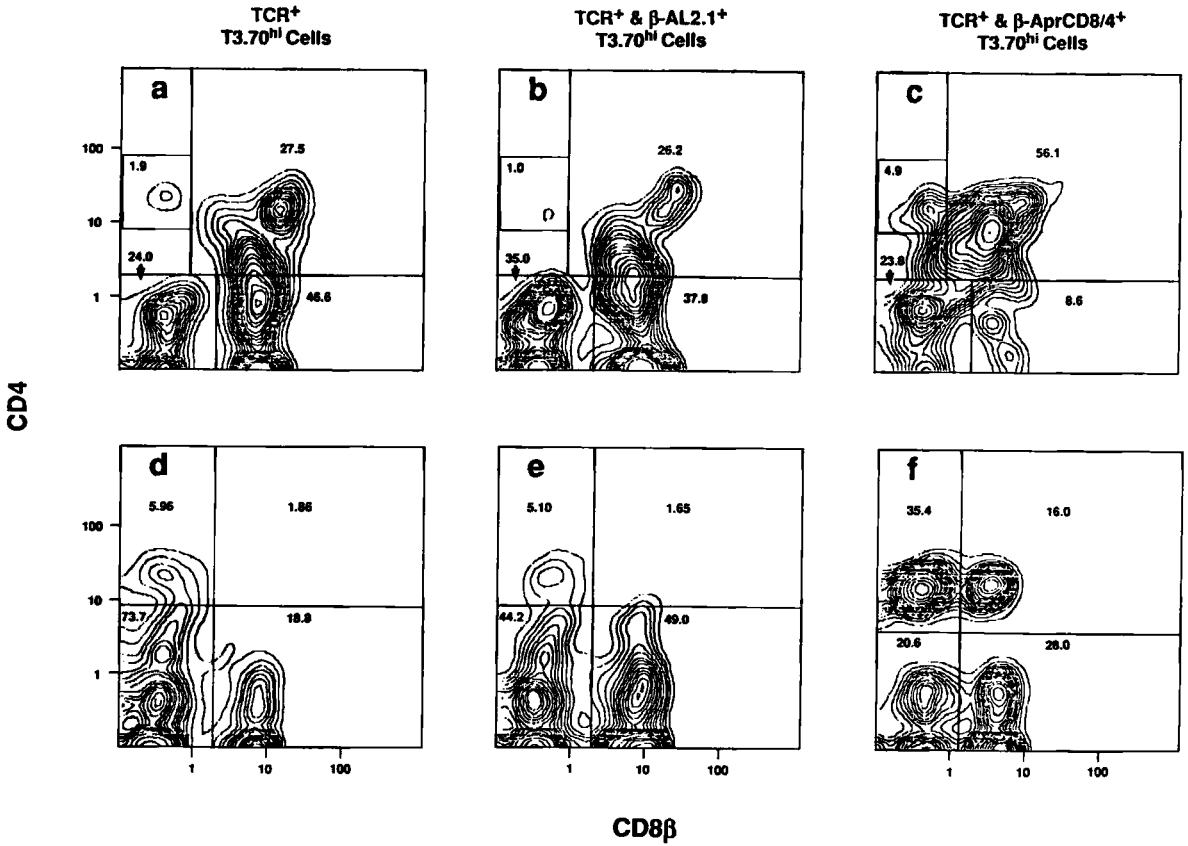


FIG. 6. Expression of a chimeric CD8/CD4 transgene leads to differentiation of T cells bearing an MHC class I-restricted TCR to the CD4 lineage. Surface expression of CD4 is shown on transgenic TCR$^+$ lymph node cells of C57BL/6J female recipients of bone marrow cells from (a) $\alpha\beta$ TCR$^+$, (b) $\alpha\beta$ TCR$^+$ plus wild-type CD8α^+ (β-AL2.1$^+$), and (c) $\alpha\beta$ TCR$^+$ CD8/CD4$^+$ chimera (β-AprCD8/4$^+$) transgenic mice. Transgenic $\alpha\beta$ TCR expression was monitored using the mAb T3.70. Bone marrow transfers (4×10^6 cells) were done from female transgenics into lethally irradiated female recipients. At 3 to 5 weeks after the transfer, single-cell suspensions of pooled lymph nodes from the recipients were prepared and stained with biotin-conjugated T3.70, followed by allophycocyanin-conjugated streptavidin and phycoerythrin-conjugated anti-CD4. Cells were analyzed on a fluorescence-activated cell sorter and the histograms show levels of CD4 staining on cells gated for high expression of the transgenic TCR. The percentages of CD4$^+$ cells are indicated. Reprinted with permission from *Nature* (Seong *et al.*). Copyright (1992) MacMillan Magazines Ltd.


TCR+
T3.70hi Cells
TCR+ & β-AL2.1+
T3.70hi Cells
TCR+ & β-AprCD8/4+
T3.70hi Cells
a
b
c
d
e
f
27.5
1.9
24.0
46.6
26.2
1.0
35.0
37.8
56.1
4.9
23.8
8.6
5.96
1.86
73.7
18.8
5.10
1.65
44.2
49.0
35.4
16.0
20.6
28.0
100
10
1
1
10
100
CD4
CD8β

didate signaling pathway is through the tyrosine kinase $p56^{lck}$, which is associated with the tails of both CD4 and CD8. However, this would present an apparent paradox: How can different signals be transmitted through the same tyrosine kinase depending on whether it is bound to CD4 or CD8? Possible explanations have already been discussed above in Section II,M and could include differences in signaling related to differences in the characteristics of the association between CD4 or CD8 and $p56^{lck}$: CD4 binds relatively more $p56^{lck}$ than does CD8 (Veillette *et al.*, 1988, 1989c; Luo and Sefton 1990); peptides mimicking regions of the cytoplasmic tails of CD4 and CD8 affect $p56^{lck}$ enzymatic activity quite differently (Bramson *et al.*, 1991); PMA causes dissociation of $p56^{lck}$ from CD4 but not CD8 (Hurley *et al.*, 1989); and crosslinking of CD4 leads to activation of the tyrosine kinase activity of $p56^{lck}$, while crosslinking of CD8 either does not or does so to a much lesser extent (Veillette *et al.*, 1989a,c, 1991; Luo and Sefton, 1990). Furthermore, differences in other associated proteins and/or available substrates might account for differential signals through CD4- versus CD8-associated $p56^{lck}$. It is also possible that the differential signals through CD4 and CD8 result not from $p56^{lck}$, but rather from a different pathway related to differences in other associations involving their unique transmembrane regions or the distinct portions of their cytoplasmic tails. For example, both CD4/$p56^{lck}$ and CD8/$p56^{lck}$ complexes have been shown to be associated with a 32-kDa GTP-binding protein (Telfer and Rudd, 1991); it could be that the latter protein is similar but distinct in the two complexes or is utilized differentially in signaling by one versus the other. The elucidation of the differences in CD4 versus CD8 signaling pathways is clearly a major focus of current interest and of critical importance in understanding the mechanisms of T cell differentiation and activation.

FIG. 7. Expression of CD4 and endogenous CD8 on thymocytes and lymph node cells in recipients of transgenic $\alpha\beta$ TCR and chimeric CD8/CD4 bone marrow. Thymocytes (a, b, and c) and lymph node cells (d, e, and f) from female C57BL/6J recipients of bone marrow from the $\alpha\beta$ TCR^+ (a and d), $\alpha\beta$ TCR^+ plus full-length $CD8\alpha^+$ (β-AL2.1$^+$) (b and e), and $\alpha\beta$ TCR^+ plus chimeric CD8/$CD4^+$ (β-AprCD8/4$^+$) (c and f) transgenic mice were analyzed for surface expression of CD4 and endogenous CD8 (as measured by CD8β expression) on cells expressing the transgenic $\alpha\beta$ TCR (detected by the mAb T3.70). Cells were stained by mAb 53-5, specific for CD8β, followed by Texas red-conjugated mouse antirat antibody. After thorough washing the cells were stained with biotin-conjugated T3.70 followed by allophycocyanin-conjugated avidin and phycoerythrin-conjugated anti-CD4. Cells were analyzed on a fluorescence-activated cell sorter. Reprinted with permission from *Nature* (Seong *et al.*). Copyright (1992) MacMillan Magazines Ltd.

ACKNOWLEDGMENTS

We are grateful to Ms. Sharon Dickow for her help in preparing the manuscript. This work was supported by NIH Grants CA46507, GM34991, and AI19512 to J.R.P. M.C.M. is the recipient of a senior postdoctoral fellowship from the American Cancer Society, California Division.

REFERENCES

Abraham, K. M., Levin, S. D., Marth, J. E., Forbush, K. A., and Perlmutter, R. M. (1991a). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 3977–3981.

Abraham, K. M., Levin, S. D., Marth, J. D., Forbush, K. A., and Perlmutter, R. M. (1991b). *J. Exp. Med.* **173,** 1421–1432.

Abraham, N., and Veillette, A. (1990). *Mol. Cell. Biol.* **10,** 5197–5206.

Abraham, N., Miceli, M. C., Parnes, J. R., and Veillette A. (1991). *Nature (London)* **350,** 62–66.

Acres, R. B., Conlon, P. J., Mochizuki, D. Y., and Gallis, B. (1986). *J. Biol. Chem.* **261,** 16210–16214.

Acres, R. B., Conlon, P. J., Mochtzuki, D. Y., and Gallis, B. (1987). *J. Immunol.* **139,** 2268–2274.

Aldrich, C. J., Hammer, R. E., Jones-Youngblood, S., Koszinowksi, U., Hood, L., Stroynowski, I., and Forman, J. (1991). *Nature (London)* **352,** 718–721.

Allen, H., Fraser, J., Flyer, D., Calvin, S., and Flavell, R. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7447–7451.

Amrein, K. E., and Sefton, B. M. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85,** 4247–4251.

Anderson, P., Blue, M. L., Morimoto, C., and Schlossman, S. F. (1987). *J. Immunol.* **139,** 678–682.

Anderson, P., Blue, M. L., and Schlossman, S. F. (1988). *J. Immunol.* **140,** 1732–1737.

Appleby, M. W., Gross, J. A., Cooke, M. P., Levin, S. D., Qian, X., and Perlmutter, R. M. (1992). *Cell (Cambridge, Mass.)* **70,** 751–763.

Ballhausen, W. G., Reske-Kunz, A. B., Tourvieille, B., Ohashi, P. S., Parnes, J. R., and Mak, T. W. (1988). *J. Exp. Med.* **167,** 1493–1498.

Bank, I., and Chess, L. (1985). *J. Exp. Med.* **162,** 1294–1303.

Bekoff, M., Kakiuchi, T., and Grey, H. M. (1985). *J. Immunol.* **134,** 1337–1342.

Berg, L. J., Fazekas de St. Groth, B., Pullen, A. M., and Davis, M. M. (1989a). *Nature (London)* **340,** 559–562.

Berg, L. J., Pullen, A. M., Fazekas de St. Groth, B., Mathis, D., Benoist, C., and Davis, M. M. (1989b). *Cell (Cambridge, Mass.)* **58,** 1035–1046.

Berg, L. J., Frank, G. D., and Davis, M. M. (1990). *Cell (Cambridge, Mass.)* **60,** 1043–1053.

Berridge, M. J. (1984). *Biochem. J.* **220,** 345–360.

Beyers, A. D., Spruyt, L. L., and Williams, A. F. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **9,** 2945–2949.

Bjorkman, P. J., Saper, M. A., Samraovi, B., Bennet, W. S., Strominger, J. L., and Wiley, D. C. (1987). *Nature (London)* **329,** 506–512.

Blackman, M., Kappler, J., and Marrack, P. (1990). *Science* **248,** 1335–1341.

Borgulya, P., Kishi, H., Muller, U., Kirberg, J., and von Boehmer, H. (1991). *EMBO J.* **10,** 913–918.

Bramson, H. N., Casnelli, J. E., Nachod, H., Regan, L. M., and Sommers, C. (1991). *J. Biol. Chem.* **266,** 16219–16225.

Burgess, K. E., Odyseos, A. D., Zalvan, D., Druker, B. L., Anderson, P., Schlossman, S. F., and Rudd, C. E. (1991). *Eur. J. Immunol.* **21,** 1663–1670.

Bustelo, X. R., Ledbettor, J. A., and Barbacid, M. (1992). *Nature (London)* **356,** 68–71.
Cammarota, G., Scheirle, A., Takacs, B., Doran, D. M., Knorr, R., Bannwarth, W., Guardiola, J., and Sinigaglia, F. (1992). *Nature (London)* **356,** 799–801.
Caron, L., Abraham, N., Pawson, T., and Veillette, A. (1992). *Mol. Cell. Biol.* **12,** 2720–2729.
Carrera, A. C., Wee, S.-B., Robert, T. M., and Pardoll, D. M. (1992). *J. Immunol.* **149,** 3142–3149.
Casnelli, J. E., Harrison, M. L., Pike, L. J., Hellstrom, K. E., and Krebs, E. G. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 282–286.
Ceredig, R., Dialynas, D. P., Fitch, F. W., and MacDonald, H. R. (1983). *J. Exp. Med.* **158,** 1654–1671.
Chalupny, N. J., Ledbetter, J. A., and Kavathas, P. (1991). *EMBO J.* **10,** 1201–1207.
Chan, A. C., Irving, B. A., Fraser, J. D., and Weiss, A. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 9166–9170.
Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992). *Cell* **71,** 649–662.
Clayton, L. K., Seih, M., Pious, D., and Reinherz, E. L. (1989). *Nature (London)* **339,** 548–551.
Clement, L. T. (1992). *J. Clin. Immunol.* **12,** 1–10.
Cockcroft, S., and Gomperts, B. D. (1985). *Nature (London)* **314,** 534–536.
Collins, T. L., Uniyal, S., Shin, J., Strominger, J. L., Mittler, R. S., and Burakoff, S. J. (1992). *J. Immunol.* **148,** 2159–2162.
Connolly, J. M., Potter, T. A., Wormstall, E., and Hansen, T. H. (1988). *J. Exp. Med.* **168,** 325–341.
Connolly, J. M., Hansen, T. H., Ingold, A. L., and Potter, T. A. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2137–2141.
Cooke, M. P., Abraham, K. M., Forbush, K. A., and Perlmutter, R. M. (1991). *Cell (Cambridge, Mass.)* **65,** 281–291.
Cooper, J. A. (1990). *In* "Peptides and Protein Phosphorylation" (B. Kemp, ed.), pp. 85–113. CRC Press, Boca Raton, Florida.
Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C., and Mathis, D. (1991). *Cell (Cambridge, Mass.)* **66,** 1051–1066.
Deans, J. P., Kanner, S. B., Torres, R. M., and Ledbetter, J. A. (1992). *Eur. J. Immunol.* **22,** 661–668.
Dembić, Z., Haas, W., Zamoyska, R., Parnes, J. R., Steinmetz, M., and von Boehmer, H. (1987). *Nature (London)* **326,** 510–511.
De Vries, J. E., Yssel, H., and Spits, H. (1989). *Immunol. Rev.* **109,** 119–141.
Dianzani, U., Lugman, M., Rojo, J., Yagi, J., Baron, J. L., Woods A., Janeway, C. A., Jr., and Bottomly, K. (1990). *Eur. J. Immunol.* **20,** 2249–2257.
Dianzani, U., Shaw, A., Al-Ramadi, B. K., Kubo, R. T., and Janeway, C. A., Jr. (1992a). *J. Immunol.* **148,** 678–688.
Dianzani, U., Redoglia, V., Malavasi, F., Bragardo, M., Pileri, A., Janeway, C. A., and Bottomly, K. (1992b). *Eur. J. Immunol.* **22,** 365–371.
Doyle, C., and Strominger, J. L. (1987). *Nature (London)* **330,** 256–257.
Emmrich, F., Strittmatter, U. and Eichmann, K. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8298–8301.
Emmrich, F., Kanz, L., and Eichmann, K. (1987). *Eur. J. Immunol.* **17,** 529–534.
Fantl, W. J., Escobedo, J. A., Martin, G. A., Turck, C. W., del Rosario, M., McCormick, F., and Williams, L. T. (1992). *Cell (Cambridge, Mass.)* **69,** 413–423.
Fleury, S., Lamarre, D., Meloche, S., Ryu, S.-E., Cantin, D., Hendrickson, W. A., and Sekaly, R.-P. (1991). *Cell (Cambridge, Mass.)* **66,** 1037–1049.
Fowlkes, B. J., and Pardoll, D. M. (1989). *Adv. Immunol.* **44,** 207–264.

Fowlkes, B. J., Schwartz, R. H., and Pardoll, D. M. (1988). *Nature (London)* **334,** 620–625.

Frank, S. J., Samelson, L. E., and Klausner, R. D. (1990). *Sem. Immunol.* **2,** 89–97.

Fung-Leung, W.-P., Schilham, M. W., Rahemtulla, A., Kundig, T. M., Vollenweider, M., Potter, J., van Ewijik, W., and Mak, T. W. (1991). *Cell* **65,** 443–449.

Fung-Leung, W.-P., Wallace, V. A., Gray, D., Sha, W. C., Pircher, H., Teh, H.-S., Loh, D. Y., and Mak, T. W. (1992). *Eur. J. Immunol.* (in press).

Gabert, J. C., Langlet, C., Zamoyska, R., Parnes, J. R., Schmitt-Verhulst, A. M., and Malissen, B. (1987). *Cell (Cambridge, Mass.)* **50,** 545–554.

Gallagher, P. F., Fazekas de St. Groth, B., and Miller, J. F. A. P. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86,** 10044–10048.

Gassman, M., Guttinger, M., Amrein, K. E., and Burn, P. (1992). *Eur. J. Immunol.* **22,** 283–286.

Gay, D., Maddon, P., Sekaly, R., Talle, M. A., Godfrey, M., Long, E., Goldstein, G., Chess, L., Axel, R., Kappler, J., and Marrack, P. (1987). *Nature (London)* **328,** 626–629.

Gilman, A. G. (1987). *Annu. Rev. Biochem.* **56,** 615–649.

Glaichenhaus, N., Shastri, N., Littman, D. R., and Turner, J. M. (1991). *Cell (Cambridge, Mass.)* **64,** 511–520.

Goldstein, S. A. N., and Mescher, M. F. (1988). *J. Immunol.* **140,** 3707–3711.

Gorman, S. D., Sun, Y. H., Zamoyska, R., and Parnes, J. R. (1988). *J. Immunol.* **10,** 3646–3653.

Grusby, M. J., Johnson, R. S., Papaioannou, V. E., and Glimcher, L. H. (1991). *Science* **253,** 1417–1420.

Guidos, C. J., Danska, J. S., Fathman, C. G., and Weissman, I. L. (1990). *J. Exp. Med.* **172,** 835–845.

Harnett, M. M., and Klaus, G. G. (1988). *Immunol. Today* **9,** 1315–1330.

Haughn, L., Gratton, S., Caron, L., Sekaly, R.-P., Veillette, A., and Julius, M. (1992). *Nature (London)* **358,** 328–331.

Hsi, E. D., Siegel, J. M., Minami, Y., Luong, E. T., Klausner, R. D., and Samelson, L. E. (1989). *J. Biol. Chem.* **264,** 10836–10842.

Hugo, P., Waanders, G. A., Scollay, R., Shortman, K., and Boyd, R. L. (1990). *Int. Immunol.* **2,** 209–216.

Hurley, T. R., Luo, K., and Sefton, B. M. (1989). *Science* **245,** 407–409.

Hyman, R., and Trowbridge, I. S. (1981). *Immunogenetics* **12,** 511–523.

Hyman, R., Trowbridge, I. S., Stallings, V., and Trotter, J. (1982). *Immunogenetics* **15,** 413–420.

Ingold, A. L., Lande, C., Knall, C., Evans, G. A., and Potter, T. A. (1991). *Nature (London)* **352,** 721–723.

Irving, B. A., and Weiss, A. (1991). *Cell (Cambridge, Mass.)* **64,** 891–901.

Janeway, C. A., Jr. (1988). *Nature (London)* **335,** 208–210.

Janeway, C. A., Jr. (1991). *Sem. Immunol.* **3,** 153–160.

Janeway, C. A., Jr. (1992). *Annu. Rev. Immunol.* **10,** 645–674.

June, C. H., Fletcher, M. C., Ledbetter, J. A., and Samelson, L. E. (1990a). *J. Immunol.* **144,** 1591–1599.

June, C. H., Fletcher, M. C., Ledbetter, J. A., Schieven, G. L., Siegel, J. N., Phillips, A. F., and Samulson, L. E. (1990b). *Proc. Natl. Acad. Sci. U.S.A.* 7722–7762.

Kanner, S. B., Deans, J. P., and Ledbetter, J. A. (1992). *Immunology* **75,** 441–447.

Kappler, J. W., Roehm, N., and Marrack, P. (1987a). *Cell (Cambridge, Mass.)* **49,** 273–283.

Kappler, J. W., Wade, T., White, J., Kushnir, E., Blackman, M., Bill, J., Roehm, R., and Marrack, P. C. (1987b). *Cell (Cambridge, Mass.)* **49,** 263–271.

Kappler, J. W., Staerz, U. D., White, J., and Marrack, P. C. (1988). *Nature (London)* **332,** 35–40.

Kappler, J. W., Kushir, E., and Marrack, P. (1989). *J. Exp. Med.* **169,** 1533–1542.

Karaki, S., Tanabe, M., Nakauchi, H., and Takiguchi, M. (1992). *J. Immunol.* **149,** 1613–
1618.

Kashishian, A., Kazlauskos, A., and Cooper, J. A. (1992). *EMBO J.* **11,** 1373–1382.

Kaye, J., Hsu, M.-L., Sauron, M. E., Jameson, S. C., Gascoigne, N. R. J., and Hedrick, S. M. (1989). *Nature (London)* **341,** 746–750.

Killeen, N., Moriarty, A., Teh, H.-S., and Littman, D. R. (1992). *J. Exp. Med.* **176,** 89–97.

Kirszbaum, L., Sharpe, J. A., Goss, N., Lahnstein, J., and Walker, I. D. (1989). *J. Immunol.* **142,** 3931–3936.

Kisielow, P., Teh, H. S., Blüthmann, H., Staerz, U. D., Steinmetz, M., and von Boehmer, H. (1988). *Nature (London)* **333,** 742–746.

Koch, C., Anderson, D., Moran, M., Ellis, C., and Pawson, T. (1991). *Science* **252,** 668–674.

Koller, B. H., Marrack, P., Kappler, J. W., and Smithies, O. (1990). *Science* **248,** 1227–1231.

König, R., Huang, L. Y., and Germain, R. N. (1992). *Nature (London)* **356,** 796–798.

Koretzky, G. A., Picus, J., Thomas, M. L., and Weiss, A. (1990). *Nature (London)* **346,** 66–68.

Koretzky, G. A., Kohmetscher, M. A., Kadleck, T., and Weiss, A. (1992). *J. Immunol.* **149,** 1138–1142.

Kruisbeek, A. M., Fultz, M. J., Sharrow, S. O., Singer, A., and Mond, J. J. (1983). *J. Exp. Med.* **157,** 1932–1941.

Kruisbeek, A. M., Mond, J. J., Fowlkes, B. J., Carmen, J. A., Bridges, S. A., and Longo, D. L. (1985). *J. Exp. Med.* **161,** 1029–1047.

Kupfer, A., Singer, S. J., Janeway, C. A., Jr., and Swain, S. L. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84,** 5888–5892.

Leahy, D. J., Axel, R., and Hendrickson, W. A. (1992). *Cell (Cambridge, Mass.)* **68,** 1145–1162.

Ledbetter, J. A., Seaman, W. E., Tsu, T. T., and Herzenberg, L. A. (1981). *J. Exp. Med.* **153,** 1503–1516.

Ledbetter, J. A., June, C. H., Rabinovich, P. S., Grossman, A., Tsu, T. T., and Imboden, J. B. (1988). *Eur. J. Immunol.* **18,** 525–532.

Ledbetter, J. A., Gilland, L. K., and Schieven, G. L. (1990). *Sem. Immunol.* **2,** 99–106.

Lee, W. T., and Vitetta, E. S. (1992). *Eur. J. Immunol.* **22,** 1455–1459.

Lee, N. A., Loh, D. Y., and Lacy, E. (1992). *J. Exp. Med.* **175,** 1013–1025.

Letourneur, F., and Klausner, R. D. (1992). *Science* **255,** 79–82.

Letourneur, F., Gabert, J., Cossen, P., Blanc, D., Davoust, J., and Malissen, B. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2339–2343.

Liaw, C. W., Zamoyska, R., and Parnes, J. R. (1986). *J. Immunol.* **137,** 1037–1043.

Lo, D., and Sprent, J. (1986). *Nature (London)* **319,** 672–675.

Luo, K. X., and Sefton, B. M. (1990). *Mol. Cell. Biol.* **10,** 5305–5313.

Luqman, M., Johnson, P., Trowbridge, I., and Bottomly, K. (1991). *Eur. J. Immunol.* **21,** 17–22.

MacDonald, H. R., Hengartner, H., and Pedrazzini, T. (1988a). *Nature (London)* **355,** 174–176.

MacDonald, H. R., Schneider, R., Lees, R. L., Howe, R. C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R. M., and Hengartner, H. (1988b). *Nature (London)* **322,** 40–43.

Margolis, B., Hu, P., Katzav, S., Li, W., Oliver, J. M., Ullrich, A., Weiss, A., and Schlessinger, J. (1992). *Nature (London)* **356,** 71–74.

Marrack, P. C., and Kappler, J. (1987). *Science* **238,** 1073–1079.

Marrack, P. C., Lo, D., Brinster, R., Palmiter, R., Burkly, L., Flavell, R. H., and Kappler,
J. W. (1988). *Cell (Cambridge, Mass.)* **53,** 627–634.

Marth, J. D., Cooper, J. A., King, S. F., Zeigler, S. F., Tinker, D. A., Overall, R. W., Krebs, E. G., and Perlmutter, R. M. (1988). *Mol. Cell. Biol.* **8,** 540–550.

Marusic-Galesic, S., Stephany, D. A., Longo, D. L., and Kruisbeek, A. M. (1988). *Nature (London)* **333,** 180–189.

Mazerolles, F., Amblard, F., Lumbroso, C., Lecomte, E., Van de Moortele, P. F., Barbat, C., Piatier Tonneau, D., Auffray, C., and Fischer, A. (1991). *Eur. J. Immunol.* **20,** 637–645.

Mazerolles, F., Hauss, P., Barbat, C., Figdor, C., and Fischer, A. (1990). *Eur. J. Immunol.* **21,** 887–894.

McCarthy, S. A., Kruisbeek, A., Uppenkamp, I. K., Sharrow, S. O., and Singer, A. (1988). *Nature (London)* **336,** 76–79.

Miceli, M. C., and Parnes, J. R. (1991). *Sem. Immunol.* **3,** 133–141.

Miceli, M. C., von Hoegen, P., and Parnes, J. R. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2623–2627.

Mittler, R. S., Goldman, S. F. J., Spitalny, G. L., and Burakoff, S. F. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86,** 8531–8535.

Mittler, R. S., Rankin, B. M., and Kiener, P. A. (1992). *J. Immunol.* **147,** 3434–3440.

Moebius, U., Kober, G., Griscelli, A. L., Hercend, T., and Meuer, S. C. (1991). *Eur. J. Immunol.* **21,** 1793–1800.

Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K.-U., Veillette, A., Davidson, D., and Mak, T. W. (1992). *Nature (London)* **357,** 161–164.

Mustelin, T., and Altman, A. (1990). *Oncogene* **5,** 809–813.

Mustelin, T., Coggeshall, K. M., Isakov, N., and Altman, A. (1990). *Science* **247,** 1584–1587.

Nada, S., Okada, M., MacAuley, A., Cooper, J., and Nakagawa, H. (1991). *Nature (London)* **351,** 69–72.

Nakayama, K.-I., and Loh, D. Y. (1992). *Science* **257,** 94–96.

Nakayama, T., June, C. H., Munitz, T. I., Sheard, M., McCarthy, S. A., Sharrow, S. O., Samelson, L. E., and Singer, A. (1990a). *Science* **249,** 1558–1562.

Nakayama, T., Singer, A., Hsi, E. D., and Samelson, L. E. (1990b). *Nature (London)* **341,** 651–654.

Newell, M. K., Haughn, L. J., Maroun, C. R., and Julius, M. H. (1990). *Nature (London)* **20,** 286–288.

Norment, A. M., Salter, R. D., Parham, P., Englehard, V. H., and Littman, D. L. (1988). *Nature (London)* **336,** 79–81.

Nikolic-Zugic, J. (1991). *Immunol. Today* **12,** 65–70.

Nikolic-Zugic, J., and Bevan, M. J. (1990). *Nature (London)* **344,** 65–67.

O'Rourke, A. M., and Mescher, M. F. (1992). *Nature (London)* **358,** 253–255.

O'Rourke, A. M., Rogers, J., and Mescher, M. (1990). *Nature (London)* **346,** 187–189.

Ostergaard, H. L., and Trowbridge, I. S. (1990). *J. Exp. Med.* **172,** 347–350.

Ostergaard, H. L., and Trowbridge, I. S. (1991). *Science* **251,** 1423–1425.
Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86,** 8959–8963.
Overduin, M., Rios, C. B., Mayer, B. J., Baltimore, D., and Cowburn, D. (1992). *Cell (Cambridge, Mass.)* **70,** 697–704.
Owens, T., Fazekas de St. Groth, B., and Miller, J. F. A. P. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9209–9213.
Park, D. J., Rho, H. W., and Rhee, S. G. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 5453–5456.
Parnes, J. R. (1989). *Adv. Immunol.* **44,** 265–311.
Parnes, J. R., von Hoegen, P., and Miceli, M. C. (1989). *CSH Symp. Quant. Biol.* **54,** 649–655.
Parsons, T. J., and Weber, M. J. (1989). *Curr. Top. Microbiol. Immunol.* **147,** 79–127.
Paterson, D. J., and Williams, A. F. (1987). *J. Exp. Med.* **166,** 1603–1613.
Pessa-Morikawa, T., Mustelin, T., and Andersson, L. C. (1990). *J. Immunol.* **144,** 2690–2697.
Pingel, J. T., and Thomas, M. L. (1989). *Cell* **58,** 1055–1065.
Pircher, H., Kurki, K., Lang, R., Hengartner, H., and Zinkernagel, R. M. (1989). *Nature (London)* **342,** 559–562.
Pircher, H., Rohrer, U. H., Moskophidis, D., Zinkernagel, R. M., and Hengartner, H. (1991). *Nature (London)* **351,** 482–485.
Potter, T. A., Boyer, C., Schmitt-Verhulst, A. M., Goldstein, P., and Rajan, T. V. (1984). *J. Exp. Med.* **160,** 317–322.
Potter, T. A., Rajan, T. V., Dick, R. F., and Bluestone, J. A. (1989). *Nature (London)* **337,** 73–75.
Rahemtulla, A., Fung-Leung, W. P., Schilham, M. W., Kundig, T. M., Sambhara, S. R., Narendran, A., Arabian, A., Wakeham, A., Paige, C. J., Zinkernagel, R. M., Miller, R. G., and Mak, T. W. (1991). *Nature (London)* **353,** 180–183.
Ramsdell, F., and Fowlkes, B. J. (1989). *J. Immunol.* **143,** 1467–1472.
Ratnofsky, S. E., Peterson, A., Greenstein, J. L., and Burakoff, S. J. (1987). *J. Exp. Med.* **166,** 1747–1757.
Resh, M. D., and Ling, H.-P. (1990). *Nature (London)* **346,** 84–88.
Reske-Kunz, A. B., and Rüde, E. (1985). *Eur. J. Immunol.* **15,** 1048–1054.
Reth, M. (1989). *Nature (London)* **338,** 383–384.
Robey, E. A., Fowlkes, B. J., and Pardoll, D. M. (1990). *Sem. Immunol.* **2,** 25–34.
Robey, E. A., Fowlkes, B. J., Gordon, J. W., Kioussis, D., von Boehmer, H., Ramsdell, F., and Axel, R. (1991). *Cell (Cambridge, Mass.)* **64,** 99–107.
Robey, E. A., Ramsdell, F., Kioussis, D., Sha, W., Loh, D., Axel, R., and Fowlkes, B. J. (1992). *Cell (Cambridge, Mass.)* **69,** 1089–1096.
Rojo, J. M., Saizawa, K., and Janeway, C. A., Jr. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86,** 3311–3315.
Rosenstein, Y., Ratnofsky, S., Burakoff, S. J., and Hermann, S. H. (1989). *J. Exp. Med.* **169,** 149–160.
Rosoff, P. M., Burakoff, S. J., and Greenstein, J. L. (1987). *Cell (Cambridge, Mass.)* **49,** 845–853.
Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L., and Schlossman, S. F. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85,** 5190–5194.
Ryu, S. E., Kwong, P. D., Truneh, A., Porter, T. G., Arthos, J., Rosenberg, M., Dai, X., Xuong, N.-H., Axel, R., Sweet, R. W., and Hendrickson, W. A. (1990). *Nature (London)* **348,** 419–426.

Saizawa, K., Rojo, J., and Janeway, C. A., Jr. (1987). *Nature (London)* **328,** 260–263.

Salter, R. D., Norment, A. M., Chen, B. P., Clayberger, C., Krensky, A. M., Littman, D. R., and Parham, P. (1989). *Nature (London)* **338,** 345–347.

Salter, R. D., Benjamin, R. J., Wesley, P. K., Buxton, S. E., Garrett, T. P. J., Clayberger, C., Krensky, A. M., Norment, A. M., Littman, D. R., and Parham, P. (1990). *Nature (London)* **345,** 41–46.

Samelson, L. E., Patel, M. D., Weissman, A. M., Harford, J. B., and Klausner, R. D. (1986). *Cell (Cambridge, Mass.)* **46,** 1083–1090.

Samelson, L. E., Phillips, A. F., Luong, E. T., and Klausner, R. D. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4358–4362.

Sanders, S. K., Fox, R. O., and Kavathas, P. (1991). *J. Exp. Med.* **174,** 371–379.

Schwartz, R. H. (1989). *Cell* **57,** 1073–1081.

Scollay, R., Wilson, A., D'Amico, A., Kelley, K., Egerton, M., Pearse, M., Wu, L., and Shortman, K. (1988). *Immunol. Rev.* **104,** 81–104.

Scott, B., Blüthmann, H., Teh, H. S., and von Boehmer, H. (1989). *Nature (London)* **338,** 591–595.

Seong, R. H., Chamberlain, J. W., and Parnes, J. R. (1992). *Nature (London)* **356,** 718–720.

Sha, W. C., Nelson, C., Newberry, R. C., Kranz, D. M., Russell, J. H., and Loh, D. Y. (1988a). *Nature (London)* **335,** 271–274.

Sha, W. C., Nelson, C. A., Newberry, R. D., Kranz, D. M., Russell, J. H., and Loh, D. Y. (1988b). *Nature (London)* **336,** 73–76.

Sha, W. C., Nelson, C. A., Newberry, R. D., Pullen, J. D., Pease, L. R., Russell, J. H., and Loh, D. Y. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 6186–6190.

Shaw, A. S., Amrein, K. E., Hammond, C., Stern, D. F., Sefton, B. F., and Rose, J. F. (1989). *Cell (Cambridge, Mass.)* **59,** 627–636.

Shaw, A. S., Chalupny, J., Whitney, J. A., Hammond, C., Amrein, K. E., Kavathas, P., Sefton, B. M., and Rose, J. K. (1990). *Mol. Cell. Biol.* **10,** 1853–1862.

Sleckman, B. P., Peterson, A., Jones, W. K., Foran, J. A., Greenstein, J. L., Seed, B., and Burakoff, S. J. (1987). *Nature (London)* **328,** 351–353.

Sleckman, B. P., Peterson, A., Foran, J. A., Gorga, J. C., Kara, C. J., Strominger, J. L., Burakoff, S. J., and Greenstein, J. L. (1988). *J. Immunol.* **141,** 49–54.

Sprent, J., Lo, D., Gao, E.-K., and Ron, Y. (1988). *Immunol. Rev.* **101,** 173–190.

Sprent, J., Gao, E.-K., and Webb, S. R. (1990). *Science* **248,** 1357–1363.

Stamenkovic, I., Sgroi, D., Aruffo, A., Sy, M. S., and Anderson T. (1991). *Cell (Cambridge, Mass.)* **66,** 1133–1144.

Stein, P. L., Lee, H. M., Rich, S., and Soriano, P. (1992). *Cell (Cambridge, Mass.)* **70,** 741–750.

Straus, D. B., and Weiss, A. (1992). *Cell (Cambridge, Mass.)* **70,** 585–593.

Swain, S. L. (1983). *Immunol. Rev.* **74,** 129–142.

Takada, S., and Engleman, E. G. (1987). *J. Immunol.* **139,** 3231–3235.

Teh, H. S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Blüthmann, H., and von Boehmer, H. (1988). *Nature (London)* **335,** 229–233.

Teh, H. S., Kishi, H., Scott, B., and von Boehmer, H. (1989). *J. Exp. Med.* **169,** 795–802.

Teh, H. S., Kishi, H., Scott, B., Borguly, P., von Boehmer, H., and Kisielow, P. (1990). *Dev. Immunol.* **1,** 1–10.

Telfer, J. C., and Rudd, C. E. (1991). *Science* **254,** 439–441.

Terry, L. A., DiSanto, J. P., Small, T. N., and Flomenberg, N. (1990). *Tissue Ant.* **35,** 82–91.

Thomas, M. L. (1989). *Annu. Rev. Immunol.* **7,** 339–369.
Tite, T. P., Sloan, A., and Janeway, C. A., Jr. (1986). *J. Mol. Cell Immunol.* **2,** 179–185.
Turner, J. M., Brodsky, M. H., Irving, B. A., Levin, S. D., Perlmutter, R. M., and Littman, D. R. (1990). *Cell (Cambridge, Mass.)* **60,** 755–765.
Ullrich, A., and Schlessinger, J. (1990). *Cell (Cambridge, Mass.)* **61,** 203–212.
Van Oers, N. S., Garvin, A. M., Davis, C. B., Forbush, K. A., Carlow, D. A., Littman, D. R., Perlmutter, R. M., and Teh, H. S. (1992). *Eur. J. Immunol.* **22,** 735–743.
Van Seventer, G. A., van Lier, R. A. W., Spits, H., Ivanyi, P., and Melief, C. J. M. (1986). *Eur. J. Immunol.* **16,** 1363–1371.
Vega, M. A., Kuo, M. C., Carrera, A. C., and Strominger, J. L. (1990). *Eur. J. Immunol.* **20,** 453–456.
Veillette, A., Bookman, M. B., Horak, E. H., and Bolen, J. B. (1988). *Cell (Cambridge, Mass.)* **55,** 301–308.
Veillette, A., Bolen, J. B., and Bookman, M. A. (1989a). *Mol. Cell. Biol.* **9,** 4441–4446.
Veillette, A., Bookman, A., Horak, E. M., Samelson, L. E., and Bolen, J. B. (1989b). *Nature (London)* **338,** 257–259.
Veillette, A., Zúñiga-Pflücker, J. C., Bolen, J. B., and Kruisbeek, A. M. (1989c). *J. Exp. Med.* **170,** 1671–1680.
Veillette, A., Abraham, N., Caron, L., and Davidson, D. (1991). *Sem. Immunol.* **3,** 143–152.
von Boehmer, H. (1986). *Immunol. Today* **7,** 333–336.
von Boehmer, H., and Kisielow, P. (1990). *Science* **248,** 1369–1373.
Walker, I. D., Murray, B. J., Hogarth, P. M., Kelson, A., and McKenzie, I. F. C. (1984). *Eur. J. Immunol.* **14,** 906–910.
Wang, J., Yan, Y., Garrett, T. P. J., Liu, J., Rodgers, D. W., Garlick, R. L., Tarr, G. E., Husain, Y., Reinherz, E. L., and Harrison, S. C. (1990). *Nature (London)* **348,** 411–418.
Wange, R. L., Tony Kong, A.-N., and Samelson, L. E. (1992). *J. Biol. Chem.* **267,** 11685–11688.
Wassmer, D., Chan, C., Lodgedeberg, L., and Shevach, E. M. (1985). *J. Immunol.* **135,** 2237–2242.
Weber, J. R., Bell, G. M., Han, M. Y., Pawson, T., and Imboden, J. B. (1992). *J. Exp. Med.* **176,** 373–379.
Wegener, A.-M. K., Letourneur, F., Hoeveler, A., Brocker, T., Luton, F., and Malissen, B. (1992). *Cell (Cambridge, Mass.)* **68,** 83–97.
Weiss, A., Koretzky, G., Schatzman, R., and Kadlecek, T. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 5484–5488.
Weyand, C. M., Goronzy, J., and Fathman, C. G. (1987). *J. Immunol.* **138,** 1351–1356.
Wheeler, C. J., von Hoegen, P., and Parnes, J. R. (1992). *Nature (London)* **357,** 247–249.
Williams, D. B., Barber, B. H., Flavell, R. A., and Allen, H. (1989). *J. Immunol.* **142,** 2796–2806.
Zamoyska, R., Derham, P., Gorman, S. D., von Hoegen, P., Bolen, J. B., Veillette, A. V., and Parnes, J. R. (1989). *Nature (London)* **342,** 278–281.
Zijlstra, M., Bix, M., Simister, N. E., Loring, J. M., Raulet, D. H., and Jaenisch, R. (1990). *Nature (London)* **344,** 742–746.
Zúñiga-Pflücker, J. C., Jones, L. A., Longo, D. L., and Kruisbeek, A. M. (1989a). *CSH Symp. Quant. Biol.* **54,** 153–160.
Zúñiga-Pflücker, J. C., Longo, D. L., and Kruisbeek, A. M. (1989b). *Nature (London)* **338,** 76–79.

Zúñiga-Pflücker, J. C., McCarthy, S. A., Weston, M., Longo, D. L., Singer, A., and Kruisbeek, A. (1989c). *J. Exp. Med.* **169,** 2085–2096.
Zúñiga-Pflücker, J. C., Jones, L. A., Longo, D. L., and Kruisbeek, A. M. (1990). *J. Exp. Med.* **171,** 427–437.
Zúñiga-Pflücker, J. C., Jones, L. A., Chin, L. T., and Kruisbeek, A. M. (1991). *Sem. Immunol.* **3,** 167–175.

This article was accepted for publication on 4 November 1992.

ADVANCES IN IMMUNOLOGY, VOL. 53

B Lymphopoiesis in the Mouse

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I. Introduction

Early development of cells along the B lymphocyte lineage pathway is marked by successive gene rearrangements in H chain, then in L chain loci, by selective expression of surface-located and intracellular markers, by differential proliferation potential *in vitro* on stromal cells in the presence of cytokines, and by a differential capacity to populate the B cell compartments of severe combined immunedeficient (SCID) mice. The molecular processes involved in immunoglobulin gene rearrangements (Taccioli *et al.*, 1992), selective expression of markers and their potential functions, and proliferative and differentiating capacities of progenitors and precursors (Kincade *et al.*, 1989; Dorshkind, 1990; Rolink and Melchers, 1991; Rajewsky, 1992) for Ly-1$^+$ and Ly-1$^-$ cells (CD5$^+$ and CD5$^-$) (Hardy, 1992) have repeatedly been updated in reviews.

When V_H and V_L gene segments are rearranged, the resulting repertoire of V_H/V_L combinations expressed in immunoglobulin molecules on the surface of B cells is exposed to selective processes. Negative selection against self antigens either deletes or anergizes self-reactive B cells (Nemazee and Bürki, 1989; Goodnow *et al.*, 1989). However, the peripheral repertoire of B cells appears also positively selected by antigen (Förster and Rajewsky, 1990; Gu *et al.*, 1991a). Positive selection might be part of an antigen-specific response of B cells to proliferation and maturation to immunoglobulin-secreting cells and memory cells, or might also be a change of migratory properties and life expectancies without division and differentiation. B cells can respond to T cell-independent and T cell-dependent antigens (Melchers and Anderson 1984; Liu *et al.*, 1992), but only the latter induce immunoglobulin class switching, somatic hypermutations, and memory (McKean *et al.*, 1984; MacLennan and Gray, 1986; Berek and Milstein, 1987; Jacob *et al.*, 1991; Schittek and Rajewsky, 1992).

This review centers on recent progress in our understanding of early B cell development from pluripotent stem cells to surface

immunoglobulin-positive (sIg^+) B cells, which are then selected to die or to live. Progress has been made in several areas of research:

1. The detection and elucidation of the structure of differentiation stage-specific markers expressed in and on B lineage cells.
2. The classification of the function(s) of some of these markers in B lymphocyte development.
3. The generation of long-term proliferating B lineage committed stem cells *in vitro* and the population of B lineage compartments in SCID mice.
4. The generation of transgenic mice, either by heterologous insertion of one or several copies of a transgene with functions in the B lineage, or by a targeted integration of a defective gene at the proper site in the genome by homologous recombination [knock-out (ko) mice] and analysis of the resulting lesions in B cell development.

The main emphasis of this review is on work done with mice. B cell development in humans, sheep, rabbits, chicken, frogs, and fish shows fascinating similarities and differences (reviewed in Litman *et al.*, 1989; Rathbun *et al.*, 1989; Zachau, 1989; Weill and Reynaud, 1992; Wilson and Warr, 1992; Du Pasquier *et al.*, 1992; Wilson *et al.*, 1992).

II. B Cells from Embryonic Stem Cells

The earliest cell with the capacity to generate B cells is an embryonic stem (ES) cell. ES cells are established from the inner cell mass of a mouse blastocyst between the 16- and 64-cell stage of development. These ES cells can be grown in tissue culture. Transfection of DNA into the ES cells can lead to either heterologous or homologous incorporation into chromosomes. ES cells can then be introduced into another blastocyst and the blastocyst can be transferred into a foster mother, who develops and gives birth to a chimeric mouse. The injected ES cells can participate in the formation of all tissues. If germ cells are included, a new, transgenic strain of mice is established. If B lineage cells are included, functions of the transgenic, mutant genes expressed in the B lineage can be assayed. In principle, the chimeric mice do not have to transmit the transgene into the germ line as long as the transgene encodes a dominant trait that can be assayed in the mouse. This should be the principle by which many detailed functions of genes are investigated without establishing a mouse strain for each mutation.

Several reports indicate that progenitors of B cells can be gener-

ated from ES cells entirely by *in vitro* techniques (Chen *et al.*, 1992; Gutierrez-Ramos and Palacios, 1992). ES cells can differentiate *in vivo* to embryoid bodies, from which B lineage cells can be established. This should eventually enable us to study the functions of many genes with functions in the B lymphocytic lineage without ever establishing a mouse strain for it. Once a pluripotent, a lymphoid-committed, or a B lineage-committed stem cell (see below) has been established in culture, immunodeficient (SCID) mice can be populated with these progenitors and precursors and the functions of the parts of the hematopoietic system, which develop from these progenitors and precursors, can be studied in the SCID host.

III. Differential Expression of Lineage-Related Markers during B Cell Development in Different Body Sites

B lymphocyte development occurs in multiple body sites during pre- and postembryonic development, prenatally in embryonic blood, yolk sac, embryonic placenta, liver, and omentum (Solvasan and Kearney, 1992), and postnatally in blood, spleen, and, most of all, bone marrow (Melchers, 1979; Ogawa *et al.*, 1988; Rolink and Melchers, 1991; Rolink *et al.*, 1993).

The enzyme terminal deoxyribonucleotidyltransferase (TdT), involved in the insertion of N-regions in D_H-to-J_H and V_H-to-D_HJ_H joints of the H chain genes, is expressed postnatally in bone marrow, but not prenatally in fetal liver (Gregoire *et al.*, 1979). Consequently, practically all joints of fetal liver-derived pre-B and B cells have no N-region diversity, whereas most of the joints of postnatal bone marrow-located and -derived pre-B and B cells do. Although the presence of N-regions is known to contribute to increased variability in the antigen-binding, complementarity-determining region 3 of H chains, the functional significance of an absence of such increased variability in fetal liver-derived H chains remains a matter of speculation (Rolink and Melchers, 1991). It appears that H chain loci with N-region diversity are selected against in preimmune B cell repertoires (Carlsson *et al.*, 1992).

The vast majority of V_L-to-J_L joints are devoid of N-regions, because the enzyme TdT is already shut off at these late stages of pre-B cell development. It is not known what turns on or off the expression of TdT, and whether productive rearrangements in the H chain loci and a subsequent expression of μH chains on the surface of pre-B cells contribute to the downregulation of expression of TdT. Nevertheless, earlier stages ($B220^-$ TdT^+ and $B220^+$ TdT^+ cells) can be

distinguished (Osmond, 1990; early and intermediate pro-B cells) from later stages ($B220^+$ TdT^-, late pro-B, and large and small pre-B cells) in bone marrow. The late stages are expected to have their H chain gene rearrangements completed, which makes some of these cells detectable by the expression of cytoplasmic μH chains. The later $B220^+$ TdT^- stages of pre-B cells are also expected to be in the process of (maybe continuous and multiple) rearrangements of their L chain loci (Feddersen and van Ness, 1990; Harada and Yamagishi, 1991).

Fetal liver-derived pre-B cells can also be distinguished from bone marrow-derived pre-B cells by the differential expression of a novel regulatory myosin light chain in the bone marrow (Oltz *et al.*, 1992) and a novel LIM-homeobox domain in fetal liver-derived cells (F. Alt, personal communication). The proteins of the surrogate L chain expressed by the pre-B cell-specific genes V_{preB} and λ_5 are expressed on early progenitors and pre-BI cells, on some pre-BII cells, and on some intermediate, immature B cells, but not in mature B cells, memory cells, or immunoglobulin-secreting plasma cells (Sakaguchi and Melchers, 1986; Kudo *et al.*, 1987, 1989, 1992; Pillai and Baltimore, 1987, 1988; Kudo and Melchers, 1987; Kerr *et al.*, 1989; Karasuyama *et al.*, 1990, 1992; Misener *et al.*, 1990, 1991; Cherayil and Pillai, 1991; Karasuyama *et al.*, 1992).

The surrogate L chain appears on the surface even before μH chains are expressed, together with a complex of proteins (p130/p55), of which one (p55) is disulfide bonded to the λ_5 protein (Karasuyama *et al.*, 1992). On subsequent cells of the B lineage differentiation pathway, the surrogate L chain can be associated by disulfide bonds to the $D_H/J_HC\mu$ protein (Reth and Alt, 1984) and to the $V_HD_HJ_H$-rearranged μH chain. It has been found on Abelson-virus-transformed pre-B cell lines of these stages of differentiation, but the expression on the corresponding normal pre-B cells needs to be investigated. It has been speculated that these immunoglobulin-like molecules on the surface of pro- and pre-B cells recognize a set of differentiation antigens on the cooperating stroma cells, and that this recognition controls critical cell divisions and subsequent steps of differentiation, including those of subsequent immunoglobulin gene rearrangements, along the pathway of B cell differentiation (Melchers *et al.*, 1989) (Fig. 1). We will discuss below where these speculations are supported or contradicted by experimental findings.

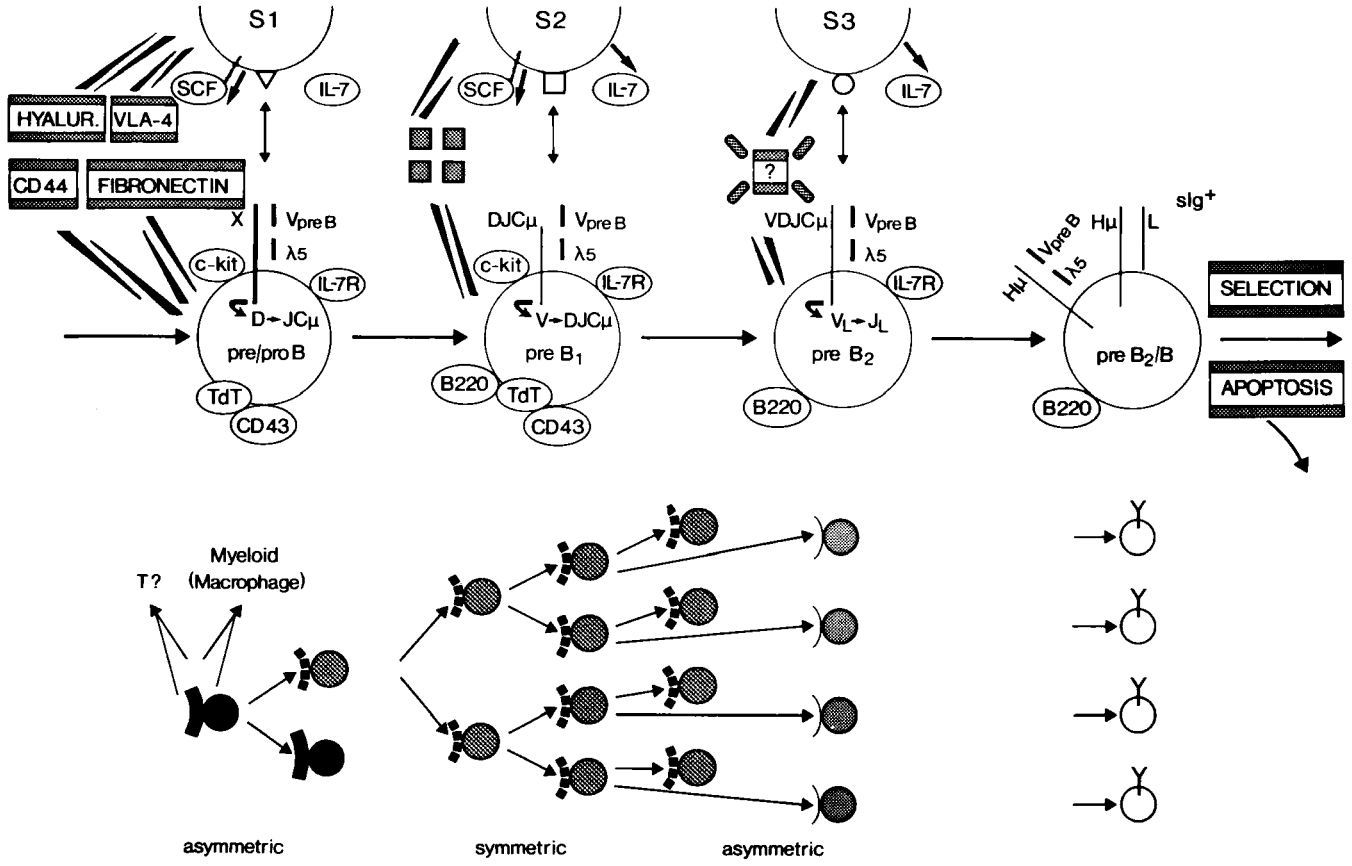
B cell differentiation has also been ordered by the differential expression of other lineage-related markers. In addition to the

high-molecular-weight form of the common leukocyte antigen B220 (CD45), which is not expressed on the earliest progenitors but is thereafter found on all B lineage cells, are other antigens: the heat-stable antigen (HSA) is not expressed on very early pro-B cells but is on pre-B cells (Hardy *et al.*, 1991); the surface-located enzyme aminopeptidase, called BP-1, is expressed on late pre-B cells but not on early pre-B cells (Hardy *et al.*, 1991); leukosialin (CD43), detected by the monoclonal antibody (mAb) S7, is detected on early, but not on late, pre-B cells (Hardy *et al.*, 1991), much the same (Rolink *et al.*, 1992) as the tyrosine kinase c-kit encoded in the *white-spotting* locus of the mouse and the receptor for the growth factor ligand stem cell factor (SCF) encoded by the *steel* locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Zsebo *et al.*, 1990; Anderson *et al.*, 1990; Copeland *et al.*, 1990; Huang *et al.*, 1990; Okada *et al.*, 1991; Ogawa *et al.*, 1991; Kodama *et al.*, 1992). As expected, the genes *RAG-1* and *RAG-2* which encode elements of the rearrangement complex, are expressed throughout all stages of pre-B cell development (Blackwell *et al.*, 1986; Lieber *et al.*, 1987; Schatz and Baltimore, 1988), as is the protooncogene N-*myc* (Zimmerman and Alt, 1990). The expression of these markers can be regulated by surface immunoglobulin in N-*myc*-transgenic pre-B and immature B cells (Ma *et al.*, 1992).

Many $B220^-$ and $B220^+$ $CD43^+$ c-kit^+ TdT^+ cells are expected to be D_HJ_H-rearranged but not $V_HD_HJ_H$-rearranged pre-BI cells, whereas many of the $B220^+$ $CD43^-$ c-kit^- BP-1^+ TdT^- cells might already be $V_HD_HJ_H$ rearranged (Rolink *et al.*, 1991a; Hardy *et al.*, 1991; Hardy and Hayakawa, 1991), but that is likely to be an oversimplification of a much more complex set of pre-B cell subpopulations differing in all these parameters. Such a predictably oversimplified picture of marker expression during early B cell development is shown in Fig. 1.

IV. Proliferation and Differentiation Capacities of B Cell Subpopulations

Proliferation and differentiation of pro- and pre-B cells require cell contact with a microenvironment of stromal cells present in the primary organs where the B cells develop (reviewed in Kincade *et al.*, 1989; Dorshkind, 1990; Rolink and Melchers, 1991). B cell development in fetal liver appears to occur in one synchronous wave, first in a stromal cell-dependent phase between days 13 and 16 of gestation, followed by a stromal cell-independent phase between days 17 and 19. Between days 16 and 17, sIg^+ cells appear; these cells become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pre B1
B220
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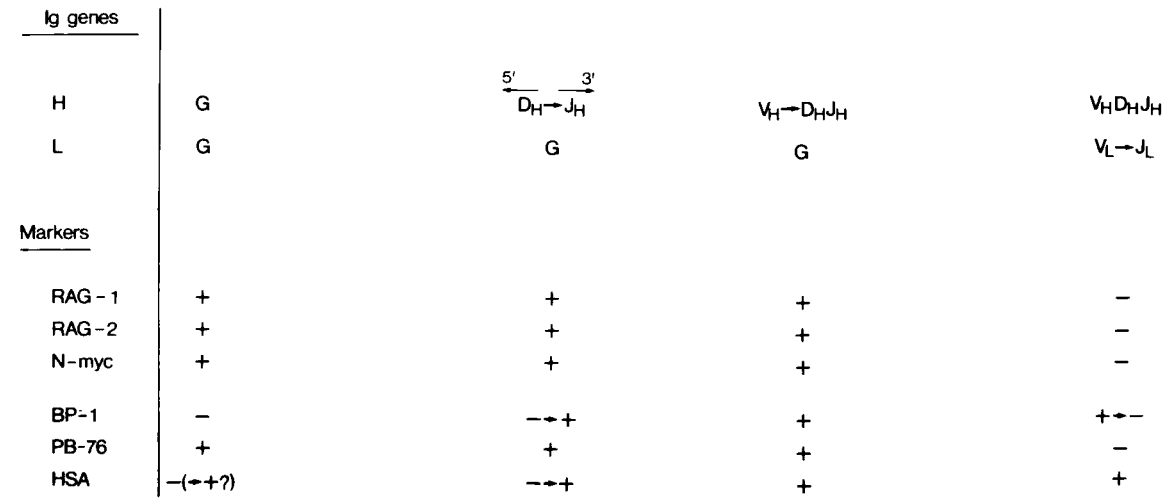

FIG. 1. B lymphopoiesis in the mouse. The figure shows four different stages of B cell development in interaction with stromal cells at different stages of immunoglobulin gene rearrangements expressing different intracellular and surface markers, some of them functionally involved in this development. It also shows the proliferative capacity of these subpopulations for asymmetric or symmetric divisions. For details, see text.

mitogen reactive between days 18 and 19 (Strasser *et al.*, 1989). These experiments suggested that in the early, stromal cell-dependent phase gene rearrangements in the immunoglobulin heavy and light chain loci take place, whereas in the later, stromal cell-independent phase sIg^+ B cells mature to mitogen-reactive cells. On primary stromal cells from fetal liver or bone marrow very little proliferation is observed *in vitro*, even in the presence of a variety of interleukins (ILs), including IL-7. This changes dramatically when cloned stromal cell lines are used in tissue culture.

We now know of a variety of stromal cells (usually preadipocytic fibroblast clones and lines from fetal liver, bone marrow, and even thymus) that support the long-term proliferation of defined subpopulations of pro- and pre-B cells from fetal liver, blood, spleen, and bone marrow. IL-7 has been identified as a major cytokine that costimulates this proliferation (Namen *et al.*, 1988), but it is likely that other cytokines such as IGF-I (Landreth *et al.*, 1992), IL-6, IL-11, GM-CSF, and *steel*-encoded SCF (McNiece *et al.*, 1991; Hirayama *et al.*, 1992; Landreth *et al.*, 1992) have costimulatory activities, maybe with different types of stromal cells, and maybe on different stages and subpopulations of pre-B cell development.

We have established lines and clones of pro- and pre-B cells from fetal liver, blood, spleen, and bone marrow that proliferate in serum-substituted media in the presence of exogenously added IL-7 and in contact with stromal cells for long periods of time (Rolink *et al.*, 1991a). They can be cloned and recloned with efficiencies near 100% and retain their stage of differentiation, as described below. They are capable of differentiation to sIg^+ B cells *in vivo* and *in vitro*, and they can populate pre-B and B cell compartments of SCID mice for long periods of time. Therefore, they have properties of B lineage-committed stem cells, which undergo equal divisions into two cells at the same stage of differentiation when they proliferate on stromal cells in the presence of IL-7, and they can undergo unequal, or differentiating, divisions into one or two differentiated, eventually mature B cells when removal from their environment of stromal cells and IL-7 induces their differentiation. Nishikawa and colleagues (Nishikawa *et al.*, 1988; Hayashi *et al.*, 1990) have defined three stages of pro- and pre-B cell development by colony assays of early cells (which need only stromal cells to proliferate), a subsequent stage (which needs stromal cells and IL-7 to proliferate), and a late stage (which needs only IL-7 to proliferate). It has been suggested that in the intermediate stage contact of pro- and pre-B cells with stromal

cells may, in fact, induce the production of IL-7 in stromal cells (Sudo *et al.*, 1989).

A. Long-Term Proliferating Pro- and Pre-B Cells Reactive to Stromal Cells and IL-7

The high cloning efficiency of pro- and pre-B cells reactive to stromal cells and IL-7 is apparently already a property of cell clones *ex vivo*. Cloning *ex vivo* from B cell-generating organs by limiting dilution cultures has identified a pro- or pre-B cell as a limiting cell. This has allowed quantitative analysis of the frequencies, and consequently of the absolute numbers, of such clonable pro- and pre-B cells in liver, blood, spleen, and bone marrow throughout the life of a mouse (Rolink *et al.*, 1993). Frequencies and numbers of clonable cells change in the different organs during a life span. A wave of clonable cells appears before birth and disappears after birth in liver (Melchers, 1979; Rolink *et al.*, 1993). Up to 2 weeks after birth, high frequencies of clonable cells are present in spleen and are also detectable in blood, but become undetectable at 6–8 weeks in these sites. In bone marrow, up to 2% of all cells are clonable early, but decrease 10- to 20-fold within 6 months after birth.

Clonable pro- and pre-B cells are enriched in the $B220^-$ c-kit^{low} as well as the $B220^+$ c-kit^+ (and $B220^+$ $CD43^+$) cell populations of bone marrow. They are depleted from $B220^+$ c-kit^- and $B220^+$ $CD43^-$ populations, and are absent in $B220^-$ c-kit^- and $B220^-$ c-kit^{high} populations of bone marrow. $B220^+$ c-kit^+ and $B220^+$ $CD43^+$ cells are likely to be the same, because long-term proliferating $B220^+$ c-kit^+ cells in culture also express $CD43^+$ (Fig. 1). The absolute numbers of clonable $B220^-$ c-kit^{low} and of $B220^+$ c-kit^+ cells drop 5- to 20-fold within 6 months after birth.

Clonable pro- and pre-B cells, capable of long-term proliferation on stromal cells in the presence of IL-7, are present in fetal liver and bone marrow of a wide variety of different inbred strains of mice, transgenic mice, and mice in which genes with functions in the B lineage pathway of differentiation have been inactivated by targeted integration of a defective gene. The frequencies and absolute numbers of such clonable cells are equal, if not higher, in severe combined immunodeficient (Bosma *et al.*, 1988) and RAG-2^{ko} mice (Shinkai *et al.*, 1992), in B cell-deficient μH chain–transmembrane^{ko} mice (Kitamura *et al.*, 1991), in λ_5^{ko} mice (Kitamura *et al.*, 1992), in B cell-deficient CBA/N mice, and in autoimmune disease-prone (NZB/

NZW)F_1 mice (see below). This indicates that the stromal cell/IL-7 reactive compartment of clonable cells includes these cells before there are rearrangements in the H chain loci, and before expression of μH chain proteins on the surface.

The frequencies of clonable pro- and pre-B cells are depressed in mice expressing transgenic μH chains (Grosschedl *et al.*, 1984). It is not clear at present whether the lower numbers of clonable cells are a result of accelerated differentiation (Reichman-Fried *et al.*, 1990; Era *et al.*, 1991) or of deletion of cells. Finally, mice expressing transgenic κL chains (Carmack *et al.*, 1991) and λL chains (Weiss and Bogen, 1991; Vasicek *et al.*, 1992) are either delayed or severely depressed in their B cell development. The numbers of clonable pro- and pre-B cells are also reduced. It remains to be seen whether the transgenic L chains, prematurely expressed in pro- and pre-B cells, in fact compete with surrogate L chains for the binding to p130/p55, to $D_HJ_HC\mu$ protein, and to μH chains, and thereby inhibit proper development before the stage of a clonable pro- or pre-B cell.

Long-term proliferating, clonable pro- and pre-B cells are either $B220^-$ or $B220^+$, BP-1^- or BP-1^+, $PB76^+$, $CD43^+$, c-kit^{low} or c-kit^+, HSA^+ or HSA^-, RAG-1^+, RAG-2^+, N-myc^+, and MHC class I^+, are reactive to IL-7 (i.e., probably express IL-7 receptors), express the surrogate L chain V_{preB} and λ_5 on their surface, and are μH chain negative, but express p130/p55 (probably on the surface) (Karasuyama *et al.*, 1992) (Fig. 1). They do not express CD3, CD4, CD5, CD8, MHC class II, or the receptors for IL-2 or IL-5 and are not reactive to mitogens such as lipopolysaccharide. Depending on whether they are bone marrow or fetal liver derived, they express (respectively, do not express) TdT and the new regulatory myosin light chain, and they do not express (respectively, express) the LIM homeobox-domain gene.

Early in the development of fetal liver and of neonatal bone marrow, at least some of the H chain loci in clonable cells are still in germ-line configuration. This can also be concluded from experiments with bone marrow cells from RAG-1^{ko} or RAG-2^{ko} mice, which have normal, if not elevated, numbers of clonable cells (see above). For the proliferation of such early pro-B cells, stromal cells may well contribute with other contacts and other cytokines, in addition to the exogenously added IL-7. Nishikawa and colleagues have identified early clonable cells that proliferate on stromal cells in the absence of IL-7 (Hayashi *et al.*, 1990). Such clonable cells might still be $B220^-$, and it remains to be investigated whether they are only B lineage committed.

Bipotent precursors for B lineage cells and macrophages have been found early in fetal liver development; these precursors derive their dual potential from a cell with all immunoglobulin loci still in germ-line configuration (Cumano *et al.*, 1992; Cumano and Paige, 1992). Similar bipotent cells have previously been found as virus-transformed early progenitor lines (Holmes *et al.*, 1986; Davidson *et al.*, 1988). In addition, $B220^-$ progenitors have been found that give rise to T and B lineage cells (Takai *et al.*, 1992)

H chain loci in germ-line configuration can still be found after 2 or 3 weeks in tissue cultures of early fetal liver- or bone marrow-derived pro- and pre-B cells, i.e., at a time when a single cell could have expanded to (2–5) $\times$ 10^6 cells. This might indicate that these early pro-B cells keep their germ-line H chain gene conformation by asymmetric divisions in which one germ-line pro-B cell gives rise to one germ-line and one D_HJ_H-rearranged cell (Fig. 1).

In the tissue culture conditions under discussion, i.e., on stromal cells in the presence of IL-7, the early pro-B and pre-B cells eventually change their properties after prolonged proliferation *in vitro*. They may remain $B220^-$ or become $B220^+$, remain HSA^- or become HSA^+, or remain $BP\text{-}1^-$ or become $BP\text{-}1^+$, but it is clear that a clone of early pro- or pre-BI cells with some H chain loci still in germ-line configuration and some already D_HJ_H-rearranged undergoes continuous, subsequent D_H-to-J_H rearrangement, maybe in both H chain loci, and until the most 5′ D_H or the most 3′ J_H segment has been used in these rearrangements (Rolink *et al.*, 1993; Haasner *et al.*, in preparation). Once all possible D_HJ_H rearrangements have been made, the resulting final D_HJ_H stably arranged pre-BI cells continue to proliferate on stromal cells in the presence of IL-7 (unless they are rearranged in reading frame II and express a $D_HJ_HC\mu$ protein on the surface (Reth and Alt, 1984; Gu *et al.*, 1991a) (see also below for the function of the surrogate L chain) and continue to display the properties of a B lineage-committed stem cell as outlined above (Fig. 1).

B. Molecular Contacts between Pre-B Cells and Stromal Cells

Some of the molecular contacts that regulate pro- and pre-BI cell proliferation in contact with stromal cells, and that may control the performance of stromal cells in providing contacts and cytokines, have been identified. Monoclonal antibodies specific for c-kit inhibit the *in vitro* proliferation of some (Rolink *et al.*, 1991c) but not all (Collins and Dorshkind, 1987; Kodama *et al.*, 1992) long-term proliferating cells. This indicates that c-kit regulates pro- and pre-B cell

proliferation, in line with the observation that the majority of all long-term proliferating pro- and pre-B cells express c-kit on their surface (see above). The *steel*-encoded stem cell factor has been found to be a costimulatory cytokine in IL-7-stimulated pre-B cell growth (McNiece *et al.*, 1991), but it remains to be seen whether the membrane-bound or the secreted form is costimulatory, and how these two forms of SCF regulate pre-B cell growth.

Other contacts between pre-B cells and stromal cells involve VLA-4 and fibronectin (Miyake *et al.*, 1991) and CD44 and hyaluronate (Miyake *et al.*, 1990a,b), but these contacts may be different for different stages of pro- and pre-B cell development, and it remains be investigated whether CD44, as one example, always uses the same splice form of its gene to be expressed for these contacts (Stamenkovic *et al.*, 1991).

Finally, we expect the surrogate L chain, in association with early complexes of p130/p55 (Karasuyama *et al.*, 1992), with $D_HJ_HC\mu$ protein, and with $V_HD_HJ_H$-rearranged μH chain, to be involved in contacts and controls affecting proliferation and differentiation of pro- and pre-B cells (Melchers *et al.*, 1989), but the partners in such contacts on stromal cells have not been identified, nor is it clear what the functional consequences of these contacts could be.

C. Precursor B Cells That Do Not Proliferate on Stromal Cells in the Presence of IL-7 for Long Periods of Time

The vast majority of pre-B cells in bone marrow are $B220^+$ $CD43^-$ c-kit^-. *In vitro* they do not proliferate in symmetric divisions for extended periods on stromal cells in the presence of IL-7. Some of them, however, are still reactive to IL-7 alone for a limited number of divisions (Hayashi *et al.*, 1990; Rolink *et al.*, 1991a). These pre-BII cells are likely to still express RAG-1 and RAG-2, as well as the surrogate L chain, but they are probably all TdT^- and have their $V_HD_HJ_H$ rearrangements completed on at least one of the two H chain loci (Osmond, 1990, 1991; Hardy *et al.*, 1991). Association of a productive $V_HD_HJ_H$-rearranged μH chain, with the surrogate L chain may, in fact, signal the cell to turn off CD43 and c-kit expression, but that remains to be investigated in greater detail. Removal of IL-7 from *in vitro* cultures of pre-BI cells certainly allows unproductive rearrangements of H and/or L chain gene loci, and this differentiation also leads to downregulation of CD43 and c-kit, to a loss of stromal cell/IL-7 reactivity, and to apoptosis of sIg^- and sIg^+ differentiated cells (Rolink *et al.*, 1991a).

It is possible that pre-BII cells represent a transitory state on the

way to mature sIg^+ or sIg^- B cells, with no interactive capacity with stromal cells and no special B cell-generating functions. Because these pre-BII cells are, however, so frequent, i.e., they represent over 90% of all $B220^+$ pre-B cells (Osmond, 1990, 1991), it is conceivable that they are occupying a bone marrow pre-B cell compartment controlled by stimuli different from those controlling pre-BI cells. Pre-BII cells may divide once every day (see below) in asymmetrical divisions to produce one new pre-BII cell and one cell on its way to L chain rearrangements and to a mature B cell (Fig. 1). *In vivo* this might occur in situations when all sites in the bone marrow are occupied and when further divisions crowd the marrow too much, so that only one of the two cells produced in the division remains attached to stroma and the other leaves the area of IL-7-production and, thereby, is induced to differentiate by initiating L chain gene rearrangements. In this scenario, contact of the pre-B cells with their ligands on stroma and with cytokine IL-7 keeps them at their given stage of differentiation, whereas loss of ligands induces differentiation. Pre-BI cells are kept at their given stage by stromal cells and IL-7; pre-BII cells may be kept at their given stage by IL-7 alone.

D. B Cell Pools and Steady States of Daily B Cell Production

It has been estimated that a mouse produces 5×10^7 mature B cells per day in adult bone marrow (Osmond, 1990), and it is assumed that this daily production is maintained at more or less the same level throughout life. These 5×10^7 cells include not only sIg^+ B cells, but also all cells that have made nonproductive rearrangements in H and/or L chain loci, yielding sIg^- mature cells. From these newly formed B cells, between 2×10^6 and 5×10^6 sIg^+ B cells are chosen daily to enter the peripheral pool of mature cells. It is evident from the combined analyses of Osmond, Hardy, Nishikawa, and their colleagues and from our own analyses that the number of $B220^-$ or $B220^+$ $c\text{-}kit^+$ $CD43^+$ pre-BI cells with long-term proliferative capacities as B lineage-committed progenitors is 10-fold too low to produce all the B cells. This assumes that such a precursor can divide once a day to yield one precursor and one more differentiated, in the end, mature B cell. Consequently, over 90% of all B cells generated per day are likely to be generated, by unequal divisions, from the $B220^+$ $CD43^-$ $c\text{-}kit^-$ TdT^- pre-BII cells, which have the appropriate pool size, if they divide once a day. In fact, Osmond (1990) has shown that this pool of late pro-B, large and small pre-B cells has the highest mitotic activity in bone marrow.

If many of these pre-BII cells are, in fact, already $V_HD_HJ_H$ rearranged, then most B cells are generated from precursors that have already committed themselves to some specificity toward an antigen, i.e., that specificity carried by the H chain alone. Because H chains are known to be able to contribute a large part of the antigen specificity of an immunoglobulin molecule, it will be interesting to see whether pre-BII cells, with μH chains and surrogate L chains on their surface, are selectable by antigen. This should have important consequences for the maintenance of B cell tolerance, for positive selection of B cells into the peripheral pool, and for possible idiotypic/antiidiotypic interactions.

It is not clear whether B cell generation continues at the same rate throughout life, i.e., whether B cell generation decreases as an animal ages. Decreased production of B cells with increasing age is found in chickens and sheep (Reynolds and Morris, 1983; Pink *et al.*, 1987). A higher daily rate of B cell production in young versus old mice is also implicit from the observation of Förster and Rajewsky (1990) that turnover of the predominant B cell population is high in young mice and low in older mice. Although the total number of $B220^+$ pre-B cells drops only twofold within the first 6 months of the life of a mouse, it is not yet known whether the *in vivo* proliferative capacities of the different pre-B cell subpopulations in the bone marrow remain the same. Our experiments indicate that the number of clonable, long-term proliferating pre-BI cells of the $B220^+$ c-kit^+ subpopulations drops by a factor of 5–10, whereas the earlier $B220^-$ c-kit^{low} progenitors and precursors drop within 6 months by 10- to 20-fold. However, the bone marrow environment (i.e., probably its stroma) does not become incompetent with age. Transplantation of young *and* old c-kit^+ B lineage precursors into *old* bone marrow (devoid of all precursors typically present in old bone marrow) allows an expansion of c-kit^+ precursors to levels that are characteristic of *young* bone marrow (Rolink *et al.*, 1993). It is likely that pre-BII cells, and maybe other cell types, compete for the sites in old bone marrow on which pre-BI cells are able to lodge and proliferate. Our finding that this ratio of pre-BII to preBI cells in bone marrow increases with age, but that it can be reversed, should be applicable to human bone marrow transplantation.

V. Possible Functions of the Surrogate L Chain in B Cell Differentiation

It has repeatedly been suggested that early differentiation along the B lymphocyte lineage pathway, marked by successive rearrange-

ments of immunoglobulin gene segments, is guided by the expression of productive rearrangements of the H chain locus, either $D_HJ_HC\mu$ proteins or as $V_HD_HJ_H$-rearranged μH chains. Deposition of the μH chains on the surface of the pre-B cells would signal the cells to begin rearranging, the next immunoglobulin gene segment along the differentiation pathway. Successful cells are thereby selected over unsuccessful cells along their way toward becoming a B cell (Melchers *et al.*, 1989; Misener *et al.*, 1991). Deposition on the surface is mediated by the disulfide-bonded association of the different forms of the μH chains with the surrogate L chain. Signaling is likely to occur via the immunoglobulin-associated molecules Ig-α and Ig-β, which are encoded by the *mb-1* and *B29* genes (Sakaguchi *et al.*, 1988; Hombach *et al.*, 1988; Hermanson *et al.*, 1988). Signaling along the B lineage differentiation pathway has been reviewed in detail elsewhere (Reth *et al.*, 1991; Alés-Martinez *et al.*, 1991).

It is evident from mice in which the λ_5 gene has been inactivated by targeted integration of a defective form of the gene (Kitamura *et al.*, 1992) that normal expression and function of the λ_5 gene is critical for normal B cell development. Although the heterozygous littermates of the λ_5^{ko} mice appear to develop their B cells normally, the homozygous λ_5^{ko} mice have an altered precursor pool and a delayed appearance of both Ly-1$^+$ and Ly-1$^-$ B cells. The Ly-1$^+$ B cell compartment appears normalized at 1 month of age, but the Ly-1$^-$ compartments fill up much more slowly, so that even 6 months after birth only around half of the normal numbers of Ly-1$^-$ B cells are present in the peripheral lymphoid organs. Even with the lower number of B cells, however, λ_5^{ko} mice mount normal immune responses to T-independent as well as T-dependent antigens.

When pro- and pre-B cells rearrange D_H segments to J_H segments, these rearrangements can occur in three reading frames. Reading frame II (rfII), but not rfI or rfIII, allows the expression of a $D_HJ_HC\mu$ protein, because the reading frame of J_H is in frame with the promoter and the start codon found upstream of most D_H elements within the H chain gene locus. This is particularly so for D_HJ_H joints made in fetal liver, where no N-region diversity is inserted (Holmberg *et al.*, 1989; Feeney, 1990; Meek, 1990; Gu *et al.*, 1990, 1991b). An analysis of the representation of rfI, rfII, and rfIII within the repertoire of pre-B cells of fetal liver has shown that rfII is suppressed in Ly-1$^-$ but not in Ly-1$^+$ B cells (Gu *et al.*, 1990, 1991b). This suppression has been interpreted to result from a suppression of all those pre-B cells expressing $D_HJ_HC\mu$ protein on their surface, and expansion on stromal cells in the presence of IL-7.

If surface deposition was mandatory for a signal to stop further expansion of rfII/D_HJ_H-rearranged pre-B cells, then pre-B cells of λ_5^{ko} mice should be unable to do so, because $D_HJ_HC\mu$ protein cannot be inserted into the surface membrane in the absence of the surrogate L chain. An analysis of the repertoire of D_HJ_H joints in pre-B cells of λ_5^{ko} mice, in fact, shows that rfII is present in approximately one-third of all joints, i.e., it is not suppressed (Melchers *et al.*, 1992). Similarly, $V_HD_HJ_H$-rearranged pre-B cells may be suppressed for further proliferation and expansion on stromal cells in the presence of IL-7, as experiments with μH chain-transgenic mice might suggest (Era *et al.*, 1991), as soon as the surrogate L chain deposits the μH chain in the surface membrane. Again, λ_5^{ko} pre-B cells should not be able to do this, and therefore accumulate $V_HD_HJ_H$-rearranged pre-B cells in the stromal cell/IL-7-reactive c-kit$^+$ compartment.

A delayed appearance of mature Ly-1$^-$ B cells in the periphery of λ_5^{ko} mice could be expected to be the result of an abnormally low rate of L chain gene rearrangements in $V_HD_HJ_H$-rearranged pre-BII cells that are incapable of inserting μH chains into their surface membrane. This assumes that rearrangements of L chain gene segments are induced in pre-B cells by the deposition of μH chains on their surface. However, our *in vitro* experiments with pre-B cell lines and clones support the notion that V_L-to-J_L rearrangements can occur without previous productive $V_HD_HJ_H$ rearrangement and insertion of μH chains into the surface membrane. Removal of IL-7 from the culture induces V_H-to-D_HJ_H and V_L-to-J_L rearrangements, even in pre-B cells that are never able to produce a productive V_H-to-D_HJ_H rearrangement (clone 18; in Rolink *et al.*, 1991b).

These findings make it unlikely that a slower rate of L chain rearrangements in λ_5^{ko} pre-B cells could explain the delayed appearance of sIg$^+$ B cells in the periphery of λ_5^{ko} mice. We expect that the surrogate L chain plays yet another role in B cell development. In fact, the surrogate L chain is found on the surface of progenitors and pre-B cells *before* μH chains are ever expressed (either as $D_HJ_HC\mu$ proteins or as $V_HD_HJ_H$-rearranged μH chains) (Misener *et al.*, 1991; Melchers *et al.*, 1992; Karasuyama *et al.*, 1992). Pre-BI cells from fetal liver with D_HJ_H-rearranged H chain loci in rfI or rfIII and stromal cell/IL-7-reactive progenitors from RAG-2^{ko} mice (Shinkai *et al.*, 1992; Melchers *et al.*, 1992) are such cells on which the surrogate L chain has been detected with the aid of monoclonal antibodies specific for V_{preB} and λ_5 (Melchers *et al.*, 1992; Karasuyama *et al.*, 1992). Immunoprecipitation with these monoclonal antibodies detects a complex of protein molecules (p130/p55) associated with the sur-

rogate L chain, of which p55 is disulfide bonded to the protein. A delayed B cell development in the λ_5^{ko} mice might be the result of the inability of the progenitor and pre-B cells of these mice to deposit this complex into the surface membrane. This might slow down either the proliferation or further development of these early cells along the B lymphocyte lineage pathway of differentiation.

There are other indications that such an early immunoglobulin-like complex might be involved in control of proliferation and/or differentiation of pro- to pre-B cells. Mice expressing transgenic κL or λL chain genes have a delayed, and in some cases very deficient, B cell development (Weiss and Bogen, 1991; Vasicek *et al.*, 1992). Surface deposition of an early complex, not with surrogate L chain but with transgenic κL or λL chains, on early progenitors may also disturb or slow down proliferation and/or further development of B lineage cells at the early stage of differentiation from pro- to pre-BI cells.

On the other hand, pre-BI-type stromal cell/IL-7-reactive cells from λ_5^{ko} mice can be cloned at normal, if not elevated, frequencies, from fetal liver or bone marrow of λ_5^{ko} mice. Even if they had their immunoglobulin H chain loci in germ-line configuration at the time of cloning *ex vivo*, they are D_HJ_H (and often also $V_HD_HJ_H$) rearranged, when they have grown from one precursor to 10^6-10^9 cells, i.e., within 30 divisions during 3–4 weeks *in vitro*. These observations do not point to a delayed capacity to rearrange the immunoglobulin H chain gene segments.

The most dramatic defect of λ_5^{ko} mice is their 40-fold reduced compartment of $B220^+$ $CD43^-$ c-kit^- pre-BII cells, leading to a delayed appearance of Ly-1^- sIg^+ B cells that is only 40% of normal, even at 6 months of age (Kitamura *et al.*, 1992). These Ly-1^- sIg^+ B cells could well be generated from approximately 5×10^5 D_HJ_H-rearranged pre-BI cells, if they generated that number of $V_HD_HJ_H$-rearranged, V_LJ_L-rearranged cells by asymmetric divisions each day and if 10% of all of these fully rearranged cells had a productive H *and* productive L chain gene and, therefore, became sIg^+, and if these B cells became long lived in the process. Half of the normal level of Ly-1^- sIg^+ B cells (i.e., 5×10^7 cells) would thus be reached in 100 days. This underlines the importance of the $B220^+$ $CD43^-$ c-kit^- pre-BII compartment for the development of a Ly-1^- sIg^+ B cell compartment in mice. The surrogate L chain plays the role of selecting from all $V_HD_HJ_H$-rearranged cells those having a productive rearrangement and, therefore, expressing μH chain protein. If the μH chain cannot be deposited in the surface membrane, as is the case in pre-B cells from RAG-2^{ko}, μH chain–transmembrane^{ko}, and λ_5^{ko} mice, this pre-

BII-compartment is greatly reduced or totally missing (Kitamura *et al.*, 1991, 1992; Kitamura and Rajewsky, 1992; Shinkai *et al.*, 1992).

VI. Differentiation of Pre-B to B Cells

A. Differentiation of Pre-B to B Cells *in Vitro*

Pre-BI cell lines and clones proliferating on stromal cells in the presence of IL-7 can be induced to undergo V_H-to-D_HJ_H and V_L-to-J_L rearrangements when IL-7 is removed from culture (Rolink *et al.*, 1991a). Within 2 to 3 days, 5–20% of all cells become sIg^+ and the rest remain sIg^-. More than 95% of the cells lose the capacity to proliferate on stromal cells in the presence of IL-7 within 2 days, indicating that all cells differentiate under these conditions. It is possible, but has yet to be proved by sequencing analyses, that the sIg^- mature cells develop from out-of-frame rearrangements in H and/or L chain genes. Extensive apoptosis of sIg^+ and sIg^- mature cells is observed in culture. One of the earliest markers of a mature B cell is the IL-2 receptor α chain (TAC antigen). Mitogen-reactive cells develop, though in lower frequencies. Compared to the frequencies of lipopolysaccharide (LPS)-reactive sIg^+ B cells of normal spleen (~1 to 3) (Andersson *et al.*, 1977), around 100-fold lower frequencies of LPS-reactive cells are observed, and these frequencies can vary from pre-B cell clone to pre-B cell clone by 5- to 10-fold. These experiments indicate that not all sIg^+ B cells are yet mature to the extent that they are mitogen reactive. Future experiments will have to evaluate what renders an originally LPS-unreactive sIg^+ B cell reactive, and whether selection forces of antigen and/or environmental cells and factors (T cells? cytokines?) play a role.

The majority (90–95%) of the sIg^+ B cells, which develop within 3 days of differentiation in culture, are $\mu^+/\kappa L^+$, whereas a small percentage (3–10%) expresses μH and λL chains. Thus, the high ratio of $\kappa L/\lambda L$ chains characteristic of the peripheral B cell repertoire of the mouse (Cohn and Langman, 1990) is already evident in the antigen-independent transition from pre-B to B cells (Rolink *et al.*, 1991a). The V_H repertoire expressed in the sIg^+ B cells is biased toward the V_H segment located 3′ in the V_H cluster (i.e., V_H7183 and V_HQ52) and, thus, resembles that of the V_H repertoires developing in fetal and neonatal B cells and in B cells during regeneration after bone marrow transplantation and before normalization (Streb, 1992).

Similar $\kappa L/\lambda L$ chain ratios and V_H repertoires develop in sIg^+ B cells

from pre-B cell clones transplanted into SCID mice *in vivo*, indicating that the normalization of the V_H repertoire to representation, according to gene segment copy numbers in the genomes, is dependent on factors (T cells?) that are not present in these SCID mice.

Differentiation of pre-B cells to sIg^+ B cells, of course, never occurs with pre-B cells of RAG-2^{ko} or μH–transmembrane^{ko} mice, though these cells enter other parts of the differentiation program by becoming unreactive to stromal cells and IL-7. Differentiation of pre-B cells from λ_5^{ko} mice to sIg^+ and LPS-reactive cells is apparently normal with respect to kinetics and ratios, indicating that V_H-to-D_HJ_H and V_L-to-J_L rearrangements can occur in pre-B cells that do not deposit productively rearranged μH chains into their surface membrane (Rolink *et al.*, in preparation).

B. Differentiation of Pre-B Cells to B Cells *in Vivo*

Transfer of long-term proliferating pre-BI cells from fetal liver into SCID mice leads to a stable population in the bone marrow of pre-B and sIg^+ B cells, to 5–10% of normal levels of sIg^+ B cells in spleen, and to normal levels of sIg^+ Ly-1^+ B cells in the peritoneum. Turnover of the pre-B and sIg^+ B cells in the bone marrow and spleen remains high for several months, although stable levels of pre-B and B cells are maintained for several months. Serum IgM levels in SCID mice become those of normal mice, whereas IgA levels are low and IgG levels remain below detection (Rolink *et al.*, 1991b; Reininger *et al.*, 1992). No donor-derived T cells or myeloid cells can be detected in the SCID hosts. This suggests that pre-BI cells are B lineage-committed stem cells, which are capable of populating at least the Ly-1^+ B cell compartments to normal levels.

Bone marrow-derived pre-BI cells lines and clones are often not capable of populating the pre-B and B cell compartments of mice in this way. These observations are in agreement with those of Hardy and Hayakawa (1991), whose experiments suggest that Ly-1^+ B cells originate from fetal liver-derived pre-B cells, whereas Ly-1^- B cells derive from pre-B cells of bone marrow. In their experiments, however, neither bone marrow- nor fetal liver-derived pre-B cells ever populate the SCID bone marrow stably with pre-B cells, so that one of the essential properties of B lineage-committed stem cells is apparently absent. It therefore remains to be investigated how the Ly-1^- B cell compartment is established, which cells and factors cooperate in B cell establishment, and how a normal level and a steady state of turnover of cells in this compartment are maintained. The pre-BI cell-populated

SCID mice offer the opportunity to see whether Ly-1$^-$ cells can be generated from an originally Ly-1$^+$ cell-oriented B cell development by the transfer of T cells and antigen into these mice.

C. B Cell Differentiation in CBA/N Immunodeficient Mice

CBA/N mice carry an X chromosome-linked immune defect (xid) that results in the inability of sIg$^+$ B cells to respond to T cell-independent antigens of type 2: these mice also have a much lower response to T cell-independent antigens of type 1, but their T cell-dependent responses are normal (Scher, 1982). CBA/N mice lack the Ly-1$^+$ B cell lineage (Hayakawa *et al.*, 1983) and have one-third of the normal number of peripheral sIg$^+$ B cells (Sprent *et al.*, 1985), although their B220$^+$, Abelson virus-infectable precursor B cell compartment appears to be normal (Kincade *et al.*, 1982; Reid and Osmond, 1985). It has recently been shown that in immunoglobulin-transgenic mice, expression of the μH and λL chains of the phosphorylcholine-specific antibody M167, under the control of the heavy chain enhancer *EμH* on the xid background, results in the elimination of most B cells from the peripheral lymphoid tissues of the xid mice, whereas only the phosphorylcholine-specific B cells are eliminated from xid mice expressing only the μH chain of the M167 antibody as a transgene (Kenny *et al.*, 1991). These results suggest that the phosphorylcholine-specific B cells that develop in μH chain-transgenic xid mice are either deleted via unknown, antigen-specific, surface immunoglobulin-mediated mechanisms, or that they fail to receive the appropriate signals to exit the bone marrow and enter the peripheral lymphoid organs. In support of this deletion is the finding that, in a μH chain-transgenic xid mouse expressing an H chain from a TNP-specific antibody (not directed against a self antigen, such as the ubiquitously present phosphorylcholine determinant), TNP-specific B cells develop normally in the periphery.

Pre-B cells from CBA/N mice proliferate normally on stromal cells in the presence of IL-7, and enter the programs of differentiation to sIg$^+$ stromal cell/IL-7-unreactive cells normally. However, these cells never become LPS reactive (Rolink *et al.*, in preparation). This suggests that the transition from immature sIg$^+$ B cells to mitogen-reactive sIg$^+$ B cells is impaired in CBA/N mice and might be involved in the initial selection of an Ly-1$^+$ B cell compartment. CBA/N mouse pre-B cells proliferating on stromal cells in the presence of IL-7 might be the appropriate tool to study the molecular and cellular bases of the xid-induced lesion that leads to the deletion of Ly-1$^+$ cells and that might be rescued by other environmental factors (T cells?) in the T cell-

dependent, Ly-1$^-$ B cell compartment. These cells might shed further light on the problem of how the Ly-1 B cell compartment is established and maintained.

D. Differentiation of Pre-B Cells to B Cells in *bcl-2*-Transgenic Cells

The *bcl-2* gene encodes a 26-kDa protein (and its 21-kDa variant form) (Tsujimoto and Croce, 1986); this protein is predominantly expressed in the inner mitochondrial membranes of mainly B and T lymphocyte lineage cells at defined stages of their differentiation (Granninger *et al.*, 1987; Reed *et al.*, 1987; Gurfinkel *et al.*, 1987; Hockenberry *et al.*, 1990; Pettersson *et al.*, 1992). It was originally identified in translocations of human follicular lymphomas as the locus (18q21) translocated to the human immunoglobulin heavy chain locus (14q32) (Tsujimoto *et al.*, 1984; Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Cleary *et al.*, 1986; Adachi *et al.*, 1990). Translocations leave the coding region intact but render the gene controllable by the immunoglobulin heavy chain locus, leading to constitutive high expression along the B lineage pathway from early precursors to plasma cells. Although the exact function of the *bcl-2* gene is still unknown, it rescues cells from programmed death (apoptosis) (Wyllie, 1980; Duvalle and Wyllie, 1986), thereby prolonging the half-life of the cells in which it is expressed. This has been shown for T lineage, B lineage, and other hematopoietic cells by transfection of the *bcl-2* gene (Vaux *et al.*, 1988; Borzillo *et al.*, 1992), and in *bcl-2*-transgenic mice (McDonnell *et al.*, 1989, 1990; Strasser *et al.*, 1990, 1991a,b; Sentman *et al.*, 1991; Nunez *et al.*, 1991). During the normal development of lymphocytes, *bcl-2* expression may play the role of changing the life span of antigen-selected lymphocytes during memory interaction to longevity (Hockenberry *et al.*, 1991; Hardie *et al.*, 1991; Pettersson *et al.*, 1992).

From all these studies it appeared reasonable to assume that pre-B cells from *Eμ/bcl-2*-transgenic mice (*bcl-2* under the control of the enhancer for the μH chain) would inhibit the apoptosis of B cells differentiating from long-term proliferating pro- and pre-B cells when IL-7 is removed from cultures of these cells on stromal cells. Indeed, proliferation of *bcl-2*-transgenic pro- and pre-B cells is normal on stromal cells in the presence of IL-7, and differentiation is normal on removal of IL-7 when the capacity to rearrange V_H to D_HJ_H and V_L to J_L is monitored, and when the loss of capacity for continued proliferation in stromal cells in the presence of IL-7 is monitored (Rolink *et al.*, in preparation). However, the differentiated cells do not die by apoptosis, and sIg$^+$ and sIg$^-$ cells are formed, the latter probably as a conse-

quence of out-of-frame rearrangements in either H and/or L chain loci. Only the sIg^+, but not the sIg^-, cells are stimulatable by the mitogen LPS to clonal proliferation, indicating that sIg expression is mandatory for LPS reactivity of the cells. During prolonged differentiation of pre-B to B cells *in vitro*, more and more of the sIg^+ cells express λL chains, so that after 1–2 weeks, in fact, up to 50% of all sIg^+ cells express λL chains, in strong contrast to the normal situation where the κL/λL ratio on sIg^+ B cells is 95:5 (Cohn and Langman, 1990; Rolink *et al.*, 1991c). A higher number of λL chain-expressing B cells might be expected in all situations, where the process of subsequent, secondary Vκ-to-Jκ rearrangements is allowed to continue (Feddersen and van Ness, 1990; Harada and Yamagishi, 1991) and where the eventual deletion of Cκ by rearrangements to the RS element downstream of Cκ deletes the κL chain locus.

Because the peripheral pool of B cells in a normal mouse is sIg^+ and does not contain sIg^- B cells, and because that is also the case in the *bcl-2*-transgenic mice, it suggests that exit from the bone marrow into the periphery and/or subsequent selection processes favor sIg^+ over sIg^- B cells.

The expression of *bcl-2* in differentiated B cells is likely to facilitate the molecular analysis and the cellular kinetics of B cell development from pre-B cells in normal, immunodeficient, and autoimmune disease-prone mice.

VII. Genetic Defects Expressed in the B Lineage that Lead to Autoimmune Disease

(NZB/NZW)F_1 (B/WF_1) mice spontaneously develop an autoimmune disease that closely resembles systemic lupus erythematosus (SLE). The disease is characterized by elevated serum levels of IgM and IgG, i.e., by hypergammaglobulinemia, and by elevated levels of IgM antinuclear antibodies (ANAs) and the appearance of IgG ANA antibodies with specificities for single-stranded as well as double-stranded DNA and for histones. In addition, IgG antibodies specific for erythrocytes and for retroviral envelope glycoprotein gp70 are detected. The animals finally succumb to fatal immune complex-mediated glomerulonephritis (Howie and Helyer, 1968; Staples and Talal, 1969; Lambert and Dixon, 1968; Steward and Hay, 1976; Moutsopoulos *et al.*, 1977; Izui *et al.*, 1978, 1979, 1981a,b; Goldings *et al.*, 1980; Gioud *et al.*, 1983; Maruyama *et al.*, 1983; Slack *et al.*, 1984). An excessive B cell activity could be one of the major abnormalities in these mice, as the parental NZB strain shows an enhanced and accelerated precursor B cell development (Ohsugi and Gershwin, 1979;

Jyonouchi and Kincade, 1984; Yoshida *et al.*, 1985, 1987; Schwieterman *et al.*, 1992).

The inheritance of the SLE-like autoimmune disease in B/WF_1 mice is complex, and multiple genes must be responsible for either accelerating or suppressing the disease, and these genes might be expressed in different cell lineages (Knight and Adams, 1978; Raveche *et al.*, 1981; Bocchieri *et al.*, 1982; Shirai, 1982; Hang *et al.*, 1983; Maruyama *et al.*, 1983; Hirose *et al.*, 1983; Kotzin and Palmer, 1987).

The B/WF_1 autoimmune disease can be transferred by hematopoietic stem cells or by lymphoid progenitors, suggesting that abnormalities leading to the disease are expressed in these cells and their descendants (Akizuki *et al.*, 1978; Yoshida *et al.*, 1987; Schwieterman *et al.*, 1992). These transfer experiments could not, however, distinguish precursor lymphocytes from the mature lymphocytes, from myeloid precursors, or from mature cells as potential sites of expression of the defects. In fact, the involvement of T cells has been indicated (Wofsy and Seaman, 1985), and it has been shown that the NZW parental genes, which contribute in a major way to the renal disease in the B/WF_1 mice, are closely linked to the MHC locus (Kotzin and Palmer, 1987; Babcock *et al.*, 1989). If it were, in fact, class I or class II genes, the latter might implicate the selection of the T cell repertoire in B/WF_1 mice as one major factor contributing to the disease.

The possibility to establish, from fetal liver, B lymphocyte lineage-committed progenitors and precursors in culture and to transfer them into SCID hosts enabled us to distinguish those B/WF_1-encoded disease-inducing and disease-propagating genes that are expressed in the B lymphocyte lineage from those that are expressed in other cell lineages (Reininger *et al.*, 1992). Progenitor and precursor B cell lines from B/WF_1 mice, reactive to stromal cells and IL-7, can be grown in long-term tissue cultures. They express B lineage-specific markers B220, PB76, V_{preB}, λ_5, and others, but do not express sIg. On removal of IL-7 from the cultures, they differentiate into sIg^+ B cells, which can be stimulated *in vitro* by mitogens to IgM-secreting cells.

They can be transferred into SCID mice, where they elicit many parameters of the lupuslike disease. Most notably, the B/WF_1 pre-B cell-populated mice develop hyperplasia of the IgM- and IgG-secreting cells in many lymphoid organs and elicit elevated levels of IgM, IgG_{2a}, and IgG_3 for the next 3 to 5 months. Classical lupus-connected ANA antibodies against single- and double-stranded DNA are found in the IgG fractions. They do *not* develop erythrocyte-specific antibodies or gp70 retroviral glycoprotein-specific antibodies, and no lymphoid infiltrations are seen in the many organs in which the B/WF_1 mouse elicits them during the progression of the disease. Up to

20% of the SCID mice populated with B/WF$_1$ pre-B cells develop proteinuria as a consequence of IgG deposits in the glomeruli of the kidney during a 7-month period after pre-B cell transfer.

These results show that genetic defects expressed in the B lineage can be expressed in SCID mice separately, and without the influence of T cells or other environmental, cooperating cells of B/WF$_1$ mice. Experiments are now in progress with pre-B cells from NZB mice (which show a modified form of the autoimmune disease, with anemia as the lethal outcome), from NZB × C57BL/6J mice (which are healthy), from NZW mice, and from recombinant inbred lines of NZB × SM. The experiments might allow an identification of genetic loci contributing to parts of the total pattern of induction and propagation of the lupuslike autoimmune disease. They should also allow a distinction of disease-eliciting and disease-suppressing factors and their genetic loci, expressed in different cell lineages. Eventually, this should lead to the identification and elucidation of the structure of these genes, which should aid the search for similar genes in humans. In principle, genetic defects contributing to human autoimmune diseases that are expressed in the human B lymphocyte lineage should be analyzable by the same strategy.

For *in vitro* isolation and propagation of human progenitor and precursor B cells from either fetal liver, cord blood, fetal spleen, or bone marrow, FACS sorting with the aid of specific markers (B220, c-kit, V_{preB}/λ_5, etc.) might enrich the proper cells. Human pre-B cells might be grown in preadipocytic stromal cells in the presence of the proper cytokines. SCF, IL-6, IL-7, IL-1, and GM-CSF are among the candidates that might induce and propagate human pre-B cell proliferation (McNiece *et al.*, 1991; Ogawa *et al.*, 1991).

One important prerequisite for the diagnosis of autoimmune disease (and immunodeficiency) defects in human lymphocyte lineage cells is the capacity of SCID mice to accept human progenitors and precursors for migration into the right organs, for population of the right sites, and for appropriate growth and differentiation to mature cells. The new SCID/SCID mouse (McCune *et al.*, 1988; Mosier *et al.*, 1988; Péault *et al.*, 1991) appears to be only a first attempt in this direction.

Our experiments with B/WF$_1$ pre-B cells eliciting lupuslike disease syndromes in SCID mice make us hope that, one day, we might be able to analyze, in this way, pre- and pro-B cells of individuals of a disease-prone family for genetic defects expressed in the B lineage. The pre-B populated SCID mice could serve to test factors (T cells?) for possible suppression and cure of the disease. In this way, this experimental system of pre-B cell transfer into SCID mice may not only diagnose the

predisposition for the disease, but may also lead to treatment and eventual therapy in the patient.

ACKNOWLEDGMENT

The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd., Basel, Switzerland.

REFERENCES

Adachi, M., Tefferi, A., Greipp, P. R., Kipps, T. J., and Tsujimoto, Y. (1990). Preferential linkage of *bcl*-2 to immunoglobulin light chain gene in chronic lymphocytic leukemia. *J. Exp. Med.* **171,** 559.

Akizuki, M., Reeves, J. P., and Steinberg, A. D. 1978. Expression of autoimmunity by NZB.NZW marrow. *Immunol. Immunopathol.* **10,** 247.

Alés-Martínez, J. E., Cuende, E., Martínez-A, C., Parkhouse, R. M. E., Pezzi, L., and Scott, D. W. (1991). Signalling in B cells. *Immunol. Today* **12,** 201.

Andersson, J., Coutinho, A., and Melchers, F. (1977). Frequencies of mitogen-reactive B cells in the mouse. I. Distribution in different lymphoid organs from different inbred strains of mice at different ages. *J. Exp. Med.* **145,** 1511.

Anderson, D. M., Lyman, S. D., Baird, A., Wignall, J. M., Eisenman, J., Rauch, C., March, C. J., Boswell, H. S., Gimple, S. D., Cosman, D., and Williams, D. E. (1990). Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell (Cambridge, Mass.)* **63,** 235.

Babcock, S. K., Appel, V. B., Schiff, M., Palmer, E., and Kotzin, B. L. (1989). Genetic analysis of the imperfect association of H-2 haplotype with lupus-like autoimmune disease. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 7552.

Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., NcBride, O. W., Epstein, A. L., and Korsmeyer, S. J. (1985). Cloning the chromosomal breakpoint of t(14:18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell (Cambridge, Mass.)* **41,** 899.

Berek, C., and Milstein, C. (1987). Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* **96,** 23.

Blackwell, T. K., Moore, M. W., Yancopoulos, G. D., Suh, H., Lutzker, S., Selsing, E., and Alt, F. W. (1986). Recombination between immunoglobulin variable region segments is enhanced by transcription. *Nature (London)* **324,** 585.

Bogen, B., and Weiss, S. (1991). A rearranged λ_2 light gene chain retards but does not exclude χ and λ_1 expression. *Eur. J. Immunol.* **21,** 2391.

Bocchieri, M. H., Cooke, A., Smith, J. B., Weigert, M., and Riblet, R. J. (1982). Independent segregation of NZB immune abnormalities in NZB × C58 recombinant inbred mice. *Eur. J. Immunol.* **12,** 349.

Borzillo, G. V., Endo, K., and Tsujimoto, Y. (1992). Bcl-2 confers growth and survival advantage to interleukin 7-dependent early pre-B cells which become factor independent by a multistep process in culture. *Oncogene* **7,** 869.

Bosma, M., Schuler, W., and Bosma, G. (1988). The scid mouse mutant. *Curr. Top. Microbiol. Immunol.* **137,** 197.

Carlsson, L., Övermo, C., and Holmberg, D. (1992). Selection against N-region diversity in immunoglobulin heavy chain variable regions during the development of pre-immune B cell repertoires. *Intl. Immunol.* **4,** 549.

Carmack, C. E., Camper, S. A., Mackle, J. J., Gerhard, W. U., and Weigert, M. G. (1991).

Influence of a Vκ8 L chain transgene on endogenous rearrangements and the immune response to the HA(SB) determinant on influenza virus. *J. Immunol.* **147,** 2024.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P., and Bernstein, A. (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature (London)* **335,** 88.

Chen, U., Kosco, M., and Staerz, U. (1992). Establishment and characterization of lymphoid and myeloid mixed-cell populations from mouse late embryoid bodies, "embryonic-stem-cell fetuses". *Proc. Natl. Acad. Sci. U.S.A.* **89,** 2541.

Cherayil, B. J., and Pillai, S. (1991). The ω/λ5-surrogate immunoglobulin light chain is expressed on the surface of transitional B lymphocytes in murine bone marrow. *J. Exp. Med.* **173,** 111.

Cleary, M. L., and Sklar, J. (1985). Nucleotide sequence of a t(14:18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 7439.

Cleary, M. L., Smith, S. D., and Sklar, J. (1986). Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2 immunoglobulin transcript resulting from the t(14:18) translocation. *Cell (Cambridge, Mass.)* **47,** 19.

Cohn, M., and Langman, R. E. (1990). The protection: The unit of humoral immunity selected by evolution. *Immunol. Rev.* **115,** 7.

Collins, L. S., and Dorshkind, K. (1987). A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis. *J. Immunol.* **138,** 1082.

Copeland, N. G., Gilbert, D. J., Cho, B. C., Donovan, P. J., Jenkins, N. A., Cosman, D., Anderson, D., Lyman, S. D., Williams, D. E. (1990). Mast cell growth factor maps near the Steel locus on mouse chromosome 10 and is deleted in a number of Steel alleles. *Cell (Cambridge, Mass.)* **63,** 175.

Cumano, A., and Paige, C. (1992). Enrichment and characterization of uncommitted B-cell precursors from fetal liver at day 12 of gestation. *EMBO J.* **11,** 593.

Cumano, A., Paige, C. J., Iscove, N. N., and Brady, G. (1992). Bipotential precursors of B cells and macrophages in murine fetal liver. *Nature (London)* **356,** 612.

Davidson, W. F., Pierce, J. H., Rudikoff, S., and Morse, H. C. I. (1988). Relationships between B cell and myeloid differentiation studies with a B lymphocyte progenitor line, HAFTL-1. *J. Exp. Med.* **168,** 389.

Dorshkind, K. (1990). Regulation of hemopoiesis by bone marrow stromal cells and their products. *Annu. Rev. Immunol.* **8,** 111.

Du Pasquier, L., Wilson, M., and Robert, J. (1992). The immune system of *Xenopus:* Special focus on B cell development and immunoglobulin genes. *In* "The Biology of *Xenopus*" (R. C. Tousley and H. R. Kohel, eds.). *Proc. Symp. Zool. Soc. (London)*, in press.

Duvalle, E., and Wyllie, A. H. (1986). Death and the cell. *Immunol. Today* **7:**115.

Era, T., Ogama, D. L., Nishikawa, S. I., Okamoto, M., Honjo, T., Akaji, K., Miyasaki, J. I., and Yamamura, K. (1991). Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. *EMBO J.* **10,** 337.

Feddersen, R. D., and van Ness, B. G. (1990). Corrective recombination of mouse immunoglobulin kappa alleles in Abelson murine leukemia virus-transformed pre-B cells. *Mol. Cell. Biol.* **10,** 569.

Feeney, A. J. (1990). Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J. Exp. Med.* **172,** 1377.

Förster, I., and Rajewsky, K. (1990). The bulk of the peripheral B cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4781.

Geissler, E. N., Ryan, M. A., and Housman, D. E. (1988). The dominant-white spotting (*W*) locus of the mouse encodes the c-kit proto-oncogene. *Cell (Cambridge, Mass.)* **55,** 185.

Gioud, M., Kotzin, B. L., Rubin, R. L., Joslin, F. G., and Tan, E. M. (1983). *In vivo* and *in vitro* production of anti-histone antibodies in NZB/NZW mice. *J. Immunol.* **131,** 269.

Goldings, E. A., Cohen, P. L., McFadden, S. F., Ziff, M., and Vitetta, E. S. (1980). Defective B cell tolerance in adult (NZB ×NZW)F1 mice. *J. Exp. Med.* **152:**730.

Goodnow, C. C., Crosbie, J., Jorgensen, H., Brink, R. A., and Basten, A. (1989). Induction of self-tolerance in mature peripheral B lymphocytes. *Nature (London)* **342,** 385.

Granninger, W. B., Seto, M., Boutain, B., Goldman, P., and Korsmeyer, S. J. (1987). Expression of bcl-2 and bcl-2-Ig fusion transcripts in normal and neoplastic cells. *J. Clin. Invest.* **80,** 1512.

Gregoire, K. E., Goldschneider, I., Barton, R. W., and Bollum, F. J. (1979). Ontogeny of terminal deoxynucleotidyl transferase-positive cells in lymphopoietic tissues of rat and mouse. *J. Immunol.* **123,** 1347.

Grosschedl, R., Weaver, D., Baltimore, D., and Costantini, F. (1984). Introduction of a mu immunoglobulin gene into the mouse germ line: Specific expression in lymphoid cells and synthesis of functional antibody. *Cell (Cambridge, Mass.)* **38,** 647.

Gu, H., Förster, I., and Rajewsky, K. (1990). Sequence homologies, N sequence insertion and J_H utilization in $V_HD_HJ_H$ joining: Implications for the joining mechanisms and the ontogenetic priming of Ly1 B cells and B-CLL progenitor generation. *EMBO J.* **9,** 2133.

Gu, H., Tarlinton, D., Müller, W., Rajewsky, K., and Förster, I. (1991a). Most peripheral B cells in mice are ligand-selected. *J. Exp. Med.* **173,** 1357.

Gu, H., Kitamura, D., and Rajewsky, K. (1991b). B cell development regulated by gene rearrangement arrest of maturation by membrane-bound Dm protein and selection of DH element reading frames. *Cell (Cambridge, Mass.)* **65,** 47.

Guitierrez-Ramos, J.-C., and Palacios, R. (1992). *In vitro* differentiation of embryonic stem cells into lymphocyte precursors able to generate T- and B-lymphocytes *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 9171.

Gurfinkel, N., Unger, T., Givol, D., and Mishinski, J. F. (1987). Expression of the bcl-2 gene in mouse B lymphocytic cell lines is differentiation stage specific. *Eur. J. Immunol.* **17,** 567.

Hang, L., Slack, J. H., Amundson, C., Izui, S., Theofilopoulos, A. N., and Dixon, F. J. (1983). Induction of murine autoimmune disease by chronic polyclonal B cell activation. *J. Exp. Med.* **157,** 874.

Harada, K., and Yamagishi, H. (1991). Lack of feedback inhibition of VK gene rearrangement by productively rearranged alleles. *J. Exp. Med.* **173,** 409.

Hardie, D. L., Gordon, J., and MacLennan, I. C. M. (1991). Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. *Eur. J. Immunol.* **21,** 1905.

Hardy, R. R. (1992). Variable gene usage, physiology and development of Ly1+ (CD5+) B cells. *Curr. Opin. Immunol.* **4,** 181.

Hardy, R. R., and Hayakawa, K. (1991). A developmental switch in B lymphopoiesis. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 11550.

Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D., and Hayakawa, K. (1991). Resolution and characterization of Pro-B and Pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* **173,** 1213.

Hayakawa, K., Hardy, R. R., Stall, A. M., and Herzenberg, L. A. (1983). The "Ly1 B" cell subpopulations in normal, immunodefective and autoimmune mice. *J. Exp. Med.* **157,** 202.

Hayashi, S. I., Kunisada, T., Ogawa, M., Sudo, T., Kodama, H., Suda, T., Nishikawa, S., and Nishikawa, S. I. (1990). Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. *J. Exp. Med.* **171,** 1683.

Helyer, B. J., and Howie, J. B. (1963). Renal disease associated with positive lupus erythematosus tests in cross-bred strain of mice. *Nature (London)* **197,** 197.

Hermanson, G. G., Eisenberg, D., Kincade, P. W., and Wall, R. (1988). B29: A member of the immunoglobulin gene superfamily exclusively expressed on B lineage cells. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6890.

Hirayama, F., Shih, J.-P., Awgulewitsch, A., Warr, G. W., Clark, S. C., and Ogawa, M. (1992). Clonal proliferation of murine lymphohemopoietic progenitors in culture. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5907.

Hirose, S., Nagasawa, R., Sekikawa, I., Hamaoki, M., Ishida, Y., Sato, H., and Shirai, T. (1983). Enhancing effect of H-2-linked NZW gene(s) on the autoimmune traits of (NZB × NZW)F1 mice. *J. Exp. Med.* **158,** 228.

Hockenberry, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korameyer, S. J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature (London)* **348,** 334.

Hockenbery, D. M., Zutter, M., Hickey, W., Nahm, M., and Korsmeyer, S. (1991). BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 6961.

Holmberg, D., Anderson, A., Carlson, L., and Forsgren, S. (1989). Establishment and functional implications of B cell connectivity. *Immunol. Rev.*, **110,** 889.

Holmes, K. L., Pierce, J. H., Davidson, W. F., and Morse, H. C. I. (1986). Murine hematopoietic cells with pre B/myeloid characteristics are generated by *in vitro* transformation with retroviruses containing res, ras, abl and src oncogenes. *J. Exp. Med.* **164,** 443.

Hombach, J., Leclercq, L., Radbruch, A., Rajewsky, K., and Reth, M. (1988). A novel 34 kd protein coisolated with the IgM molecule in surface IgM-expressing cells. *EMBO J.* **7,** 3451.

Howie, J. B., and Helyer, B. J. (1968). The immunobiology and pathology of NZB mice. *Adv. Immunol.* **9,** 215.

Huang, E., Nocka, K., Beier, D. R., Chu, T. Y., Buck, J., Lahm, H. W., Wellner, D., Leder, P., and Besmer, P. (1990). The hematopoietic growth factor KL is encoded by the *Sl* locus and is the ligand of the c-kit receptor, the gene product of the *W* locus. *Cell (Cambridge, Mass.)* **63,** 225.

Izui, S., McConahey, P. J., and Dixon, F. J. (1978). Increased spontaneous polyclonal activation of B lymphocytes in mice with spontaneous autoimmune disease. *J. Immunol.* **121,** 2213.

Izui, S., McConahey, P. J., Theofilopoulos, A. N., and Dixon, F. J. (1979). Association of circulating retroviral gp70 immune complexes with murine systemic lupus erythematosus. *J. Exp. Med.* **149,** 1099.

Izui, S., McConahey, P. J., Clark, J. P., Hang, L. M., Hara, I., and Dixon, F. J. (1981a). Retroviral gp70 immune complexes in NZB × NZW F2 mice with murine lupus nephritis. *J. Exp. Med.* **154,** 517.

Izui, S., McConhay, P. J., and Dixon, F. J. (1981b). Identification of retroviral gp70 and anti-gp70 antibodies involved in circulating immune complexes in NZB × NZW mice. *J. Exp. Med.* **153,** 1151.

Jacob, J., Kelsoe, G., Rajewsky, K., and Weiss, U. (1991). Intraclonal generation of antibody mutants in germinal centres. *Nature (London)* **354,** 389.

Jyonuchi, H., and Kincade, P. W. (1984). Precocious and enhanced functional maturation

of B lineage cells in New Zealand Black mice during embryonic development. *J. Exp. Med.* **159,** 1277.

Karasuyama, H., Kudo, A., and Melchers, F. (1990). The proteins encoded by Vpre-B and λ5 pre-B cell-specific genes can associate with each other and with mu heavy chains. *J. Exp. Med.* **172,** 969.

Karasuyama, H., Shinkai, Y., Oltz, E. M., Alt, F. W., Rolink, A., and Melchers, F. (1992). Three different partners associated with V_{preB}/λ_5 surrogate light chains on the surface of precursor B cell lines at different developmental stages. *J. Exp. Med.* (submitted).

Kenny, J. J., Stall, A. M., Sieckmann, D. G., Lamers, M. C., Finkelman, F. D., Finch, L., and Longo, D. L. (1991). Receptor-mediated elimination of phosphocholine-specific B cells in X-linked immune-deficient mice. *J. Immunol.* **146,** 2568.

Kerr, W. G., Cooper, M. D., Feng, L., Burrows, P. D., and Hendershot, L. M. (1989). Mu heavy chain can associate with a pseudo-light chain complex (jL) in human pre-B cells *"in vitro". Proc. Natl. Acad. Sci. U.S.A.* **85,** 4473.

Kincade, P. W., Jyonouchi, H., Landreth, K. S., and Lee, G. (1982). B lymphocyte precursors in immunodeficent mice. *Immunol. Rev.* **64,** 81.

Kincade, P. W., Lee, G., Pietrangeli, C. E., Hayashi, S. I., and Gimble, J. M. (1989). Cells and molecules that regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* **7,** 111.

Kitamura, D., and Rajewsky, K. (1992). Targeted disruption of μ chain membrane exon causes loss of heavy-chain allelic exclusion. *Nature (London)* **356,** 154.

Kitamura, D., Roes, J., Kühn, R., and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature (London)* **350,** 423.

Kitamura, D., Kudo, A., Schaal, S., Müller, W., Melchers, F., and Rajewsky, K. (1992). A critical role of λ5 protein in B cell development. *Cell (Cambridge, Mass.)* **69,** 823.

Knight, J. G., and Adams, D. D. (1978). Three genes for lupus nephritis in NZB × NZW mice. *J. Exp. Med.* **147,** 1653.

Kodama, H., Nose, M., Yamaguchi, Y., Tsunoda, J.-I., Suda, T., Nishikawa, S., and Nishikawa, S.-I. (1992). *In vitro* proliferation of primitive hemopoietic stem cells supported by stromal cells: Evidence for the presence of a mechanism(s) other than that involving c-kit receptor and its ligand. *J. Exp. Med.* **176,** 351.

Kotzin, B. L., and Palmer, E. (1987). The contribution of NZW genes to lupus-like disease in (NZB × NZW)F1 mice. *J. Exp. Med.* **165,** 1237.

Kubagawa, H., Cooper, M. D., Carroll, A. J., and Burrows, P. D. (1989). Light-chain gene expression before heavy-chain gene rearrangement in pre B cells transformed by Epstein–Barr virus. *PNAS* **86,** 2356.

Kudo, A., and Melchers, F. (1987). A second gene VpreB in the λ5 locus of the mouse, which appears to be selectively expressed in pre B lymphocytes. *EMBO J.* **6,** 2267.

Kudo, A., Sakaguchi, N., and Melchers, F. (1987). Organization of the murine Ig-related λ5 gene transcribed selectively in pre B lymphocytes. *EMBO J.* **6,** 103.

Kudo, A., Bauer, S. R., and Melchers, F. (1989). Structure, control of expression and putative function of the pre-B cell-specific genes Vpre-B and lambda 5. *Prog. Immunol.* **7,** 339.

Kudo, A., Thalmann, P., Sakaguchi, N., Davidson, W. F., Pierce, J. H., Kearney, J. F., Reth, M., Rolink, A., and Melchers, F. (1992). *Int. Immunol.* **4,** 831.

Lambert, P. H., and Dixon, F. J. (1968). Pathogenesis of glomerulonephritis of NZB/W mice. *J. Exp. Med.* **127,** 507.

Landreth, K. S., Narayanan, R., and Dorshkind, K. (1992). Insulin-like growth factor I (IGF-I) regulates pro-B cell differentiation. *Blood* **80,** 1207.

Lieber, M. R., Hesse, J. E., Mizuuchi, K., and Gellert, M. (1987). Developmental stage specificity of the lymphoid (V(D)J recombination activity. *Genes Dev.* **1,** 751.

Litman, G. W., Shamblott, M. J., Haire, R., Amemiya, C., Nishikata, H., Hinds, K., Harding, F., Litman, R., and Varner, J. (1989). Evolution of immunoglobulin gene complexity. *In* "Progress in Immunology" (F. Melchers, ed), p. 361. Springer-Verlag, New York.

Liu, Y.-J., Johnson, G. D., Gordon, J., and MacLennan, I. C. M. (1992). Germinal centres in T-cell-dependent antibody responses. *Immunol. Today* **13,** 17.

Ma, A., Fisher, P., Dildrop, R., Oltz, E., Rathbun, G., Achacoso, P., Stall, A., and Alt, F. W. (1992). Surface IgM mediated regulation of RAG gene expression in Eμ-N-myc B cell lines. *EMBO J.* **11,** 2727.

MacLennan, I. C. M., and Gray, D. (1986). Antigen-driven selection of virgin and memory B cells. *Immunol. Rev.* **91,** 61.

Maruyama, N. F., Furukawa, F., Nakai, Y., Sasaki, Y., Ohta, K., Ozaki, S., Hirose, S., and Shirai, T. (1983). Genetic studies of autoimmunity in New Zealand mice. IV. Contribution of NZB and NZW genes to the spontaneous occurrence of retroviral gp70 immune complexes in (NZB × NZW)F1 hybrid and the correlation to renal disease. *J. Immunol.* **130,** 740.

McCune, J. M., Namikawa, R., Kaneshima, H., Shultz, L. D., Liberman, M., and Weissman, I. L. (1988). The SCID-hu mouse: Murine model for the analysis of human hematolymphoid differentiation and function. *Science (Washington, D.C.)* **241,** 1632.

McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P., and Korsmeyer, S. J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell (Cambridge, Mass.)* **57,** 79.

McDonnell, T. J., Nunez, G., Platt, J. M., Hockenberry, D., London, L., McKearn, J. P., and Korsmeyer, S. J. (1990). Deregulated bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. *Mol. Cell. Biol.* **10,** 1901.

McKean, D., Huppi, K., Bell, M., Staudt, L., Gerhard, W., and Weigert, M. (1984). Generation of antibody diversity in the immune response of Balb/c mice to influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3180.

McNiece, I. K., Langley, K. E., and Zsebo, K. M. (1991). The role of recombinant stem cell factor in early B cell development. *J. Immunol.* **156,** 3785.

Meek, K. (1990). Analysis of junctional diversity during B lymphocyte development. *Science* **250,** 820.

Melchers, F. (1979). Three waves of B lymphocyte development during embryonic development in the mouse. *INSERM Symp.* **10,** 281.

Melchers, F., and Andersson, J. (1984). B cell activation: Three steps and their variations. *Cell (Cambridge, Mass.)* **37,** 715.

Melchers, F., Strasser, A., Bauer, S. R., Kudo, A., Thalmann, P., and Rolink, A. (1989). Cellular stages and molecular steps of murine B-cell development. *Cold Spring Harbor Symp. Quant. Biol.* **LIV,** 183.

Melchers, F., Karasuyama, H., Haasner, D., Reiniger, L., and Rolink, A. (1992). Birth, life and death of a B cell. *Prog. Immunol.* **8** (in press).

Misener, V., Jongstra-Bilen, J., Young, A. J., Atkinson, M. J., Wu, G. E., and Jongstra, J. (1990). Association of Ig L chain-like protein λ5 with a 16-kilodalton protein in mouse pre-B cell lines is not dependent on the presence of Ig H chain protein. *J. Immunol.* **145,** 905.

Misener, V., Downey, G. P., and Jongstra, J. (1991). The immunoglobulin light chain related protein λ5 is expressed on the surface of mouse pre-B cell lines and can function as a signal transducing molecule. *Int. Immunol.* **3,** 1129.

Miyake, K., Medina, K. L., Obo, S., Hamaoka, T., and Kincade, O. W. (1990a). Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *J. Exp. Med.* **171,** 477.

Miyake, K., Underhill, C. B., Lesley, J., and Kincade, P. W. (1990b). Halyuronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* **172,** 69.

Miyake, K., Weissman, I. L., Greenberger, J. S., and Kincade, P. W. (1991). Evidence for a role of the integrin VLA-4 in lympho-hemapoiesis. *J. Exp. Med.* **173,** 599.

Mosier, D. E., Gulizia, R. J., Baird, S. M., and Wilson, D. B. (1988). Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature (London)* **335,** 256.

Moutsopoulos, H. M., Boehm-Truitt, M., Kassan, S. S., and Chused, T. M. (1977). Demonstration of activation of B-lymphocytes in New Zealand Black mice at birth by immunoradiometric assay for murine IgM. *J. Immunol.* **119,** 1639.

Namen, A. E., Lupton, S., Hjerrild, K., Wagnall, J., Mochuzuki, D. Y., Schmierer, A., Mosley, B., March, C., Urdal, D., Gillis, S., Cosman, D., and Goodwin, R. G. (1988). Stimulation of B cell progenitors by cloned murine interleukin 7. *Nature (London)* **333,** 571.

Nemazee, D. A., and Bürki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti MHC class I antibody genes. *Nature (London)* **337,** 562.

Nishikawa, S. I., Ogawa, M., Nishikawa, S., Kumisada, T., and Kodama, H. (1988). B lymphopoiesis on stromal cell clones: Stromal cell clones acting on different stages of B cell differentiation. *Eur. J. Immunol.* **18,** 1767.

Nunez, G., Hockenberry, D., McDonnell, T. J., Sorensen, C. M., and Korsmeyer, S. J. (1991). Bcl-2 maintains B cell memory. *Nature (London)* **353,** 71.

Ogawa, M., Nishikawa, S., Ikuta, K., Yamamura, F., Naito, M., Takahashi, K., and Nishikawa, S. I. (1988). B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST-2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. *EMBO J.* **7,** 1337.

Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S. I., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H., and Nishikawa, S. I. (1991). Expression and function of c-kit in hemopoietic progenitor cells. *J. Exp. Med.* **174,** 63.

Ohsugi, Y., and Gershwin, M. E. (1979). Studies of congenitally immunologic mutant New Zealand Black mice. III. Growth of B lymphocyte clone in congenitally athymic and hereditary asplenic ($D^{h/+}$) NZB mice: A primary B cell defect. *J. Immunol.* **123,** 1260.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Nishikawa, S. I., Miura, Y., and Suda, T. (1991). Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood* **78,** 1706.

Oltz, E. M., Yancopoulos, G. D., Morrow, M. A., Rolink, A., Lee, G., Wong, F., Kaplan, K., Ollies, S., Melchers, F., and Alt, F. A. (1992). A novel regulatory myosin light chain gene distinguishes pre B cell subsets and is IL-7 iducible. *EMBO. J.* **11,** 2759.

Osmond, D. G. (1990). B cell development in bone marrow. *Semin. Immunol.* **2,** 173.

Osmond, D. G. (1991). Proliferation kinetics and the lifespan of B cells in central and peripheral lymphoid organs. *Curr. Opin. Immunol.* **3,** 179.

Péault, B., Weissman, I. L., Baum, C., McCune, J. M., and Tsukamoto, A. (1991). Lymphoid reconstitution of the human fetal thymus in SCID mice with $CD34^+$ precursor cells. *J. Exp. Med.* **174,** 1283.

Pettersson, M., Jernberg-Wiklund, H., Larsson, L.-G., Sundström, C., Givol, I., Tsuji-

moto, Y., and Nilsson, K. (1992). Expression of the bcl-2 gene in human multiple myeloma cell lines and normal plasma cells. *Blood* **79,** 495.

Pillai, S., and Baltimore, D. (1987). Formation of disulphide-linked M_2W_2 tetramers in pre-B cells by the 18K ω-immunoglobulin light chain. *Nature (London)* **329,** 172.

Pillai, S., and Baltimore, D. (1988). The omega and iota surrogate immunoglobulin light chains. *Curr. Top. Microbiol. Immunol.* **137,** 136.

Pink, J. R. L., Lassila, O., and Vainio, O. (1987). B lymphocytes and their self-renewal. *In* "Avian Immunology: Basis and Practice" (A. Toivanen and P. Toivanen, eds.), p. 65. CRC Press, Boca Raton.

Rajewsky, K. (1992). Early and late B-cell development in the mouse. *Curr. Opin. Immunol.* **4,** 171.

Rathbun, G., Berman, J., Yancopoulos, G., and Alt, F. W. (1989). Immunoglobulin light-chain genes of the κ type in man and mouse. *In* "Immunoglobulin Genes" (T. Honjo, F. W. Alt, and T. H. Rabbitts, eds.), p. 63. Academic Press, Orlando.

Raveche, E. A., Novotny, E. A., Hansen, C. T., Tijo, J. H., and Steinberg, A. D. (1981). Genetic studies in NZB mice. V. Recombinant inbred lines demonstrate that separate genes control autoimmune phenotype. *J. Exp. Med.* **153,** 1187.

Reed, J. C., Tsujimoto, Y., Alpers, J. D., Croce, C. M., and Newell, P. C. (1987). Regulation of bcl-2 proto-oncogene expression during normal human lymphocyte proliferation. *Science* **236,** 1295.

Reichmann-Fried, M., Hardy, R. R., and Bosma, M. J. (1990). Development of B lineage cells in the bone marrow of scid/scid mice following the introduction of functionally rearranged immunoglobulin transgenes. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2730.

Reid, G. K., and Osmond, D. G. (1985). B lymphocyte production in the bone marrow of mice with X-linked immunodeficiency (xid). *J. Immunol.* **135,** 2299.

Reininger, L., Radaszkiewicz, T., Kosco, M., Melchers, F., and Rolink, A. (1992). Development of autoimmune disease in SCID mice populated with long-term *in vitro* proliferating (NZB × NZW)F1 pre B cells. *J. Exp. Med.* **176,** 1343.

Reth, M., and Alt, F. W. (1984). Novel immunoglobulin heavy chains are produced from DJH gene segment rearrangements in lymphoid cells. *Nature (London)* **312,** 418.

Reth, M., Hombach, J., Wienands, J., Campbell, K. S., Chien, N., Justement, L. B., and Cambier, J. C. (1991). The B-cell antigen receptor complex. *Immunol. Today* **12,** 196.

Reynolds, J. D., and Morris, B. (1983). The evolution and involution of Peyer's patches in fetal and postnatal sheep. *Eur. J. Immunol.* **13,** 627.

Rolink, A., and Melchers, F. (1991). Molecular and cellular origins of B lymphocyte diversity. *Cell (Cambridge, Mass.)* **66,** 1081.

Rolink, A., Streb, M., and Melchers, F. (1991a). The κ/λ ratio in surface immunoglobulin molecules on B lymphocytes differentiating from DHJH-rearranged murine pre-B cell clones *in vitro*. *Eur. J. Immunol.* **21,** 2895.

Rolink, A., Kudo, A., Karasuyama, H., Kikuchi, Y., and Melchers, F. (1991b). Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive mitogen-reactive B cells *in vitro* and *in vivo*. *EMBO J.* **10,** 327.

Rolink, A., Streb, M., Nishikawa, S. I., and Melchers, F. (1991c). The c-kit encoded tyrosine kinase regulates the proliferation of early pre-B cells. *Eur. J. Immunol.* **21,** 2609.

Rolink, A., Haasner, D., Nishikawa, S. I., and Melchers, F. (1993). Change in frequencies of clonable pre B cells during life in different lymphoid organs of mice. *Blood* (in press).

Sakaguchi, N., and Melchers, F. (1986). λ5, a new light-chain related locus selectively expressed in pre-B lymphocytes. *Nature (London)* **324,** 579.

Sakaguchi, N., Kashiwamura, S. I., Kimoto, M., Thalmann, P., and Melchers, F. (1988). B

lymphocyte-lineage-restricted expression of *mb-1*, a gene with CD3-like structural properties. *EMBO J.* **7,** 3457.

Schatz, D. G., and Baltimore, D. (1988). Stable expression of immunoglobulin gene V(D)J recombinase activity by gene transfer into 3T3 fibroblasts. *Cell (Cambridge, Mass.)* **53,** 107.

Scher, I. (1982). CBA/N immune defective mice: Evidence for the failure of a B cell subpopulation to be expressed. *Immunol. Rev.* **64,** 117.

Schittek, B., and Rajewsky, K. (1992). Natural occurence and orgin of somatically mutated memory B cells in mice. *J. Exp. Med.* **176,** 427.

Schwieterman, W., Wood, G. M., Scott, D. E., and Steinberg, A. D. (1992). Studies of bone marrow progenitor cells in lupus-prone mice. I. NZB marrow cells demonstrate increased growth in Whitlock-Witte culture and increased splenic colony-forming unit activity in the Thy-1-lineage-population. *J. Immunol.* **148,** 2405.

Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S. J. (1991). bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell (Cambridge, Mass.)* **87,** 879.

Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelson, M., Charron, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to intiate (V(D)J) rearrangement. *Cell (Cambridge, Mass.)* **68,** 855.

Shirai, T. (1982). The genetic basis of autoimmunity in murine lupus. *Immunol. Today* **3,** 187.

Slack, J. H., Hang, L., Barkley, J., Fulton, R. J., D'Hoostelaere, L., Robinson, A., and Dixon, F. J. (1984). Isotypes of spontaneous and mitogen-induced autoantibodies in SLE-prone mice. *J. Immunol.* **132,** 1271.

Solvason, N., and Kearney, J. F. (1992). The human fetal omentum: A site of B cell generation. *J. Exp. Med.* **175,** 397.

Sprent, J., Bruce, J., Ron, Y., and Webb, S. R. (1985). Physiology of B cells in mice with X-linked immunodeficiency. I. Size, migratory properties, and turnover of the B cell pool. *J. Immunol.* **134,** 1442.

Stamenkovic, I., Aruffo, A., Amiot, M., and Seed, B. (1991). The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO J.* **10,** 343.

Staples, P. J., and Tatal, N.)1969). Relative inability to induce tolerance in adult NZB and NZB/NZW F1 mice. *J. Exp. Med.* **129,** 123.

Steward, M. W., and Hay, F. C. (1976). Changes in immunoglobulin class and subclass of anti-DNA antibodies with increasing age in N/ZBW F1 hybrid mice. *Clin. Exp. Immunol.* **26,** 363.

Strasser, A., Rolink, A., and Melchers, F. (1989). One synchronous wave of B cell development in mouse fetal liver changes at day 16 of gestation from dependence to independence of a stromal cell environment. *J. Exp. Med.* **170,** 1973.

Strasser, A., Harris, A. W., Vaux, D. L., Webb, E., Bath, M. L., Adams, J. M., and Cory, S. (1990). Abnormalities of the immune system induced by dysregulated bcl-2 expression in transgenic mice. *Curr. Top. Mircrobiol. Immunol.* **166,** 175.

Strasser, A., Harris, A. W., and Cory, S. (1991a). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell (Cambridge, Mass.)* **67,** 888.

Strasser, A., Whittingham, S., Vaux, D. L., Bath, M. L., Adams, J. M., cory, S., and Harris, A. W. (1991b). Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc. Natl. Acad. U.S.A.* **85,** 5881.

Streb, M. (1992). Ph. D. Thesis, Phil. II Faculty, University of Basel, Switzerland.

Sudo, T., Ito, M., Ogawa, Y., Iizuka, M., Kodama, H., Kunisada, T., and Hayashi, S. I.

(1989). Interleukin 7 production and function in stromal cell-dependent B cell development. *J. Exp. Med.* **170,** 333.

Taccioli, G. E., Rathbun, G., Shinkai, Y., Oltz, E. M., Cheng, H., Whitmore, G., Stamato, T., Jeggo, P., and Alt, F. W. (1992). Activities involved in V(D)J recombination. *Curr. Top. Microbiol. Immunol.* **182,** 107.

Takai, Y., Sakata, T., Iwagami, S., Tai, X. G., Kita, Y., Hamaoka, T., Sakaguchi, N., Yamagishi, H., Tsuruta, T., Teraoka, H., and Fujiwara, H. (1992). Identification of IL-7-dependent bone marrow-derived thy−, B220−, lymphoid cell clones that rearrange and express both Ig and T cell receptor genes. *J. Immunol.* **148,** 1329.

Tsujimoto, Y., and Croce, C. M. (1986). Analysis of the structure, transcripts and protein products of bcl-2, the gene involved in human follicular lymphomas. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 5214.

Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., and Croce, C. M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14:18) chromosome translocation. *Science* **226,** 1097.

Vasicek, T. J., Levinson, D. A., Schmidt, E. V., Campos-Torres, J., and Leder, P. (1992). B-less: A strain of profoundly B cell-deficient mice expressing a human λ transgene. *J. Exp. Med.* **175,** 1169.

Vaux, D. L., Cory, S., and Adams, J. M. (1988). bcl-2 gene promotes haemopoietic cell survival and co-operates with c-myc to immortalize pre-B cells. *Nature (London)* **335,** 440.

Weill, J.-C., and Reynaud, C.-A. (1992). Early B cell development in chickens, sheep and rabbits. *Curr. Opin. Immunol.* **4,** 177.

Wilson, M. R., and Warr, G. W. (1992). Fish immunoglobulins and the genes that encode them. *Annu. Rev. Fish Diseases* **2** (in press).

Wilson, M., Hsu, E., Marcuz, A., Courtet, M., Du Pasquier, L., and Steinberg, C. (1992). What limits affinity maturation of antibodies in *Xenopus*—the rate of somatic mutation or the ability to select mutants? *EMBO J.* **11.** 4337.

Wofsy, D., and Seaman, W. E. (1985). Successful treatment of autoimmunity in NZB/NZW F1 mice with monoclonal antibody to L3T3. *J. Exp. Med.* **161,** 378.

Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature (London)* **284,** 555.

Yoshida, Y., Yoshida, M., Izui, S., and Lambert, P. H. (1985). Distinct clonotypes of anti-DNA antibodies in mice with lupus nephritis. *J. Clin. Invest.* **76,** 685.

Yoshida, S., Dorshkind, K., Bearer, E., Castles, J. J., Ahmed, A., and Gershwin, M. E. (1987). Abnormalities of B lineage cells are demonstrable in long term lymphoid bone marrow cultures of New Zealand Black mice. *J. Immunol.* **139,** 1454.

Zachau, H. G. (1989). Immunoglobulin light-chain genes of the κ type in man and mouse. *In* "Immunoglobulin Genes" (T. Honjo, F. W. Alt, and T. H. Rabbitts, eds.), p. 91. Academic press, Orlando.

Zimmerman, K., and Alt, F. W. (1990). Expression and function of myc family genes. *Crit. Rev. Oncogenesis* **2,** 75.

Zsebo, K. M., Wypych, J., McNiece, I. K., Lu, H. S., Smith, K. A., Karkare, S. B., Sachdev, R. K., Yuschenkoff, V. N., Birkett, N. C., Williams, L. R., Satyagal, V. N., Tung W., Bosselman, R. A., Mendiaz, E. A., and Langely, K. E. (1990). Identification, purification and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell (Cambridge, Mass.)* **63,** 195.

This article was accepted for publication on 6 October 1992.

ADVANCES IN IMMUNOLOGY, VOL. 53

Compartmentalization of the Peripheral Immune System

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Si sbuccia l'universo como una cipolla, e una cipolla è tutta buccia, immaginiamoci una cipolla infinita, che abbia il centro da ogni parte e la circonferenza in nessun luogo, o fatta ad anello di Moebius. (Umberto Eco, "Il Pendulo de Foucault." Bombiani, Milano, p. 492)

One peels the universe like an onion, and an onion is all peel; let us imagine an infinite onion that has its center everywhere and its circumference nowhere or is constructed as a Moebius' ring.

I. Introduction

Whereas neurobiologists always have in mind the exact localization of a neuron under study, immunologists tend to underscore the mobility of circulating lymphocytes, thus justifying that *in vitro* studies may be extrapolated to the *in vivo* behavior of the system. Mononuclear cell suspensions that are enriched for certain lymphocyte populations contained in the murine spleen or lymph nodes, as well as in human peripheral blood, are subjected to phenotypic and functional *ex vivo* analysis and their function *in vitro* is widely thought to reflect what is actually happening in the intact organism—just as if higher vertebrates were a refined suspension culture for lymphocytes. As an example, the finding that splenic or lymph node lymphocytes will not proliferate in response to a given antigen is valuated as "anergy" and is interpreted as a sign of *in vivo* nonresponsiveness. Several facts indicate that *in vivo* extrapolations of *in vitro* experiments are curtailed by a series of caveats. It cannot be neglected that B and T lymphocytes have to receive contact-dependent activation signals from sessile cells *in situ* and that they exert the majority of their functions via direct intercellular interactions. Cytokines secreted by T lymphocytes have a short half-life and therefore have also a limited range of action *in vivo*. In addition, lymphocytes are influenced in their behavior by local antigens and metabolites and are embedded in a complex network of interactions with neighboring accessory cells. Although it is often assumed that the extracellular

matrix is an inert structure, functioning only as supportive tissue, it appears that matrix (glyco)proteins continuously interact with signal-transducing receptors on lymphoid cells. In these circumstances, the immunologic universe splits up into myriads of near-to-independent microenvironments, linked only by (re)circulating lymphocytes and a colloid solution composed of immunoglobulins and ubiquitous self-antigens. Attempts must be undertaken to place lymphocytes into a spatiotemporal context, although considerable methodological and conceptual difficulties may arise from this posture. The term "compartment" does not only apply to the anatomical entity in which a lymphocyte is located, but also has a chronological connotation. Most lymphocytes and their products continuously decay at a relatively fast rate and, depending on the ontogenetic phase, they change their functional capabilities. Thus, different cell populations are not only hindered from interacting by anatomical barriers, but also by different ontogenetic and temporal contexts. This review, however, will concentrate on the purely spatial aspect of compartmentalization. Moreover, the focus will be on the distribution and site-dependent function of B and T lymphocytes at the expense of other, "accessory" cell types.

The common immunological jargon defines lymphocytes located outside of the "central" lymphoid organs (thymus and bone marrow; i.e., the primary differentiation sites of immature T and B lymphocytes) as "peripheral" cells, a term that leads to an overestimation of the homogeneity of such populations. Peripheral lymphocytes are not only contained in the classical lymphoid organs (spleen, lymph nodes, tonsils, and Peyer's patches), all of which have rather disparate functions, but are also frequently in the epidermis and in entodermal nonsquamous epithelia (mucosae of the gastrointestinal, respiratory, and female reproductive tracts), as well as in mesoderm derivates (e.g., pleuroperitoneal cavity) (Table I). Instead, they are lacking in "immunoprivileged" organs not normally accessible to immune cell traffic, e.g., the central nervous system beyond the blood–brain barrier and the anterior chamber of the eye. Although interstitial lymphocytes represent a quantitatively important population—for instance, lymphocytes in the lung interstitium are as numerous as those of the circulating blood pool (*1*)—few data on such cells are available. In the present survey, differences in the phenotypic composition, repertoire, and function of lymphocytes contained in different peripheral localizations will be enumerated. These data will be employed to illustrate that the periphery of the immune system is made up of rather contrasting microenvironments. The mechanisms

TABLE I
ANATOMIC COMPARTMENTS COLONIZED BY LYMPHOCYTES OR LYMPHOCYTE PRECURSORS[a]

Type of Organ	Site
Precursors	Periaortic mesoderm (chicken)
	Yolk sack (days 7–8)[b]
	Omentum (day 13)
	Liver (day 10)
	Bone marrow (perinatal)
Central organs for B cell differentiation	Bursa fabricii (chicken)
	Ileal Peyer's patches (sheep)
	Bone marrow (rodents)
	Pleuroperitoneal cavity (rodents)?
Central organ for T cell differentiation	Thymus (day 11)
Peripheral lymphoid organs	Spleen
	Lymph nodes (mucosal, superficial)
	Tonsils
	Appendix
	Peyer's patches of the gut
Nonlymphoid organs	Intraepithelial lymphocytes (skin, intestine, bronchi, etc.)
	Lamina propria lymphocytes (e.g., uterus)
	Omental milky spots
	Various interstitia (e.g., lung, exocrine glands)
Circulating lymphocytes	Peripheral blood lymphocytes
	Thoracic duct lymphocytes

[a] The classification of organs is purely didactic. All sites of the body, including the central lymphoid organs and nonlymphoid organs, contain peripheral lymphocytes (i.e., cells that have been exported from the central site of differentiation). Pluripotential precursors for T and NK cells reside not only in the prethymic compartments, but also in the thymus. Some lymphoid organs are commonly grouped together due to their anatomic localization, e.g., the gut-associated lymphoreticular tissue (including Peyer's patches, appendix, gut lamina propria, and intraepithelial lymphocytes) and the bronchus-associated lymphoid tissue.

[b] Numbers refer to the first appearance (gestational age) during mouse embryonic development.

targeting lymphocytes to a given anatomical structure and/or selecting a special repertoire at a given site, as well as the implications of the compartmentalization of the peripheral immune system in the maintenance of immune homeostasis, will be discussed.

II. T Lymphocytes in Peripheral Compartments

T cells not only produce a nearly infinite antigen receptor repertoire via somatic diversification processes, including gene rearrangements and somatic mutation, but can also be classified into a multitude of subpopulations that differ in the expression of classes of the T

cell receptor (TCR; α/β or γ/δ heterodimers) and cluster of differentiation (CD) antigens, in the activation state, or in functional terms (reviewed in Refs. 2–7). Thus, for instance, the differentiation antigens CD4 and CD8, which are coexpressed during thymic ontogeny on the surface of thymocytes, are found on mutually exclusive α/β T lymphocyte subsets in the periphery. $CD4^+$ α/β T cells are predominantly of the helper phenotype, whereas CD8 (usually a heterodimer composed of CD8α and CD8β) is mainly expressed on cytotoxic and suppressor T cells. This functional distinction is not absolute, because some $CD4^+$ T lymphocytes can effect cytotoxicity and suppression, and a more stringent correlation exists between CD4/CD8 expression and major histocompatability complex (MHC) gene products expressed by target or antigen-presenting cells (APCs). $CD4^+$ T cells interact with cells expressing class II, whereas $CD8^+$ cells are class I restricted (*5, 8–10*). Expression of the CD4 and CD8 molecules also correlates, though not in an absolute fashion, with a helper versus cytotoxic phenotype in γ/δ T cells (*11*). Thus, although a majority of the γ/δ T cells, in contrast with the α/β subset, do not express any of the two coreceptors, a significant fraction displays CD8 and exerts a suppressor or cytotoxic function (*12*). A minor poulation of γ/δ T cells (mostly found at early stages of development) (*13*) that express CD4, however, exhibits a helper phenotype (*11*). In spite of these differences, the restricting elements of the γ/δ T cells remain to be defined.

In addition to the canonical "single-positive" T cell subsets ($CD3^+CD4^+CD8^-$ or $CD3^+CD4^-CD8^+$), other classes exist; some peripheral T cells lack both CD4 and CD8 expression ("double-negative" T cells) and some express the CD8α chain as a homodimer but lack the β chain (*vide infra*). Double-positive ($CD4^+CD8^+$) T cells, which are thought to represent an immature differentiation stage, are infrequent outside of the thymus in most mammalian species. $CD4^+CD8^-$ α/β T lymphocytes may be subdivided into different classes of T helper cells (T_{H0}, T_{H1}, T_{H2}, and T_{HX}) that produce different patterns of lymphokines (*14–16*) and may be classified as naive and memory cells according to the expression of alternatively spliced products of the CD45 common leukocyte antigen (*17*). In addition, T cells may differ in their activation state, which may or may not be reflected by the expression of "activation markers" (*18*). These and other phenotypic and functional differences, together with the highly diversified repertoire of T cell receptor α/β and γ/δ chains, mean that T cells are extremely heterogeneous in specificity, activation requirements, life span, and functional properties. In the following discussions these features will be correlated with the anatomic

localization of T lymphocytes. In addition to the thymus, organs abundantly populated by T cells comprise blood, lymph, the periarteriolar sheath of spleen, the paracortex and deep cortex of lymph nodes, the interfollicular cortex of Peyer's patches, bone marrow, liver sinusoids, the pleuroperitoneal cavities, and epithelia and subepithelial tissues.

A. Spleen and Lymph Nodes

The best characterized lymphocyte populations are those contained in the spleen or lymph nodes of mice and, for obvious reasons, in the peripheral blood of humans. Both the spleen and the lymph node split up into different anatomic microcompartments (*19*), differing in the composition of B and T lymphocyte subsets. Thus, the splenic white pulp consists of three compartments: (1) the thymus-dependent periarteriolar lymphocyte sheath (PALS) and two compartments preferentially, though not exclusively, populated by B cells, namely (2) the follicles and (3) the marginal zone. Lymph nodes contain the T cell-dependent cortical areas and sinus, as well as follicles dominated by B cells. The different T cell subsets are inhomogeneously distributed in the different zones of spleen and lymph nodes. As an example, the majority of T lymphocytes contained in the afferent lymph vessels have a "memory" phenotype, whereas most cells circulating in the efferent vessel belong to the "naive" phenotype (*20*). Although the spleen and lymph node are anatomically and functionally different—lymph nodes have a more local role than the spleen, which is dedicated to purge the blood from old or altered cells—roughly the same Vβ repertoire is found in mononuclear cell suspensions derived from both organs. In other words, self-tolerance-related clonal deletions that reflect the physical elimination of thymocytes expressing self-superantigen-reactive Vβ gene products have equal repercussions in the spleen and in lymph nodes (*21,22*), given that the vast majority of T cells contained in these organs are thymus derived (*23*). One exception from this rule is the recent finding that old BALB/c mice (>17 months) exhibit an elevated percentage of T cells commonly deleted in young (<12 months) BALB/c mice (expressing products of the Vβ3$^+$, Vβ5$^+$, or Vβ11$^+$ TCR gene families) in the lymph nodes, not in the spleen (G. Kroemer, M. de Alborán, J. A. Gonzalo, and C. Martinez-A., 1993, in preparation). The few γ/δ T cells (mostly CD4$^-$CD8$^-$) encountered in the spleen or lymph nodes preferentially express Vγ4–Jγ1Cγ1 (*24*) [nomenclature of TCR γ genes according to Raulet (*25*)] and Vγ1.1–Jγ4Cγ4 (*26*) genes, and are thymus derived.

Lymphocytes isolated from the spleen or different lymph nodes

also exhibit divergent features. Primary lymphocyte cultures of various peripheral organs, set up in the absence of serum, thus avoiding the presence of platelet-derived growth factor (PDGF), a polypeptide that inhibits the production of interleukin-4 (IL-4), IL-5, and interferon-γ (IFN-γ), show major differences in the lymphokine production pattern. Whereas splenocytes and nonmucosal (axillary, brachial, and inguinal) lymph node cells produce equal amounts of IL-2, IL-4, IL-5, and IFN-γ, lymphocytes recovered from lymph nodes that receive afferent lymphatic drainage from mucosal tissues (parathymic, mesenteric, periaortic, and deep cervical lymph nodes) produce three- to fivefold less of the T_{H1} lymphokines, IL-2 and IFN-γ, but significantly more of the T_{H2} factors, IL-4 and IL-5, *in vitro* stimulation (*27,28*). Lymph node T cells isolated from the mandibular lymph nodes adjacent to the local salivary gland contain spontaneously IL-5- and IFN-γ-secreting cells, whereas cervical lymph nodes do not produce these cytokines, unless stimulated with T cell mitogen (*29*). This T_{H1}/T_{H2} dichotomy also applies when the activated immune system is analyzed. In *Trichinella spiralis*-infected mice, IFN-γ-producing cells predominate in the spleen, whereas IL-5 producers prevail in mesenteric lymph nodes (*30*). Several explanations for these differences may be forwarded. Thus, the source of lymphocytes that drain to a lymph node could decide which particular type of T helper cell predominates (*vide infra*). In addition, local differences in steroidogenesis may determine whether IL-2 or IL-4 is produced. Thus, nonmucosal lymph nodes contain comparatively high amounts of the macrophage-associated enzyme dehydroepiandrosterone (DHEA) sulfatase, which converts adrenal DHEA sulfate to its active metabolite DHEA, a substance well known to enhance IL-2 production (*27*).

Differential requirements for antigenic or mitogenic stimulation of splenic and lymph node cells have also been observed. Thus, intravenous injection of *Staphyloccus aureus* enterotoxin B (SEB) fails to induce the expression of the IL-2 receptor light chain (IL-2Rα) in splenocytes, though this treatment induces vigorous proliferation of the $V\beta8^+$ SEB-reactive subset (*31*); in contrast, local and intraperitoneal injection of SEB induces expression of IL-2Rα in regional lymph nodes (*32,33*). Stimulation with concanavalin A (ConA) and SEB induces IFN-γ synthesis in spleen cells but not in lymph node cells cultured in the presence of fetal calf serum (which contains PDGF). This difference probably resides in the splenic adherent cell population, because such cells allowed for induction of IFN-γ production when cocultured with lymph node cells (*34*). The adherent

cells stimulating the T cell-dependent production of IFN-γ in this system probably are macrophages. Normal murine splenic T_H cells are activated by SEB to undergo blast transformation and proliferation in the presence of macrophages and other splenic APCs, such as B cells. However, the expression of the cytokines IFN-γ and, to a lesser degree, IL-2 depends on the presence of splenic macrophages. This suggests that B cells only induce clonal expansion of T_H cells, whereas splenic macrophages additionally promote terminal differentiation of activated T_H cells into T_H effector cells (35).

The differences described above for lymph node and spleen cells may in part be ascribed to the differential function of different types of "accessory" cells (e.g., B cells and macrophages) that intervene in T cell activation by presenting antigen in the context of MHC molecules and provide additional, costimulatory signals. The unequal distribution of different types of accessory cells (*vide infra*) may account for some of the differences that are observed in primary suspension cultures derived from different organs. These results underscore the need to evaluate the behavior of purified lymph node and splenic T lymphocytes in the context of different accessory cells.

B. Intestinal Intraepithelial T Lymphocytes

The microenvironment of the intestinal epithelium, an endodermal derivate similar to thymic epithelium, appears to be uniquely adapted to the regulation and possibly induction of intraepithelial lymphocyte (IEL) differentiation. Mouse intestinal intraepithelial lymphocytes (i-IELs) are interspersed between the villous epithelial cells of the small intestine and express the γ/δ T cell receptor (TCR) on the majority of cells. γ/δ i-IELs are unique to their phenotype (predominantly $CD5^-CD8\alpha^+CD8\beta^-$), thus differing from ordinary γ/δ T cells, which are $CD4^-CD8^-$, from CD8 molecules expressed in the bulk of T cells and thymocytes, which are α/β heterodimers (Lyt-2, Lyt-3), not α_2 homodimers, and from T cells located in spleen, lymph nodes, or peripheral blood, which almost all express CD5 (36). Moreover, i-IELs express CT1, a carbohydrate antigen associated with CD45 that is not found on any other peripheral T cell subset (37). The presence of an intestinal microflora may accelerate the expression of Thy-1 on γ/δ i-IELs, an expression that is absent on γ/δ i-IELs of young mice, as well the aquisition of the cytotoxic effector function (38). However, $Thy\text{-}1^-CD8\alpha^+CD8\beta^-$ γ/δ i-IELs arise in germ-free mice (39). γ/δ i-IELs predominantly express a $V\gamma5\text{–}J\gamma1C\gamma1$ γ chain together with one of several δ chains ($V\delta4 > V\delta5$, $V\delta6$, and $V\delta7$), both chains showing significant junctional diversity, including

extensive N regions and the use of both D elements in many of the δ chains (*40,41*). A minor subset of murine i-IELs express the α/β TCR heterodimer.

The γ/δ i-IELs develop extrathymically (Table II) based on the following lines of evidence: (1) they exist in athymic mice, as well as in mice with severe combined immunodeficiency (SCID) (*42*); (2) they develop in irradiated thymectomized mice reconstituted with T-depleted bone marrow or day 15 fetal liver (*12,39,43,44*); (3) Vγ5 rearrangements occur on day 11 of *in utero* development both in the gut and in the liver, prior to T cell colonization of the thymus (*45*); (4) i-IELs, in contrast to lymph node T cells, contain mRNA for the RAG protein (*39,46*), which is required for the rearrangement of TCR genes (*47,48*); and (5) the selection mechanisms operative in thymus-derived γ/δ T cells do not apply to γ/δ i-IELs. Thus, in mice that bear a self-reactive transgenic TCR γ/δ (specific for a product of the TL region of MHC $H\text{-}2^k$ and $H\text{-}2^b$), potentially autoaggressive intraepithelial lymphocytes are deleted in the thymus and are undetectable in the spleen (*49*) but are present in the gut and in the skin (*50*). When i-IELs of $H\text{-}2^{d/d}$ and $H\text{-}2^{b/d}$ (autoantigen-bearing) donors are compared in their reactivity to $H\text{-}2^b$-bearing stimulator cells, it becomes evident that cells derived from autoantigen-bearing donors fail to produce IL-2, IL-3, and IFN-γ and do not express the IL-2Rα chain, thus displaying an "anergic" phenotype. Only with increasing age, self-specific IELs decrease in frequency (*50*). Thus, in this system, self-specific thymus-dependent γ/δ T cells are clonally deleted,

TABLE II
ORIGIN OF T LYMPHOCYTES FROM DIFFERENT LYMPHOID COMPARTMENTS

	Phenotype of T cells[b]	
Organ[a]	Thymic origin	Extrathymic origin
Lymph node	Most α/β and γ/δ T cells	Vγ4–Dγ2Jγ1
i-IEL	α/β $TCR^+CD4^-CD8\alpha^+\beta^+$	α/β $TCR^\pm CD8\alpha^+\beta^-$;γ/δ TCR^+
s-IEL	Vγ3–Jγ1–Cγ1–Vδ1–Dδ3–Jδ2	Most other γ/δ T cells
Lung	—	Vγ5–Dγ2Jγ1
Liver	α/β TCR^{high}	α/β $TCR^{dull}CD4^-CD8^-$;γ/δ TCR^+
Bone marrow	α/β $TCR^+CD4^+/CD8^+$	α/β $TCR^+CD4^-CD8^-$
Peritoneum	—	α/β $TCR^+CD4^-CD8^-$

[a] i-IEL, Intestinal intraepithelial lymphocyte; s-IEL, skin intraepithelial lymphocyte.

[b] The predominant phenotypes of cells thought to require a thymus-dependent differentiation step, as well as of cells that arise in athymic animals, have been listed. For details and references, see text.

whereas autoreactive cells arising extrathymically are rendered functionally tolerant. Nonetheless, γ/δ i-IELs are susceptible to the induction of programmed cell death based on the significant portion of γ/δ i-IELs (not of α/β i-IELs) that rapidly succumbs to apoptosis after *ex vivo* isolation (*51*). Apparently, γ/δ i-IEL cells undergo extrathymic selection processes, because Vγ4 is expressed on a greater percentage of IELs in mice carrying the MHC *H-2*^{k} haplotype (50–70%) than in those homozygous for *H-2*^{d} (30%), irrespective of the presence of a thymus. This feature is probably controlled by the *H-2* I-E molecule (*44*).

Mouse α/β i-IELs and α/β T cells located in the lamina propria are either phenotypically similar to thymus-derived T cells (Thy-1$^+$CD5$^+$CD8α^+CD8β^+) (roughly 50%) or different (Thy-1$^+$CD5$^+$ CD8α^+CD8β^- or Thy-1$^-$CD5$^+$CD8α^+CD8β^-; approximately 20% each). Thy-1$^+$CD5$^+$CD8α^+CD8β^+ i-IELs are thymodependent (Table II), as shown by their absence in nude mice, have undergone self-superantigen-related Vβ deletions (*52*), and are absent in germfree mice (the *nu* mouse mutation leads to congenital athymia). In contrast, α/β TCR$^+$CD8α^+CD8β^- i-IELs may differentiate in a thymo-independent fashion, since they become detectable in the gut of old (12 months) athymic nude mice. Among i-IELs from normal BALB/c mice, an appreciable fraction (approximately 5%) is CD4$^+$CD8$^+$ and thus could represent an immature stage of extrathymic T cell differentiation (*53,54*). Thy-1$^-$CD8$\alpha^+\beta^-$ IELs express TCR Vβ regions that are deleted in peripheral T cells and in CD8$\alpha^+\beta^+$ IELs, e.g., Vβ3 and Vβ11 in BALB/c mice (*52*) and Vβ6 in AKR/J mice (*54*). This finding is reminiscent of athymic nude or neonatally thymectomized mice, in which T cells differentiating in the absence of a thymus also display a nondeleted TCR Vβ repertoire (55–59) (Table III), and therefore is interpreted as an argument in favor of the thymo-independence of CD8$\alpha^+\beta^-$ i-IELs. CD8$\alpha^+\beta^-$ i-IELs from BALB/c mice respond normally to *S. aureus* enterotoxin A (SEA, a superantigen that specifically stimulates Vβ3$^+$ and Vβ11$^+$ cells), but fail to proliferate with syngeneic spleen cells that express Mls-2^a and I-E and therefore may be expected to activate Vβ3$^+$ and Vβ11$^+$ cells. Thus, forbidden CD8$\alpha^+\beta^-$ IEL cells are either anergic to self-superantigens or have been selected in the gut for reactivity with bacterial superantigens, but for low affinity to self-superantigens (*54*).

Thus far, little is known about the function of i-IELs, cells that are located at the borderline of a milieu that is heavily colonized by saprophytary microorganisms and parasites. At this singular site, the im-

TABLE III
FORBIDDEN α/β T CELLS IN THE PERIPHERAL IMMUNE SYSTEM OF BALB/C MICE

Organ	Manipulation	Forbidden T cells[a]	Phenotype[b]
Spleen	None, 1–6 months	No	—
	None, 18 months	No	—
	Neonatal thymectomy	Yes	$CD4^+$ or $CD8^+$ (SP), anergic[c]
	Irradiation + CsA[d]	No	—
Lymph node	None, 1–6 months	No	—
	None, 18 months	Yes	—
	Neonatal thymectomy	Yes	SP, anergic
	Irradiation + CsA	No	—
Peritoneum	None	No	—
	Neonatal thymectomy	Yes	DN or SP, anergic
	CsA	No	—
	Irradiation + CsA	Yes	DN, not anergic
i-IEL	None	Yes	$CD8\alpha^+\beta^-$, not anergic[e]
Bone marrow	None	Yes	DN, not anergic

[a] Self-superantigen-reactive TCR Vβ gene products that normally are clonally deleted in single-positive T cells from spleen and lymph nodes of BALB/c mice (Vβ3, Vβ5, and Vβ11) are considered as "forbidden."

[b] SP, Single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$); DN, double negative ($CD4^-CD8^-$).

[c] Anergy is defined as the incapacity to proliferate in response to matrix-bound antibodies specific for the forbidden Vβ.

[d] Sublethal irradiation (750 rad), followed by daily injections of cyclosporine A (CsA).

[e] Cells proliferate in response to the superantigen *Staphylococcus aureus* enterotoxin A, which stimulates cells bearing TCR Vβ3 or Vβ11 products (*54*).

mune system is additionally challenged with the task of avoiding unnecessary and potentially harmful immune reactions against alimentary compounds whose incompletely digested breakdown products are resorbed via the mucosae. This special feature may be involved in the induction of oral tolerance (*60,61*). Gut-associated lymphoid tissue (GALT) arose during evolution prior to the development of the thymus. If ontogeny recapitulates phylogeny, then it may be expected that the GALT would represent a more "primitive" compartment that fulfills functions that are important early in life, e.g., induction of oral tolerance and surveillance of the bacterial colonization in the gut (*62*). The finding that γ/δ i-IELs have a relatively restricted repertoire is in line with the idea of a "primitive" function. The i-IELs from mice orally immunized with sheep red blood cells (SRBCs) contain T cells that exhibit T contrasuppressor activity and, on adoptive transfer, reverse the humoral anti-SRBC tolerance of mice orally tolerized with SRBCs. This function is restricted to a population of γ/δ TCR^+ i-IELs that binds the lectin of *Vicia villosa* (*63*), irrespective of

whether they express CD8α^+ (*62*). In contrast, α/β i-IELs function as T helper cells for *in vitro* anti-SRBC antibody responses (*63*).

T cells isolated from the gut also display particular growth requirements and lymphokine productions patterns. Thus, freshly isolated α/β or γ/δ i-IELs fail to proliferate and to express IL-2 receptor α chains on stimulation with solid-phase anti-CD3ε, anti-α/β TCR, or anti-γ/δ TCR, no matter whether accessory cells, IL-2, IL-4, or phorbol ester are added to the culture (*64*). In contrast, they exhibit CD3/TCR-mediated redirected killing activity because they are cytotoxic for FcγR-bearing target cells in the presence of anti-CD3ε, anti-α/β TCR, or anti γ/δ TCR antibodies (*37,43,64*). Accordingly, i-IELs contain granules that are identical to those observed in natural killer (NK) or cytotoxic T cells in that they contain serine esterases of the granzyme family and perforin (*39*). Human i-IEL lines exhibit a predominant α/β TCR$^+$CD4$^-$CD8$^+$ phenotype, have a restricted repertoire, and exhibit cytotoxic activity specific for class I-like CD1 molecules (*65*). However, the class Ib restriction initially postulated for γ/δ T cells has not been confirmed in other experimental systems. In humans, the duodenal mucosa contains a significantly higher frequency of spontaneously IFN-γ-secreting cells, as compared to peripheral blood, inflamed gingival tissue, and bone marrow (*66*). Both γ/δ and α/β i-IELs expressing CD8α of the mouse spontaneously produce IL-5 and IFN-γ (*67*), and this has also been reported for α/β TCR/CD3$^+$CD4$^+$CD8$^-$ T cells from the submandibular salivary gland (*29*). T_{H2}-type CD4$^+$ T cells that produce IL-5 and IL-6 are more frequent in the lamina propria than in other tissues (*68,69*), a finding that could be related to the high production of T_{H2} lymphokines by mucosal lymph node cells (*28*). Moreover, the local production of IL-5 and IL-6 might condition the terminal differentiation of surface IgA$^+$ lamina propria B lymphocytes to IgA-secreting plasma cells in the gut and in salivary glands. Accordingly, depletion of local T cells with anti-CD4 antibodies results in a reduction in the number of mature IgA plasma cells present in the lamina propria of the intestine, as well as in the overall size of germinal centers contained in the Peyer's patches (*70*). In synthesis, it appears that cells regulating oral tolerance or mediating T cell help and cytotoxicity are present in the gut.

C. Invariant Intraepithelial γ/δ T Cells in the Skin and in the Female Reproductive Tract

Two classes of intraepithelial lymphocytes, those contained in the skin (s-IELs) and those present in the female reproductive tract (endometrium, myometrium, and vagina) and tongue (r-IELs), are char-

acterized by the preponderance of the same invariant γ/δ TCR chains, irrespective of the mouse strain. Because, moreover, the γ/δ TCR lacks any junctional diversity, the repertoire of s-IELs and r-IELs is largely monomorphic. s-IELs are sessile Thy-1$^+$CD4$^-$CD8$^-$ dendritic epidermal cells (dECs) expressing identical Vγ3–Jγ1Cγ1 and Vδ1–Dδ3–Jδ2 γ and δ chain sequences (*71,72*), whereas r-IELs bear the Vγ4–Jγ1Cγ1 γ chain in conjunction with the same δ chain as s-IELs (*73*). Thus, these cells differ from variable γ/δ cells contained in the spleen and lymph nodes that express different γ and δ chains (mainly Vγ2), display considerable junctional diversity, and appear later in ontogeny (*74–76*). Moreover, invariant s-IELs and r-IELs differ from another invariant population that expresses Vγ5–Dγ2Jγ1 on the majority of BALB/c lung and lymph node γ/δ T lymphocytes in the sense that only these latter cells are subjected to strain-dependent selection processes (*77*).

Vγ3$^+$ s-IELs are thymus-derived based on their absence in athymic nude mice and their presence after engraftment with fetal thymic lobes (*78*), whereas the invariant BALB/c Vδ5–Dδ2Jγ1 lymph node and lung population may arise from an extrathymic differentiation pathway (*77*). The s-IELs from nude mice express other γ/δ gene products (Vγ1.1–Jγ4–Cγ4 and Vδ6–Jδ1–Cδ) that have been speculated to originate from the liver (*79*) and display junctional diversity, and thus do not belong to the invariant series (*80*). The rearrangement of the γ/δ TCR V genes expressed on invariant intraepithelial γ/δ T cells (Vγ3/Vδ1 followed by Vγ4/Vδ1 corresponding to s-IELs and r-IELs, respectively) takes place during early fetal thymocyte development, before variable γ/δ T cells arise (*72,73*) and before the enzyme responsible for N-nucleotide addition, terminal desoxyribonucleotidyltransferase, is expressed, thus explaining the lack of nucleotide additions in the junctional region. The low expression of an exonuclease implicated in imprecise joining could furthermore contribute to the generation of an invariant complementarity-determining region 3 of the TCR. In later stages of fetal development, the subsequent waves of γ/δ T cells arising in the thymus not only differ in the employment of the TCR gene segments and in a major junctional diversity, but also in their capacity to home to specific peripheral sites (*81*). In spite of the very markedly site-dependent TCR repertoire (*74*), the TCR per se does not guide γ/δ T cells to a particular site, because T cells expressing a transgenic γ/δ TCR home to any lymphoid compartment, irrespective of the specificity of the antigen receptor (*82*).

Little information is available on the function of s-IELs and r-IELs.

The s-IELs proliferate in response to ConA or anti-TCR/CD3 antibodies via an IL-2/IL-2 receptor-mediated pathway and can mediate cytolysis of anti-CD3 or anti-TCR-coated targets (*83*). Although no evidence is available that s-IELs are activated *in situ*, such cells (Thy-1$^+$CD4$^-$CD8$^-$) respond *in vitro* to a keratinocyte-specific peptide by producing IL-2. The specific recognition of keratinocytes is mediated by the invariant Vγ3$^+$ TCR (*84*). It can be speculated that the expression or presentation of such a keratinocyte-specific self-antigen would be induced by infection, transformation, or other stimuli. Variant extrathymically arising s-IELs express a TCR that has been implicated in the response to mycobacterial antigens and self-heat-shock proteins (*80,85*). Similarly, invariant r-IELs could be specific for autologous "stress" antigens, thus exerting a "trauma signal surveillance" (*76*). It is possible that invariant IELs represent a more primitive phylogenetic class of immune defense than do variable γ/δ T cells. A subset of presumably thymus-derived s-IELs express Vγ1–Jγ1Cγ4 in conjunction with Vδ6Cδ (*86*) and spontaneously secrete cytokines (IL-4 and GM-CSF). This may be attributed to the expression of an autoreactive γ/δ TCR, because α/β loss-mutant T cells transfected with this γ/δ TCR spontaneously start to produce IL-2 in culture (*87*). Activation of these autoreactive cells requires the interaction of the vitronectin receptor with extracellular matrix proteins expressing the RGDS motif, suggesting a complex regulation of their activity (*88*). The recognition of cell type-specific or ubiquitous self-antigens could reflect a primitive immune function that, instead of detecting foreign antigens shed by intruding microorganisms, marks changes in the self, thus alarming inflammatory cells and triggering the first steps to a specific immune response that would be mounted by nonsessile lymphocytes recruited from the circulation.

D. T Lymphopoiesis in the Liver

The fetal liver is a major source of hematolymphoid stem cells, including precursors for T, B, and NK lymphocytes as well as myeloid and erythroid colony-forming units. In addition, the hepatic microenvironment allows for T lymphopoiesis after birth. The liver sinusoids of normal 15- to 20-week-old mice (C3H/He, BALB/c) contain a high number of unusual T lymphocytes that phenotypically resemble the *lpr* cell (*89*), i.e., a cell type expanded in mice homozygous for the lymphoproliferation (*lpr*) mutation (*90,91*). Whereas the spleen and lymph nodes contain $<1\%$ α/β TCR$^+$CD4$^-$CD8$^-$ cells, $<1\%$ Thy-1$^+$B220$^+$ cells and $<20\%$ Pgp-1^{high} cells, such cells represent in the liver approximately 5, 5, and 40–50%, respectively, of cell

types. The α/β $TCR^+CD4^-CD8^-$ and $Pgp\text{-}1^{high}$ subset found in the liver is confined to a α/β TCR^{dull} subset not seen in spleens and lymph nodes (89). Hepatic α/β TCR^{dull} cells arise from thymo-independent differentiation, given that athymic nude mice contain such T cells in the liver, though they lack TCR^{high} cells. In spite of the local T cell differentiation, no $CD4^+CD8\alpha^+$ cells (which, in the thymus, are considered as maturating T cells) are detectable in the liver (54). Products of the Vβ8 gene family are overrepresented among α/β TCR^+ cells (50% in the bone marrow as compared to 30% in lymph nodes and spleen), especially among the α/β TCR^{dull} (>70%) and the α/β $TCR^+CD4^-CD8^-$ subsets (>90%) (89). This preponderance of Vβ8 is also found among α/β $TCR^+CD4^-CD8^-$ thymocytes (92,93) and *lpr* cells (21,94,95). No "forbidden" T cell receptors (e.g., $V\beta3^+$ or $V\beta11^+$ cells in BALB/c mice) are enriched among the hepatic α/β $TCR^+CD4^-CD8^-$ cells in normal mice (54) (Table III). In contrast, estrogen injection into male 6- to 8-week-old C3H/He mice results in an increase of hepatic α/β TCR^{dull} cells, including forbidden T cell oligoclones (Mls-2^a-reactive $V\beta3^+$ cells) (96). Bacterial stimulation also causes an increase in self-superantigen-reactive hepatic T cells (97), but the biologic significance of these findings remains elusive.

During the first weeks after birth the liver contains an increasing number of γ/δ T cells that are not detectable during the fetal or perinatal phases. Such cells have a lymphoblastic morphology, can spontaneously proliferate *in vitro*, are phenotypically $CD4^-CD8\alpha^+CD8\beta^-$, and predominantly express Vγ1 or Vγ2 in conjunction with Vδ6. As α/β TCR^{dull} lymphocytes, γ/δ T cells apparently have an extrathymic origin because they are not reduced in athymic nude mice (79).

In summary, the liver could be a major source of thymus-independent T cell generation, but the function of hepatic T cells remains obscure. It is tempting to speculate that the hepatic α/β $TCR^+CD4^-CD8^-$ subset is involved in the pathogenesis of autoimmunity, given that it is not only expanded in MRL/MP–*lpr/lpr* mice (98), but also in other autoimmunity-prone inbred strains, including C3H/HeJ–*gld/gld*, MRL-+/+, (NZB × W)F_1, and BXSB (99). Kinetic studies of the uptake of tritiated thymidine revealed that hepatic α/β $TCR^+CD4^-CD8^-$ cells from MRL/Mp–*lpr/lpr* mice proliferate *in vivo* and are exported to the hyperplastic lymph nodes (98). Hepatic T lymphopoiesis is also accentuated in mice treated with estrogen (96), subjected to bacterial stimulation (heat-inactivated *Propisnebacterium acnes* or *Escherichia coli*, i.p.) (97), or injected with syngeneic

tumor cells (*100*), revealing that an increase in liver T cells per se is not indicative of the posterior development of autoimmune lesions.

E. Bone Marrow T Cells

In the adult bone marrow, two-thirds of $CD3^+$ cells are double negative ($CD4^-CD8^-$) with a predominance of the α/β TCR (70–80%) over the $\gamma\delta$ TCR. In contrast, double-negative cells constitute a minor population (1–2%) and are mainly positive for the $\gamma\delta$ TCR in peripheral lymphoid tissues (spleen, lymph nodes, blood, peritoneum) of normal adult BALB/c, C57BL/6, or CBA/J mice. Unlike *lpr* or *gld* cells, but similar to splenic α/β $TCR^+CD4^-CD8^-$ cells from normal mice (*101*), double-negative T cells derived from the normal adult bone marrow do not express B220 or J11d and are Thy-1 and Ly-1 positive (*102*). In contrast to immature double-negative thymocytes (*92,93*), to hepatic T lymphocytes (*89*), and to *lpr* cells (*21,94,95*), as well as to splenic double-negative T cell (*103*), among which products of the Vβ8 TCR gene family are overrepresented (*vide supra*), depending on the mouse strain, only 14–35% of the $CD3^+CD4^-CD8^-$ bone marrow cells express Vβ8.

The α/β $TCR^+CD4^-CD8^-$ cells from the bone marrow are highly unique in several aspects. They develop in a thymus-independent fashion, because they are present in the bone marrow of athymic nude mice (*104*) and appear to develop directly from bone marrow precursors without rearranged β chain genes during a 48-hour *in vitro* culture (*105*). A significant proportion of α/β $TCR^+CD4^-CD8^-$ bone marrow lymphocytes bear TCR Vβ gene products that are deleted among single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) T cells from lymph nodes or the spleen, i.e., Vβ11 (which recognizes a product of the endogenous retrovirus Mtv9 in the context of I-E), Vβ3 and Vβ5 (reactive with Mtv9 and Mtv6) (*106*) in BALB/c mice ($I\text{-}E^+$, $Mtv6^+$, $Mtv9^+$), and Vβ8.1 (Mtv7) and Vβ3 (Mtv6) in DBA/2 ($I\text{-}E^+$, $Mtv6^+$, $Mtv7^+$) and CBA/J mice. The presence of a significant percentage (1–5%) of forbidden Vβ3, Vβ5, Vβ8.1, and Vβ11 in the bone marrow of BALB/c, DBA/2, or CBA/J mice indicates that neither these cells nor their precursors have undergone a self-superantigen-related clonal deletion (*102,105*). These results are in agreement with recent evidence that only a portion of double-negative T cells from the thymus have undergone self-tolerance-related clonal deletion (*107*) and confirm the physiological existence of extrathymic T cells expressing an at least partially self-reactive TCR repertoire. Lack of selection among $CD4^-CD8^-$ cells is also suggested by recent molecular studies. In mice carrying a transgenic TCRα chain, the repertoire of TCRβ chains

coexpressed with the transgenic α chain is limited in $CD4^+$ or $CD8^+$ T cells, not in $CD4^-CD8^-$ T cells. This indicates that certain structural or functional constraints do not apply to the population of $CD4^-CD8^-$ T cells (*108*).

The α/β $TCR^+CD4^-CD8^-$ cells from the bone marrow are different from a similar splenic T cell subset that does exhibit Mls-1^a- and I-E-induced clonal deletion, although it exhibits strong autoreactivity in syngeneic mixed lymphocyte reactions (*101*). Fluorescence-activated cell sorting (FACS)-purified $CD3^+CD4^-CD8^-$ cells from the bone marrow of BALB/c mice proliferate in response to monoclonal antibodies (mAbs) specific for anti-Vβ11 and anti-Vβ5 and produce IL-2, IL-3, IL-4, and IL-5 (*102*), indicating that these cells are not refractory (anergic) to such *in vitro* stimuli, as is the case with *lpr* cells (*90,91*) and forbidden T cells contained in the spleen or lymph nodes of athymic (neonatally thymectomized or constitutively athymic *nu/nu*) BALB/c mice (*56,57,109*). Furthermore, sorted $CD3^+CD4^-CD8^-$ bone marrow cells respond to anti-CD3 antibodies (*102*) (Table IV) whereas splenic $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells fail to do so in the

TABLE IV

DIFFERENTIAL EFFECTS OF TCR CROSSLINKING IN $CD4^-8^-$ BONE MARROW AS COMPARED TO $CD4^+8^-$ AND $CD4f1055^-8^+$ CELLS FROM THE SPLEEN[a]

Stimulus	Pretreatment	Addition of accessory cells	Proliferation of responder population		
			$CD4^-8^-$	$CD4^+8^-$	$CD4^-8^+$
αCD3	None	No	+	−	−
αCD3 + IL-2	None	No	+	+	+
αCD3	Sham	No	+	−	−
αCD3	Sham	Yes	+	+	+
αCD3	αCD3	No	+	−	−
αCD3	αCD3	Yes	+	−	−
αCD3	αCD3 + IL-2	No	ND	−	−
αCD3	αCD3 + IL-2	Yes	ND	+	+

[a] Double-negative bone marrow cells and single-positive $CD4^+$ or $CD8^+$ T cells from BALB/c mice were purified by FACS and cultured in the presence or absence of irradiated (2000 rad) syngeneic total spleen cells. Cells were stimulated with plastic-bound anti-CD3 antibody (145.C11) in the presence or absence of recombinant IL-2 (20 U/ml). After 3 days the uptake of tritiated thymidine was assessed. Where indicated, cells were pretreated with an anti-CD3 antibody (145.C11) or antihuman CD1 (sham; NA1/34) adsorbed to the polystyrene surface of microtiter plates during an 18-hour period, washed, and tested for their proliferative activity. Significant proliferation ($p < 0.01$; Δcpm > 10,000) as compared to unstimulated control cultures is indicated by a plus symbol. ND, not determined.

absence of accessory cells or exogenous IL-2 (*110*). Finally, anti-CD3 promotes down-modulation of the α/β TCR/CD3 complex, but only in $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells, and to a much lesser degree in double-negative cells (*102*). Because the reduction in TCR/CD3 expression has been suggested to account for nonresponsiveness *in vivo* (*111,112*), this may explain why double-negative T cells isolated from the bone marrow are relatively resistant to the induction of unresponsiveness. Accordingly, pretreatment of bone marrow T cells with anti-CD3 fails to induce a posterior refractoriness to stimulation with anti-CD3 antibody, as this is observed in canonical single-positive splenic T cells (Table IV).

The functional status of peripheral $CD4^-CD8^-$ T cells is unique insofar as these cells have not been subjected to, or are resistant to, clonal deletion mediated by self-superantigens, induction of proliferative anergy, and CD3/TCR down-modulation. Given that, in spite of the lack of an accessory molecule, double-negative T cells may well exert effector functions, these findings shed serious doubts on the role of self-superantigens and superantigen-related clonal deletion and anergy of $V\beta$ gene products for the preservation of self-tolerance (*113*). Human double-negative α/β T cells have been found to exhibit class I-dependent cytotoxic activity (*114,115*) or helper function for B lymphocytes (*116,117*), including the production of nephritogenic anti-DNA autoantibodies (*118*). Such human T cell lines are functional analogs of double-negative T cells from various lupus-prone inbred mouse strains that induce the production of pathogenic oligoclonal anti-DNA autoantibodies (*119,120*). Apparently, T cells that are neither clonally deleted nor rendered anergic in response to superantigens encoded by endogenous retroviruses may exist in normal mice without causing any autoimmune pathology. This could be related to the fact that purified $CD4^-CD8^-$ α/β T cells derived from the bone marrow inhibit mixed lymphocyte reactions, thus exerting a suppressor rather than helper function (*105*).

F. Peritoneal T Cells

The pleuroperitoneal cavity has been clearly demonstrated to possess a lymphocytotropic nature for Ly1/$CD5^+$ B cells (*vide infra*). Recent evidence from studies in normal mice as well as in experimentally manipulated animals has revealed differences in the phenotype and repertoire of peritoneal T lymphocytes as compared to splenic or lymph node cells.

In normal, untreated BALB/c mice, cells positive for the 3G11 epitope, probably a carbohydrate moiety on the Thy-1 glycoprotein, rep-

resent the vast majority of $CD4^+$ peripheral blood lymphocytes, 60–80% in the spleen and lymph nodes, but only a minority in the peritoneum. Splenic $CD4^+3G11^-$ cells have been shown to exhibit a memory phenotype ($CD34RB/C^{low}$, $Mel\text{-}14^{low}$, $CD44^{high}$), to be relatively resistant to the short-term mitogenic effect of ConA, to lack IL-2 production, and to be enriched for IL-4, IFN-γ, and IL-5 producers (*121*), but the functional properties of peritoneal $CD4^+3G11^-$ cells remain to be elucidated. Splenic $CD4^+3G11^+$ cells have been suggested to be recent thymic emigrants that give rise to $CD4^+3G11^-$ cells (*121*), but the exact origin of peritoneal T cells is unclear.

Experiments involving neonatally thymectomized mice suggest that the peritoneum may serve as a site of thymo-independent T cell differentiation. Whereas neonatal thymectomy of CBA/H mice results in a diminution of T cells in the spleen and lymph nodes, it causes a marked transient (8–12 weeks) increase in $Thy\text{-}1^+CD3^+$ cells in the peritoneal cavity. During the initial phase (4–8 weeks) after thymectomy peritoneal T cells exhibit predominantly a double-negative ($CD4^-CD8^-$) phenotype, but these cells are outnumbered by single-positive T cells in subsequent stages (>12 weeks). Such double-negative T cells do not arise in lymph nodes or spleen of thymectomized animals nor are they detectable in the peritoneum of euthymic controls. At any stage investigated (6–24 weeks after thymectomy), products of the TCR Vβ11 gene family (i.e., an I-E-reactive TCR normally deleted in I-E-bearing CBA/H mice) are selectively overexpressed in the peritoneum as compared to other lymphoid organs (*58,122*). In contrast to forbidden double-negative T cells from the bone marrow (*102*), forbidden T cells from the peritoneum of thymectomized mice display an anergic behavior and fail to proliferate on crosslinking of the forbidden TCR Vβ product (*122*). Nonetheless, the numeric increase in the peritoneal T cell population subsequent to thymectomy indicates that, under athymic conditions, T cell differentiation and/or accumulation occurs in the peritoneal cavity. Accordingly, in mice with severe combined immunodeficiency, $CD3^+$ T cells are found more often, and in a higher percentage of animals, in the peritoneum than in the spleen (*123*). Human peripheral blood $CD4^+$ and $CD8^+$ T cells injected intraperitoneally into SCID mice persist up to 50 days in the peritoneum, but recirculate scarcely, if at all, into the spleen and peripheral blood (*124–126*). This finding again points to the peritoneum as a specialized environment for T lymphocyte recruitment.

Along the same line, treatment of BALB/c mice with cyclosporine A (CsA) establishes a clear-cut dichotomy between peritoneal and other

lymphoid compartments traditionally defined as peripheral with respect to the central organ for T cell differentiation, the thymus. It has been reported that CsA perturbs intrathymic T cell development, interfering with the differentiation of immature $CD3^-CD4^+CD8^+$ thymocytes to mature $CD3^+CD4^+CD8^-$ or $CD3^+CD4^-CD8^+$ T cells. In contrast to other lymphoid organs (spleen, lymph nodes), daily CsA injections into irradiated BALB/c mice leads to a sixfold increase in the number of peritoneal T cells (*127*). Moreover, BALB/c mice treated by sublethal irradiation and receiving high CsA doses, not controls that are only irradiated or CsA-injected alone, exhibit a selective increase in forbidden T cells in the peritoneum (*127*). Depending on the mouse strain analyzed, CsA treatment of sublethally irradiated mice leads to a surge of forbidden T cells, i.e., T cells that, due to their unwarranted specificity for endogenous superantigen (retroviral products presented by MHC molecules), are clonally deleted in the thymus. This increase may be detected at the level of forbidden TCR Vβ gene products that are expressed in peripheral lymphoid organs (lymph nodes and spleen) of C57BR or (C57BL/6 × CBA)F_1 animals (*128,129*), but is not detected in BALB/c mice (*130,131*). In strict contrast with the data obtained in the spleen and lymph nodes, however, CsA treatment does result in expression of the forbidden TCR gene products Vβ5 and Vβ11 in the peritoneum of BALB/c mice, accompanied by a significant absolute and relative increase in peritoneal $CD3^+$, Thy-1^+, and $CD4^+$ or $CD8^+$ cells. Whereas no $CD4^+CD8^+$ (double positive) cells become detectable, a significant portion of peritoneal T cells from CsA-injected animals exhibits a $CD3^+CD4^-CD8^-$ (double negative) phenotype (*127*). The increase in forbidden ($V\beta5^+$ and $V\beta11^+$) T lymphocytes, as well as in double-negative ($CD3^+CD4^-CD8^-$) cells, in the peritoneum depends on prior irradiation of mice. Only in conditions in which T cells have to repopulate their usual habitats from the precursor population does CsA-driven expansion/enrichment of total T cells in the peritoneum occur. In contrast to cells retrieved from neonatally thymectomized animals (*122*), peritoneal T cells recovered from CsA-treated animals readily proliferate on crosslinking with solid-phase anti-Vβ5 and anti-Vβ11 antibodies in the absence of IL-2 (*127*), indicating that these potentially autoaggressive T cells are not anergic, probably because CsA inhibits the induction of nonresponsiveness (*132*). In spite of the presence of a nondeleted, nonanergic T cell population, no local or systemic signs of inflammation are detectable (*127,131*).

Recent studies on the expression of transgene-encoded α/β or γ/δ TCR products revealed that the repertoire expressed by peritoneal T

cells differs significantly from that contained in the spleen or lymph nodes. Transgenic TCR α or β chains inhibit the rearrangement and expression of the endogenous TCR α and β loci, respectively, a phenomenon that is referred to as allelic exclusion. Thus, female C57BL/6 (*H-2D*^{b}) mice bearing α and β TCR transgenes obtained from a male antigen H-Y-specific class I *H-2D*^{b}-restricted cytolytic CD4$^-$CD8$^+$ T cell clone express the transgenic β TCR (β_T) (recognized by the antibody F23.1) on the vast majority (>85%) of CD3$^+$ T cells in the thymus and in the periphery (spleen, lymph nodes, and peritoneum), as it efficiently suppresses the rearrangement of the endogenous TCR β loci (*133*). In contrast, the transgenic α chain (α_T) is not expressed on all T cells and two fractions of T cells exist in which β_T either pairs with α_T or associates with endogenous α chains (α_E) (*134*). The proportion of $\beta_T\alpha_T$ (male antigen specific) and $\beta_T\alpha_E$ changes between the thymus and other lymphoid organs due to central and peripheral selection processes (*135*). Whereas 50–60% of β_T^+ thymocytes are α_T^+, this percentage is lower in the periphery, ranging from 15 to 40% in spleen and lymph nodes. Peritoneal T cells practically lack α_T expression (<3% of β_T^+cells), indicating a selection in favor of α_E (*136*) (Fig. 1; Table V). This finding was obtained for both male and female mice, illustrating that the (auto)antigen does not intervene in the peritoneal selection/accumulation of cells expressing endogenous genes. The stronger tendency in favor of endogenous α chain expression, as compared to endogenous β, can be related to the fact that allelic exclusion by the β transgene is much more efficient than that by α (*137*).

In agreement with the markedly organ-dependent expression of $\beta_T\alpha_E$ versus $\beta_T\alpha_T$ TCR chains, the analysis of γ/δ transgenic mice (*138*) revealed a similar finding. Because γ/δ T cells populate their habitat (skin, gut epithelia, lungs, etc.) irrespective of the γ/δ transgene they express (*82*), and the expression of endogenous γ/δ products is negligible, the comparison of the γ/δ TCR expression among all CD3$^+$ T cells can be interpreted as an indication of the selection of γ/δ T cells. The γ/δ T cells are nearly equally distributed in the thymus, spleen, and lymph nodes (60–80% of all T cells). In contrast, γ/δ T cells represent only 15–30% in the peritoneum (Fig. 1). Thus, both the transgenic γ/δ TCR and the transgenic α TCR chains are selectively regulated in a hierarchical level of expression according to the following order: thymus $\leq$ lymph node $\leq$ spleen $\ll$ peritoneum. In consequence, selection of the immune repertoire appears to operate in a different way in various lymphocyte compartments. At present, no definitive explanation may be forwarded for this phenomenon. The peritoneum might either serve as a preferential site for extrathymic T cell differentiation

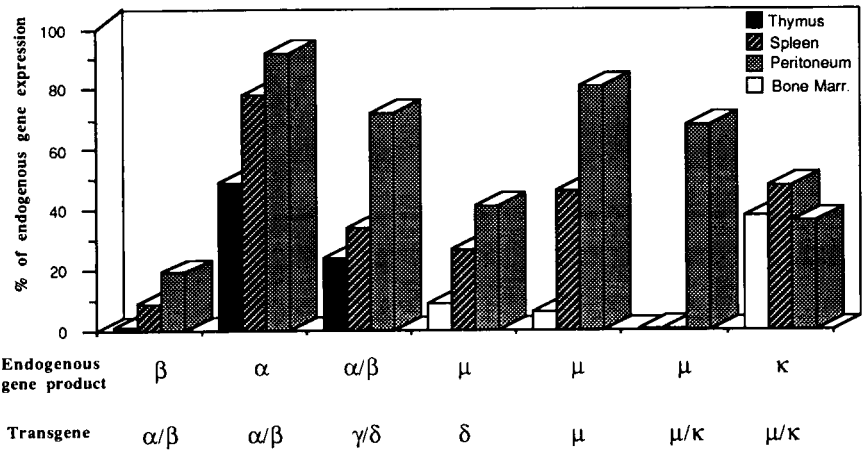

FIG. 1. Peritoneal lymphocytes exhibit a low degree of allelic exclusion. Animals bearing transgenes encoding an α/β TCR specific for the male-specific H-Y antigen (female mice analyzed), a γ/δ TCR specific for products of the MHC-TL^a product (TL^b mice analyzed), an immunoglobulin heavy μ chain derived from a trinitrophenyl-specific hybridoma, an immunoglobulin heavy δ chain with the same V/D/J region cloned 5′ to the Cδ exons, or both a μ and a κ chain (specific for $H\text{-}2K^b$; $H\text{-}2K^d$ mice analyzed) were analyzed at 3–5 months of age. The percentage of endogenous versus transgene expression was determined by two-color immunofluorescence analysis using appropriate antibodies that recognize transgenic gene products (F23.1, specific for the transgenic β chain; T3.70, which recognizes the α chain; and antibodies recognizing the γ/δ TCR or the CD8α chain, anti-IgD^a, anti-IgM^a, and 54.1, which reacts with the combined idiotype formed by the transgenic μ and κ chains) or endogenous gene products (anti-IgM^b). Values give the percentage of T (CD3$^+$) or B (IgM$^+$) lymphocytes that express endogenous gene products in the thymus, spleen, peritoneum, or bone marrow. For details and references, see text.

—perhaps a nonconventional T cell lineage—or might provide a microenvironment that favors the expansion and/or accumulation of a particular T cell repertoire.

III. B Cells in Peripheral Compartments

In analogy to T lymphocytes, the distribution of B cells follows a nonrandom pattern. Surface IgA-bearing lymphocytes are highly represented in the mucosa-associated lymphoid structures (lamina propria and Peyer's patches); the nonkeratinizing external surfaces of the body (gut and exocrine glands, including the lactating mammary gland, urogenital epithelia, and upper respiratory tract) attract predominantly IgA-secreting plasma cells. In nonmucosal sites (peripheral lymph

TABLE V
PERITONEAL BIAS IN ENDOGENOUS GENE EXPRESSION IN TRANSGENIC MICE[a]

Transgene	Endogenous gene studied	Cell population	Peritoneal bias in endogenous gene expression
α/β	α	T	Yes
α/β	β	T	Yes
γ/δ	α/β	T	Yes
CD8	CD4	T	No
δ	μ	B	Yes
μ	μ	B	Yes
μ/κ	μ	B	Yes
μ/κ	κ	B	No

[a] Animals bearing transgenes encoding an α/β TCR specific for the male-specific H-Y antigen, a γ/δ TCR specific for products of the MHC *TL*[a] product, an immunoglobulin heavy μ chain derived from a trinitrophenyl-specific hybridoma, an immunoglobulin heavy δ chain with the same V/D/J region cloned 5′ to the Cδ exons, or both a μ and a κ chain (specific for *H-2K*[b]) were analyzed at 3–5 months of age. Animals lacked the relevant self-antigen (female, *TL*[b], or *H-2K*[d] donors, respectively), thus excluding antigen-dependent selection as the driving force of peritoneal expression of endogenous gene products. Endogenous versus transgene expression was determined by two-color immunofluorescence analysis using appropriate antibodies (Fig. 1). A peritoneal bias in endogenous gene expression is determined by the comparison with splenic and lymph node populations derived from the same individual ($p < 0.01$, paired Student t test).

nodes, spleen, and skin), IgA-secreting cells are infrequent and most plasma cells secrete IgM or IgG. Similarly, distinct differentiation and activation stages of B lymphocytes are discontinuously distributed in different zones of lymph follicles. The expression of different V_H gene families is also inhomogeneous, resulting, e.g., in an increased representation of the V_H J558 family in peripheral lymph nodes and of the V_HX24 family in intestinal Peyer's patches (*139*). In view of the ongoing polemics on the "conventional bone marrow-derived" $CD5^-$ and the "peritoneal" $CD5^+$B cell subsets, the $CD5^+/CD5^-$ dichotomy of the B cell system will be discussed in detail. New insights into the functional anatomy of lymphoid follicles will also be briefly mentioned. However, it is beyond the scope of this survey to review the structure and function of the lymphoid tissues involved in the secretory or mucosal immune system, i.e., the lymph follicles and adjacent structures organized in the gut and bronchus-associated lymphoid tissues. The reader is referred to the excellent reviews by Mestecky and McGhee (*140*) and Sminia and co-workers (*141*).

A. THE $CD5^+/CD5^-$ DICHOTOMY

B cells may be divided into two classes according to the expression of CD5, a signal-transducing receptor (*142*) that interacts with the B

cell surface structure CD72/Lyb-2 (*143*). According to the official nomenclature, B1 cells represent the $CD5^+$ ($Ly\text{-}1^+$) subset and B2 cells represent the "conventional" type (*144*). $CD5^+$ B cells differ from conventional B cells with respect to the phenotype [$IgM^{high}IgD^{low}$-Mac-1(CD11b/CD18)$^+CD45^{low}Fc\varepsilon R^-IL\text{-}5R^+$]. The $CD5^-$ "sister" population has a phenotype similar to that of the $CD5^+$ B cells except that it lacks CD5 (*145–150*). $CD5^+$ B cells predominate in the coelom-derived peritoneal, pericardial, and pleural cavities (*145,151,152*), thymus (*153,154*), and tonsils, but are a minority of B cells in adult lymph nodes and spleen. Cells of the sister type are preferentially located in the marginal zone, not in the follicles of the murine spleen (*148*). In the fetal spleen all B cells express CD5, but soon after birth $CD5^-$ B cells predominate. Similarly, human fetal and umbilical cord blood B lymphocytes are $CD5^+$. This percentage declines and stable adult levels (25–35% of total B cells) are reached in late adolescence (*155*).

$CD5^+$ B cells are endowed with the capacity of self-renewal, i.e., they may expand in the absence of any cell input from IgM^- precursors, unlike conventional B cells that are replenished from the bone morrow throughout life (*147,156,157*). Possibly they are derived from a source of stem cells independent of the bone marrow that gives rise to conventional B2 cells (*149*). The fetal liver contains precursors for both B1 and B2 cells. In contrast, the fetal omentum (13 days) reconstitutes the $CD5^+$, as well as the $CD5^-$ sister, but not the conventional B cell subset when transplanted under the kidney capsule of SCID mice. Moreover, it gives rise to IgA^+ plasma cells in the lamina propria of the gut and IgM^+ plasma cells in the speen (*158*). Similarly, transfer of peritoneal B cells into Ig allotype congenic mice revealed that many of the plasma cells located in these sites are derived from precursors in the peritoneal cavity (*159*). Fetal omentum (13 days) contains, in addition to B1 precursors, T cell precursors whose differentiation depends on the simultaneous transplantation of a fetal thymus (*158*). The peritoneal cavity also contains precursors of the $CD5^+$ population as revealed by transfer to allotype-congenic SCID mice (*160*). It thus appears that this region contains pluripotent precursors and that it may well constitute an equivalent of the aortic mesoderm that gives rise to the earliest hematopoietic stem cells in birds (*161*). Recent studies of the human system confirm the presence of B cell precursors and a high percentage of $CD5^+$ mature B cells in the fetal omentum (*162*).

Recent studies of the murine system revealed that the bone marrow contains B1 and B2 precursors. Bone marrow B cells from adult normal mice reconstitute $CD5^+$ B cells in the spleen and peritoneum of allotype-congenic and neonatal SCID recipients (*163*). Human

but not murine $CD5^-$B cells express CD5 following phorbol ester treatment (*164*). This observation, together with the recent finding that treating murine $CD5^-$ splenic B cells with a combination of antiimmunoglobulin plus IL-6 induces $CD5^+$ expression and the $CD23^-IgD^-CD45^{low}J11d^{high}$ phenotype of typical $CD5^+$ peritoneal B cells (*165*), introduces a question mark into the idea that the two different B cell lineages are separated from each other at an early precursor stage (*vide supra*). This dilemma might be resolved if it is assumed that at any stage B cells are faced with the option to give rise to a B2 or, after activation, B1 phenotype, the commitment to the B1 "lineage" being irreversible. B cell precursors that carry an unmutated autoreactive and highly connective immunoglobulin repertoire (*vide infra*) would be triggered to become $CD5^+$ B cells by receiving appropriate stimuli via the antigen receptor.

Both $CD5^+$ and $CD5^-$ splenic B cells manifest ontogenetic D-proximal V_H preferences, i.e., they start with equivalent repertoires, being mostly restricted to V_H7183 and V_HQ52 gene products in the mouse. Both populations partially lose this preference with age (*166*). In addition, adult (not neonatal) $CD5^+$ B cells express immunoglobulin heavy chains exhibiting the same characteristic N-nucleotide additions found in antibody genes transcribed in adult $CD5^-$ B cells and lacking in neonatal B cells (*167*). These findings may be interpreted as evidence in favor of local, age-dependent cellular selection mechanisms rather than genetically programmed differences in the repertoire expressed by $CD5^+$ and $CD5^-$ cells. Accordingly, both conventional splenic $Ig\mu^+\gamma^+$ B cells and peritoneal $CD5^+$ B cells derived from 4- to 6 month-old donors exhibit selected overlapping sets of germ line V_HJ558 gene products as opposed to the unselected bone marrow pre-B and the neonatal peritoneal $CD5^+$ V_HJ558 repertoire (*168–170*). Murine B1 cells exhibit a relative increase in λ light chain expression (*151*) and a biased use of V_H3-like sequences, i.e., V_H gene elements that have been phylogenetically conserved (*171,172*). Peritoneal and splenic $CD5^+$ B cells from adult mice show a certain bias toward V_H11 gene expression at the adult stage, as compared to $CD5^-$ cells, regardless of location (*166,173*). Similarly, peritoneal $CD5^+$ T cells display an elevated expression of V_H12 (*174,175*), antiphosporylcholine antibodies bearing the T15 idiotype (*176*), and particular V_HJ558 genes expressed only in pre-B cells but not in mature $Ig\mu^+\delta^+$ splenic B cells (*168,170*). By analogy, human $CD5^+$ B cells recovered from fetal tissues, cord blood, or adult peripheral blood have been reported to express preferentially V_H gene elements (V_H4,5,6) that are overrepresented in the preimmune B cell repertoire and in polyreactive antibod-

ies (*177,178*). The majority of antibodies produced by B1 cells have been found to be encoded by germ line genes without somatic hypermutation (*179*).

The fact that the same V_H elements, especially phylogenetically conserved ones, are preferentially rearranged in the B1 subset and during early life (*180–182*) suggests to some authors that the B1 subset represents a primitive polyreactive immune system that could provide the first line of defense against infections (*183*). Mouse $CD5^+$ B cells produce immunoglobulins with inherent low affinities for self-antigens, e.g., for ssDNA, bromelain-treated erythrocytes and thymocytes, and the so-called natural IgM antibodies that display multireactivity and harbor specificities for certain bacterial coat antigens (e.g., α1-3 dextran, phosphatidylcholine, and undefined *E. coli* determinants) (*184,185*). Accordingly, polyreactive human IgM autoantibodies (anti-ssDNA, antichondroitin sulfate, and antithyroglobulin) are produced by FACS-sorted $CD5^{high}$ B cells, but not by $CD5^-$ B cells from the human adolescent spleen. In contrast, fetal splenic $CD5^{high}$ and $CD5^-$ B cells are functionally uniform, both producing IgM autoantibodies (*155*). Although the majority of $CD5^+$ B cells express unmutated Ig, these cells, however, can undergo somatic mutations. Polyreactive antibodies with high connectivity derived from $CD5^+$ B cells may become antiidiotypic antigen-mimicking antibodies by somatic hypermutation (*186,187*) and have been speculated to be disconnected from the idiotypic network if mutations change the framework region III. Such cells that will select conventional pre-B cells for specificity rather than connectivity would expand after immunization and carry memory in the form of the imprint of the immune system's experience with antigen, thus contributing to a hypothetical network of idiotypic and antiidiotypic interaction that can pattern the expression of both T and B cell receptor specificities (*179,188,189*). Beyond any speculation, it is known that murine $CD5^+$ B cells exert a feedback regulation of their development (*190*) and peritoneal B cells (mostly $CD5^+$) reduce the number of small pre-B cells and B cells in the bone marrow on passive transfer, provided the donor and the recipient share the same immunoglobulin allotype (*191*). Thus, this effect appears to be mediated by immunoglobulins and not by lymphokines such as IL-10, which are selectively produced by $CD5^+$ B cells (*192*).

Peritoneal $CD5^+$ B cells are thought to escape selectively from clonal selection because transgenic expression of a μ gene with specificity for the hapten 4-hydroxy-3-nitrophenyl (NP) results in a strong depletion of bone marrow pre-B cells and of splenic B cells, whereas the $CD5^+$ B cell lineage is unaffected (*193*). In the same line, no

reduction in peritoneal $CD5^+$ cells expressing a transgene-encoded autoreactive (antierythrocyte) μ/κ antibody is observed, whereas a large portion of peritoneal and splenic $CD5^-$ cells is deleted (*194*). Scott and collaborators (*195*) recently reported that, as compared to conventional splenic B cells, peritoneal B cells (containing both $CD5^+$ and $CD5^-$ sister cells) were relatively resistant to the induction of nonresponsiveness (cell death?) in the IgM responses by overnight incubation with antimouse Ig. This was observed for the IgM responses against the haptens fluorescein and trimethylammonium, the latter being dependent on the skewed V_H11 usage of peritoneal $CD5^+$ B cells. The resistance of peritoneal B cells probably relies on blunted initial Ca^{2+} responses in response to anti-Ig crosslinking; phorbol myristate acetate and/or ionomycin treatment restores the responses of both peritoneal and splenic B cells to lipopolysaccharide. Thus, antibody production by peritoneal B cells is not subject to conventional tolerance pathways (*195*). However, it is not clear from these data whether this relative resistance is a particularity of $CD5^+$ B cells, irrespective of their location, or specifically applies for peritoneal B lymphocytes.

Many authors have been tempted to implicate $CD5^+$ B cells in autoimmune processes, given that they frequently produce polyreactive autoantibodies and are resistant to tolerance induction (*vide supra*). $CD5^+$ B cells are found at increased frequencies in autoimmune disease-prone strains of mice, e.g., NZB and (NZB × NZW)F_1 (*145,151,184*), and in senescent normal mice (*196*), as well as in patients with rheumatoid arthritis and Sjögren's syndrome (*197*). B cells of mice expressing the motheaten mutation (me^v), which predisposes to the development of autoimmune disease, are exclusively $CD5^+$ (*198*). When introduced into NZB mice, the *xid* (X-linked immunodeficiency) mutation inhibits the generation of $CD5^+$ B cells as well as the development of B cell hyperreactivity and autoantibodies (*151*). Conversely, treatment of CBA/N mice that bear *xid* mutation with cyclosporine A, following a protocol that induces severe systemic autoimmunity, causes an increase in $CD5^+$ B cells (*199*). Immunogenetic studies, however, allowed for the segregation of $CD5^+$ B cell expansion and autoimmune disease. The high frequency of $CD5^+$ B cells in NZB, NZW, and (NZB × NZW)F_1 mice is controlled by the homozygosity of a locus or cluster of loci closely linked to the MHC $H\text{-}2^z$ allele of the NZW strain. The finding that the expansion of splenic $CD5^+$ B cells (enhanced by $H\text{-}2^{z/z}$) does not correlate with autoimmune symptoms (mitigated by $H\text{-}2^{z/z}$ as compared to $H\text{-}2^{d/z}$) (*200*) argues against a simple quantitative correlation between the frequency of

$CD5^+$ B cells and the incidence of autoaggression. It is also doubtful that polyreactive low-affinity ("natural") autoantibodies produced by $CD5^+$ B cells are really pathogenic. Finally, B1 cells have been postulated to play a critical role in the selection of the T cell repertoire (*201*) (*vide infra*).

B. Peritoneal B Cells

In most studies, peritoneal $CD5^+$ cells are compared with adult splenic $CD5^-$ B cells. This experimental strategy cannot distinguish whether eventual differences between the two populations are due to the CD5 phenotype or to the compartment from which the cells originate. This difficulty is aggravated by the fact that a significant portion of $CD5^-$ cells contained in the peritoneum largely belongs to the sister population (*vide supra*). In this section evidence will be presented that peritoneal B cells differ from splenic B cells, regardless of whether they express CD5.

The repertoire of both $CD5^+$ and $CD5^-$ peritoneal B cells differs from that of splenic B cells in adult C57BL/6 mice. Irrespective of the expression of CD5, the frequency of V_H11^+ B or $V\kappa22^+$ cells and the frequency of cells producing antibodies specific for bromelain-treated erythrocytes or ssDNA are higher in the peritoneum (*166*). The adult peritoneum allows adult splenic B cells (which are largely $CD5^-$) to persist for over 2 weeks after intraperitoneal transfer, whereas most cells that home to the adult (not neonatal) spleen subsequent to intravenous injection rapidly decay (within 1 week) (*202*). During the 10 days after transfer, the number of cells expressing J558, V_H11, S107, and X24 remains constant, whereas the percentage of cells expressing the D-proximal or J606 and 36–60 gene families tends to be lower. This correlates well with the local enrichment of V_H11, S107, and X24 gene products in the peritoneal cavity of normal mice and suggests that the differences in the repertoire between $CD5^+$ and $CD5^-$ cells that have been described are not determined by lineage difference, but rather by the local environment (*203*). In spite of their near-to-absolute lymphopenia, SCID mice display in the peritoneum practically normal levels of IgM^+B220^+ cells (*123*). This finding also suggests that the peritoneum is a privileged site for B lymphocyte survival and/or replication. Accordingly, human B cells were found to persist in the peritoneal cavity of SCID mice for several months (*204*).

Recent experiments employing transgenic mice underscore intrinsic differences in the repertoires of peritoneal and splenic B cells. It is well established that in normal B cells only one allelle of immunoglobulin heavy and light chain loci is found functionally rearranged

(*205,206*). Similarly, the expression of a rearranged μ or δ immunoglobulin heavy chain gene introduced into the germ line of mice inhibits the rearrangement of endogenous genes, a phenomenon that is referred to as "allelic exclusion" (*207,208*). Transgenic expression of an IgM heavy chain gene with specificity for the hapten 4-hydroxy-3-nitrophenyl causes an obstruction of B cell development that becomes manifest in a strong depletion of bone marrow pre-B cells and of splenic B cells of the conventional type. In contrast, the peritoneum-derived (Ly-1) B cell lineage is unaffected (*193*). This finding led to the conclusion that the transgene affects the rearrangement and expression of endogenous immunoglobulin in conventional bone marrow-derived B cells but not in $CD5^+$ B cells (*209*).

We have recently studied the expression of transgenes and endogenous immunoglobulin loci at the single-cell level in transgenic mice carrying μ or δ transgenes. In agreement with the rule of allelic exclusion, a majority of B cells obtained from the bone marrow or spleen of C57BL/6 mice ($Igh\text{-}1^b$) transgenic for the rearranged μ chain obtained from a BALB/c ($Igh\text{-}1^a$) anti-TNP hybridoma (*207;* A. Iglesias, unpublished) express the transgene, as recognized by a monoclonal antibody specific for the IgM^a allotype. However, a majority of B cells contained in the peritoneum are IgM^b and thus express the endogenous immunoglobulin locus (Fig. 1). Similarly, in transgenic mice carrying a δ chain (IgD^a) (*208*) that contains the same VDJ segment as the μ transgenic mice, endogenous IgM (IgM^b) is expressed on a minority of B cells from the bone marrow and the spleen, but on a majority of peritoneal T cells (Fig. 1). Analogous results were obtained in animals bearing a transgene that encodes both a μ heavy chain and a κ light chain that in combination recognize the $H\text{-}2K^b$ MHC class I product (*210*). In transgenic mice that bear the $H\text{-}2K^d$ MHC haplotype (and hence lack the relevant autoantigen), endogenous IgM (IgM^b) is expressed in neither bone marrow nor in spleen, whereas in the peritoneum up to 20% of the IgM-positive cells express endogenous IgM. In contrast, no evidence could be obtained in favor of an increased expression of endogenous κ chains, using a monoclonal antibody (54.1) that recognizes an epitope formed by the combination of the transgenic μ and κ chains (*136*). The ratio between $IgM^b54.1^+$ cells (i.e., lymphocytes that express both the transgenic μ and κ chains) and $IgM^b54.1^-$ cells (lymphocytes that express the transgenic μ chain in the context of endogenous κ chains) is the same for peritoneal and bone marrow B lymphocytes (Fig. 1, Table V). The basis for the specific compartmentalization in the expression of endogenous heavy, not light, chains remains elusive. If selection is operating on those cells, it probably is not via self-

recognition, because in μ/κ (anti-H-2^b) transgenic mice carrying an $H\text{-}2K^{b \times d}$ background self-specific cells (i.e., μ/κ transgene-expressing cells recognized by the antibody 54.1) are not significantly over- or underrepresented among peritoneal B cells. No selective overexpression of Ly-1/CD5 on B cells positive for endogenous immunoglobulin μ gene products is found. Both in μ/κ transgenic mice bearing the autoantigenic MHC haplotype $H\text{-}2K^b$ and in mice negative for $H\text{-}2K^b$, only a minor portion of peritoneal B cells expressing the endogenous IgM allotype (IgM^b) expressed Ly-1/CD5 (15–21% and 11–19%, respectively). This percentage is not significantly higher among peritoneal B lymphocytes positive for the transgenic IgM allotype (IgM^a) or reacting with the antiidiotypic antibody 54.1 (range, 10–30%). Thus, no correlation between the peritoneum-specific immunoglobulin repertoire and CD5 expression can be established, indicating that the enhanced expression of endogenous immunoglobulin heavy chains is an intrinsic property of the peritoneal B cell population.

The finding that the frequency of splenic and peritoneal $CD5^+$ B cells is regulated by different genetic loci also argues in favor of organ-specific rather than cell type-specific differences between the two compartments. Homozygosity for the $H\text{-}2^z$ MHC haplotype from the NZB mouse is associated with an increased frequency of $CD5^+$ cells in the spleen, not in the peritoneum (*200*). This suggests that MHC-related microenvironments for $CD5^+$ propagation differ between the two compartments.

Little is known on the function of the peritoneal lymphocyte compartment. As discussed, both T and B cells differing in their phenotype, function, and repertoire from the quantitatively dominant lymphocytes located in the spleen, lymph nodes, and peripheral blood are noted. Because peritoneal lymphocytes constitute a comparatively long-lived population, they may fulfill a memory function, intervening in immunoregulation, as speculated by UytedeHaag and Coutinho and co-workers (*189,191,211*).

C. Lymph Follicles

Lymphoid follicles or nodules are found inside the spleen, in lymph nodes, or in the mucosal-associated tissue along the digestive, respiratory, or genitourinary tracts (tonsils, Peyer's patches, appendix, etc.). Whereas primary lymph follicles lack a clear-cut zonal organization, secondary lymph follicles that arise from primary nodules in a T cell-dependent fashion on antigenic stimulation have a complex architecture in several concentric or convex zones, namely a corona or mantle zone, and the germinal center, which may be subdivided into the outer

zone, the apical light zone, the basal light zone, and the dark zone (*212,214*). Each zone is characterized by a particular phenotype of follicular dendritic cells and B cells and correlates with a particular pattern concerning the repertoire, proliferation, mutation, and selection of B cells (*214*). In transgenic mice carrying a rearranged antilysozyme IgM/IgD transgene, as well as a lysozyme transgene, tolerant lysozyme-reactive B cells persist within the follicular mantle zones in the spleen, lymph nodes, and Peyer's patches, but are eliminated from the splenic marginal zones. The selective accumulation of tolerant B cells within the follicular mantle zone suggests unique physiologic roles for this lymphoid microenvironment (*215*). It remains open that anergic B cells could present self-antigen to T cells that would be rendered tolerant (*216*).

Germinal centers contained in secondary lymph follicles are foci of oligoclonal and hypermutating (*217*) lymphoblasts, probably providing a microenvironment suitable for heavy chain switching and the differentiation of memory B cells. Germinal center lymphoblasts are $IgD^{-}IgG^{low}$ B cells in nonmucosal lymph nodes or preferentially $IgD^{-}IgA^{low}$ B cells in Peyer's patches, indicating that lymphoid follicles that are exposed to different antigenic microenvironments (and T helper types?) favor the production of different immunoglobulin classes. It has been recently shown that transient germinal center reactions elicited by the same antigen preferentially induce a specific IgM/IgA response in Peyer's patches and a IgM/IgG_1 response in lymph nodes, indicating intrinsic antigen-independent differences between the mucosal and nonmucosal humoral immune systems (*218*). The follicular mantle contains small recirculating B cells (*219*) and the central dark zone contains proliferating and mutating (*217,220*) centroblasts that replenish the centrocyte population, which in turn, are selected by antigen presented on follicular dendritic cells in the conjunction with CD23 (and other stimuli, such as IL-1β?) either to succumb to programmed cell death or to differentiate into plasma blasts or memory B cells (*214*). It can be speculated that B cells that have lost their antigen specificity would not be positively selected by follicular cells presenting the relevant antigen and thus would commit apoptotic suicide. Follicular dendritic cells contained in germinal centers of secondary lymph follicles have been shown to absorb passively antigen–antibody complexes and MHC class II–peptide complexes shed from B cells, thus serving as long-term antigen depots (*221,222*) that could be important for the maintenance of immunological memory (223). The fixation of immune complexes is achieved via Fc or C3b receptors without endocytosis (*213,221*), whereas the mechanism of MHC class II absorption remains elusive.

IV. Generation of Compartments

Differences in the phenotypic composition, repertoire, and function of different local lymphocyte populations may be attributed to several principally different, though mutually nonexclusive, mechanisms (Fig. 2). First, precursors belonging to different lineages may be targeted *a priori* to different tissues, guided by spatiotemporal sequences of morphoregulatory molecules that are expressed in a tissue-specific fashion, a possibility that is illustrated by the topobiology of T lymphocyte migration and could involve chemotaxis, haptotaxis, and contact guidance. Second, immature precursors could undergo local induction processes that lead to tissue-specific differentiation. This could be the case with T cells developing extrathymically in the context of epi- or mesothelia. Third, after an initial random distribution of lymphocyte populations and specificities, a selection step could lead to the preferential expansion and maintenance of some cells, whereas others are diluted out or physically eliminated. Stimulation by ubiquitous or tissue-specific antigens presented by different types of accessory cells

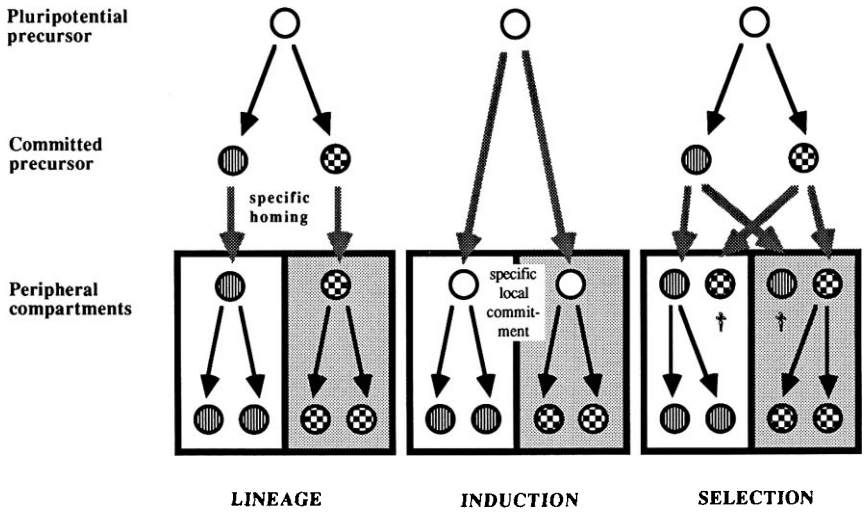

FIG. 2. Mechanisms leading to an inhomogeneous distribution of lymphocytes in different compartments. First, pluripotential precursors may be committed to a predetermined differentiation pathway, thus forming different lineages that specifically home to different sites. As a second possibility, undifferentiated and noncommitted precursors that arrive in a predetermined compartment are conditioned by the microenvironment, thus being induced to differentiate. Third, randomly homing cells that are already committed to express a predetermined phenotype and repertoire are locally selected, thus being either expanded or deleted. Note that in all three cases the final outcome is the same. For details, explanations, and examples, see text.

may contribute to the local proliferation or depletion of T and B lymphocytes. In addition, the costimulatory signals provided by local factors, including the extracellular matrix and cytokines, may influence the fate of a lymphocyte. In this section examples for each of these possibilities will be mentioned.

A. Different Lineages

As discussed above, during the differentiation process T and B lymphocytes may be instructed to migrate to a predetermined organ (Fig. 3). This is particularly well documented in the case of γ/δ T lymphocytes, which arise in the thymus in distinct "waves" bearing γ/δ TCRs encoded by different combinations of Vγ and Vδ gene segments (25). Predetermined TCR γ/δ genes are rearranged during a brief period and most of the T cells carrying a predetermined γ/δ TCR are exported to a specific site in the periphery. Vγ3-expressing thymocytes predominate from day 14 to day 16 and are exported to the skin and mucosae of the reproductive tract; later Vγ1.1, Vγ1.2, and Vγ2 rearrangements are detectable in thymocytes that will home to lymph nodes and spleen (*224–226*). As pointed out above, the TCR per se does not guide γ/δ T cells to a particular location, given that T cells expressing a transgenic γ/δ TCR home to any lymphoid compartment (*82*). Raulet and co-workers (*75*) have proposed that progenitors of different γ/δ lineages that differ in their homing patterns are programmed to rearrange specific V genes, rather than being selected by thymic ligands. In support of this hypothesis, during thymic ontogeny γ/δ T cell precursors transcribe those Vγ genes that are still in the germ-line configuration and will undergo gene rearrangement. Because this germ-line transcription, though indispensable for the rearrangement, does not give rise to translatable products, no TCR-mediated selection can intervene in the regulation of this process. Moreover, γ/δ T cells of a given subtype exhibit nonproductive rearrangements of the same Vγ gene that they express on the surface (*75*). Nonetheless, Tonegawa and co-workers (*227,228*) favor the idea that such T cells are positively selected in the thymus. γ/δ TCRs generated in fetal thymus cultures bear canonical γ/δ TCRs, but treatment of cultures with anti-γ/δ TCR antibodies results in an increase in noncanonical in-frame junctions (*228*). Along the same line, expression of the invariant TCR expressed on epidermal IELs (Vγ3–Jγ1Cγ1/Vδ1–Dδ2–Jδ2)—normally only found on fetal thymocytes—as a transgene in the adult thymus of C57BL/6 mice leads to a dramatic depletion of transgene-expressing thymocytes (*229*). Thus, developmental changes in the thymic environment may impose the γ/δ TCR repertoire ex-

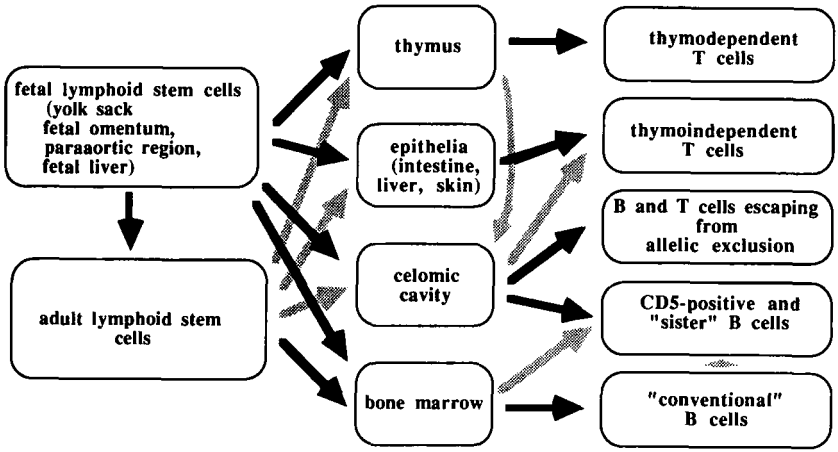

FIG. 3. Precursor product relationships in the immune system. Black arrows indicate major pathways, whereas minor or hypothetical relationships between different compartments are represented by shaded arrows.

pressed at a given stage via positive and negative selection processes. Irrespective of these possibilities, the molecular mechanism that targets a given γ/δ population to a determined site outside of the thymus remains obscure. It is possible that, by analogy to γ/δ lymphocytes, the α/β T cell population also is subdivided into different lineages, e.g., a thymus-dependent and a thymus-independent one. However, the exact precursor product relationships remain elusive and it is yet unclear whether γ/δ T cells give rise to an α/β TCR^+ progeny.

As far as B cells are concerned, two different lineages have been postulated, the conventional B2 lineage that stems from the bone marrow, and the Ly-1^+/$CD5^+$ B1 lineage. Whereas the fetal liver contains precursors for both B1 and B2 cells, the fetal omentum (13 days) has been reported to reconstitute the $CD5^+$, as well as the $CD5^-$ sister, but not the conventional B cell subset (*158,160*). Nonetheless, it is possible that the two lineages do not definitely separate at an early precursor stage and that a B2 → B1 transition may be triggered at later stages of B cell development (*164,165*).

B. Selective Migration and Extravasation

A large proportion of mature lymphocytes continuously traffic from the bloodstream into lymphoid organs and tissue, then to the collecting efferent lymphatics, and eventually back to the bloodstream, a pattern that evokes the expression "recirculation." Lymphocyte migration fol-

lows a nonrandom pattern and it appears that combinations of molecules expressed on a particular cell subset permit a lock-and-key recognition with a suitable combination of receptors expressed on specialized endothelial cells. This has been best investigated at the level of memory and naive T cells, whose antigen-nonspecific extravasation is subjected to a differential regulation. Naive T cells migrate into lymph nodes, whereas memory T cells traffic preferentially into nonlymphoid tissue (*20,230–232*).

Memory T cells ($CD45RO^+$) selectively traverse flat and inflamed endothelium in the peripheral vascular bed, attaching initially to the luminal surface in appropriate places, sticking strongly enough to the endothelial lining not to be swept away by the blood flow, and then migrating through the vessel wall into the surrounding tissue, draining to local lymph nodes via afferent lymphatic vessels. This interaction involves three ligands that are expressed at a higher density on memory, as compared to naive, T cells: (1) VLA-4 (very late antigen 4; synonym for integrin $\alpha_4\beta_1$ and CD49d/CD29), which binds to VCAM-1 (vascular cell adhesion molecule 1; INCAM-110; a molecule that is expressed upon epithelial cell activation); (2) CD44 (Pgp-1, Ly-24, Hermes), which interacts with the glycosaminoglycan hyaluronate; and (3) LFA-1 (leukocyte function-associated molecule 1; integrin $\alpha_L\beta_2$; CD11a/CD18), which binds to ICAM-1 (intercellular adhesion molecule 1; CD54; induced by endothelial cell activation) and ICAM-2 (constitutively expressed on resting endothelium) (223–239). In addition, a carbohydrate receptor exclusively expressed in a subpopulation of resting $CD4^+$ memory cells interacts with cytokine-induced E-selectin (endothelial leukocyte adhesion molecule 1; ELAM-1) which is predominantly expressed in inflamed endothelium of the skin (*238,240*).

It is predominantly memory T cells that localize to nonlymphoid tissues, for example, skin (*241*), the epithelial surface of the lung, and the lamina propria of the gut (*242*). T cells activated *in vitro* home to the gut and skin and not to lymph nodes (*243*). A subset of human memory T cells expresses the cutaneous lymphocyte-associated antigen (CLA) (*240*), a carbohydrate ligand that interacts with ELAM-1 expressed on inflamed endothelium of the skin (*244*). In the mouse, the integrins LPAM-1 (lymphocyte Peyer's patch high endothelial venule adhesion molecule), an α/β heterodimer ($\alpha_4\beta_P$) related to human VLA-4 ($\alpha_4\beta_1$), and LPAM-2 participate in lymphocyte binding to mucosal postcapillary venules (*245*). Memory cells are subdivided into subpopulations that are biased to home to nonlymphoid tissues related to the one in which they were originally stimulated (*246*), a behavior that

could serve to reencounter an antigenic insult (e.g., tissue-specific microorganisms) in the same (or a similar) anatomical location (*20,232*). The precise location (gut, lung, liver, skin, synovium, etc.) to which a memory cell homes is probably determined by an alphabet of multiple combinations of interacting pairs of molecules specifically expressed in determined tissues and T cell subpopulations. Thus, the integrin $\beta_7\alpha_{290}$ is specifically expressed on mucosal intraepithelial lymphocytes (*247*). In addition, differentially expressed isoforms of receptors, such as CD44, have been described to arise from alternative splicing (*248*).

In contrast to memory T cells, blood-borne naive T cells ($CD45RA^+$) preferentially cross a histologically distinctive postcapillary venule termed the high endothelial venule (HEV) that serves as the portal of entry into lymph nodes. Morphologically similar HEVs also exist in Peyer's patches, tonsils, and inflammatory reactions, although they must be functionally different. B cells bind better to HEVs of Peyer's patches, whereas T cells bind better to inguinal node HEVs and both bind equally well to mesenteric lymph node HEVs (*249*). i-IELs and mesenteric node lymphoblasts bind with exquisite selectivity to Peyer's patch HEVs and poorly if at all to lymph node HEVs (*249*). In the lymph node, naive T cells mix with a minority of memory cells during an 18- to 20-hr residency and make up the majority of T lymphocytes that recirculate via efferent vessels to the blood (*20*). The selective adhesion process between naive T cells and HEVs is guided by L-selectin (MEL-14; leukocyte adhesion molecule 1, LAM-1, LECAM-1; leu-8; TQ1) (*250,251*), which interacts with a vascular addressin specifically expressed on HEVs of peripheral lymph nodes. L-Selectin is expressed on naive resting T cells and is down-regulated on activated and memory T cells (*252,253*). The molecule contains an amino-terminal lectinlike domain (*254*), and mannose-containing compounds such as the phosphomannan monoester core from *Pichia holstii* exopolysaccharide (PPME) or a PPME-derived pentasaccharide with terminal mannose-6-phosphate block the entry of fluorescence-labeled lymphocytes into lymph nodes at the lymphocyte level (*254*). In addition to L-selectin, CD31, a molecule that is also preferentially expressed on naive T cells, may participate in the interaction with HEVs (*232*). Naive T cells, newly exported from the mouse thymus, express the L-selectin homing receptor for lymph node HEVs and recirculate via the lymph nodes to the blood (*255*). This traffic makes evolutionary sense, because these naive T cells thus get the chance to encounter specialized APCs in a lymph node that drains antigen from local tissues.

Anionic carbohydrate recognition via lectinlike receptors plays a role in lymphocyte traffic. Although the phosphomannan monoester core from *P. holstii* preferentially blocks the entry of lymphocytes into lymph nodes, unphosphorylated mannan from yeast is more effective in blocking lymphocyte entry into the spleen *in vivo*. Pretreatment of lymphocytes before injection with either PPME or mannan suggests that PPME acts on the lymphocyte level (probably via L-selectin), whereas mannan acts at some other site (*254*). Interestingly, neither PPME nor mannan modifies the splenic localization pattern of fluorescence-labeled lymphocytes, whereas another carbohydrate, fucoidin (a sulfated polysaccharide rich in L-fucose), displaces fluorescent lymphocytes from the periateriolar lymphocyte sheath into the marginal zone region and red pulp of the spleen (*254*). After neuraminidase treatment lymphocytes are retained in the liver because they are targets for the hepatic asialoglycoprotein receptor (*256*).

In synthesis, it appears that multiple receptors expressed on lymphocytes interact with a suitable set of complementary ligands on specific endothelial cells. Although none of the molecules implicated in lymphocyte trafficking is highly selective, the interplay (activation) between lymphocytes and endothelium, as well as cross-talk among the receptors, might refine the regulation of adhesion. Thus, specificity does not arise from high selectivity on the part of individual receptors but from the coupling of multiple adhesion molecules in a variety of ways (*257*). Also, the fact that adhesion receptors may be expressed in alternatively spliced isoforms [e.g., CD44 (*248*) and various integrins; reviewed in Ref. 258] or may differ in the extent of N-linked carbohydrate processing (e.g., ICAM-1 (*259*) may contribute to this combinatorial diversity.

In view of the capacity of endothelial cells to present antigen and to trigger the TCRs during a first attachment phase, it is possible that antigen specific mechanisms participate in the extravasation of T lymphocytes (*230,260*). TCR-mediated signaling may change the adhesive properties of the integrin LFA-1 (*233*), a molecule that is involve in the trafficking to peripheral lymph nodes and Peyer's patches. This might be important in the local accumulation of pathogen-reactive T cells and could have some importance in the extravasation of myelin basic protein-specific T cells that initiate demyelinating lesions in experimental allergic encephalitis, a rodent model of multiple sclerosis.

C. Local Selection

Transfer of splenic T cells into syngeneic nude recipients results in an expansion of these T cells. The Vβ repertoire recovered from the

spleen of such reconstituted nude mice is very similar to that of the injected cell population, even when FACS-sorted populations enriched or deleted in T cells expressing a defined Vβ gene product are used as a source of donor cells (*261*). This could suggest that the composition of the splenic α/β T cell repertoire is dictated by the influx of T cell progenitors rather than by local selection processes. In sharp contrast, transfer of adult splenic B cells into the peritoneum leads to a change in the local repertoire (relative increase of V_HJ558, V_H11, S1-7, and X24) that mimics the repertoire of B cells normally present in the peritoneal cavity of untreated mice (*203*). It is also tempting to invoke local selection processes in view of the fact that B and T lymphocytes expressing transgenic and endogenous antigen receptors are unevenly distributed in the peripheral immune system. Thus, B cells that evade allelic exclusion and express endogenous immunoglobulins in mice carrying μ or (μ + κ) transgenes represent a small percentage of bone marrow cells, a larger fraction of resting surface IgM^+ B cells in the spleen, and constitute the majority of immunoglobulin-secreting splenic B cells (*262,263*) and a even higher percentage of peritoneal B cells (*136*). The same peritoneal predilection in endogenous gene expression is observed in mice carrying TCR α/β or IgD heavy chain transgenes (*136*) (Table V). These findings could suggest a positive selection in favor of cells expressing endogenous gene products, although transfer experiments will have to confirm this hypothesis.

At the local level, tissue-specific antigens may be important in selection processes. Thus, the high frequency of antigen-specific lymphocytes present at the site of an immune response may be partially attributed to selection processes. It has been shown that splenic cells from transgenic mice bearing an α/β TCR recognizing the male-specific H-Y peptide give rise to a different repertoire when adoptively transferred to male or female recipients. Thus, cells expressing endogenous α gene products preferentially expand in the spleen of female mice, whereas male-specific T cells (expressing both the transgenic β and α chains) were selected positively in male animals (*135,264*). Similarly, positive selection of self-MHC restriction occurs not only in the thymus, but also extrathymically, as shown by transfer experiments involving nude recipients (*265*). In addition, several authors have described negative selection (clonal deletion) that is not a prerogative of the central sites of lymphocyte differentiation, but that also occurs in peripheral locations, e.g., in athymic mice in response to retroviral or bacterial superantigens (*266–271*). Clonal deletion has also been suggested to occur in transgenic mice expressing neoautoantigens in peripheral locations, e.g., an *H-2K*^{b}

transgene (*111*) or a hybrid transgene consisting of the α1 and α2 domains of the *Q10* gene and the α3 transmembrane and intracytoplasmic domains of *H-2L*^{d} selectively expressed in hepatocytes under the control of the albumin promoter (272). Thus, local superantigens as well as conventional peptidic antigens may modulate the lymphocyte repertoire.

In addition, the function of antigen-presenting cells, differences in the composition of the extracellular matrix, and local mediators may determine the selective expansion or depletion of predetermined lymphocyte subpopulations, as will be discussed in the following sections.

1. Antigen-Presenting Cells

The antigen-specific interaction between T cells and antigen-presenting cells has been compared in its complexity with mating rituals (273) inasmuch as a bilateral exchange of signals that overcomes repulsion and enforces adhesion finally leads to the excitation of both partners of the ritual, not only the lymphocyte, but also the opposing APC. The interaction between T cells and APCs involves at least four different molecule couples [MHC I/II–CD4/8, CD58(LFA3)–CD2, CD54(ICAM)–CD11a(LFA1), and B7/BB1–CD28] whose roles are distinct from the interaction between the TCR and the presented antigen (*274*), and further interacting pairs of molecules remain to be discovered.

Both the antigen-presenting and the costimulatory capabilities vary, depending on the accessory cell type, namely B cells, monocytes, and dendritic cells from different anatomic localizations. To take up antigen, B lymphocytes take advantage of immunoglobulin molecules expressed on the surface, whereas macrophages rely on phagocytosis; conversely, dendritic cells have no efficient system for the concentration of antigen. In contrast to B cells and macrophages, dendritic cells constitutively display costimulatory activity for CD4$^+$ T cells (*274–276*). Accordingly, dendritic cells are 10- to 50-fold more potent than monocytes or B cells in inducing T cell responses to a panel of superantigens and require lower doses than do other APCs to stimulate T cells (*277*). Dendritic cells are more efficient inducers of proliferation and cytokine secretion (IL-1, IL-2, and IFN-γ) in allogeneic mixed lymphocyte reactions as compared to macrophages and B cells (*278*). These functional differences may reflect the specialization of each type of antigen-presenting cell. Macrophages and B lymphocytes have to receive activation signals (bacterial walls, li-

popolysaccharide, mannan, glycans, etc.) to become costimulatory (279) and to present productively antigens from intracellular or extracellular pathogens, respectively. In contrast, dendritic cells, which may be specialized in the expression of viral antigens, display a constitutive costimulatory activity for T_{H1} cells. Accordingly, antigen-pulsed dendritic cells are capable of inducing antibody responses *in vivo* much in the same way as the native antigen, whereas antigen-pulsed low-density B cells only weakly prime mice *in vitro* (280).

The functional capacity of macrophages or dendritic cells also depends on the site from which they have been isolated. In allogeneic mixed lymphocyte reactions, splenic macrophages are more stimulatory than peritoneal macrophages (278). Accordingly, macrophages located in different tissues differ in their phenotype. Monocytes and peritoneal macrophages express less sialadhesin than do splenic marginal metallophils and subcapsular lymph node macrophages (281); exudate and resident peritoneal macrophages express different glycoforms of macrosialin (282). Dendritic cells are also encountered in very different anatomic localizations (blood, interdigitating cells of the thymic medulla and T cell areas of the spleen and lymph nodes, Langerhans cells of the skin, intraepithelial cells of the airways, gut mucosae, bile ducts, and interstitial tissues) and differ in the expression of adhesion molecules, Fc receptors, antigen-presenting capacities, and stimulation of autologous mixed lymphocyte cultures (283). The capacity of antigen storage attributed to follicular dendritic cells that are found in the germinal centers of secondary lymph follicles was discussed above. Such cells can take up antigen in the form of immune complexes and MHC class II–peptide complexes and can conserve nondenatured antigen for long periods, thus contributing to the maintenance of immunological memory.

Tissue-specific "nonprofessional" (i.e., not bone marrow derived) antigen-presenting cells, e.g., MHC class II-expressing enterocytes of the small intestine (284), as well as keratinocytes (which express class II after stimulation with, for example, IFN-γ) (285) may exert important tolerizing functions by causing an abortive activation of specific T_H cells and a concomitant anergic state, given that costimulatory signals provided by "professional" antigen-presenting cells (activated B cells and macrophages as well as dendritic cells) for complete and productive activation of T cells are not delivered by epithelial cells. Accordingly, class II^+ hapten-modified keratinocytes induce specific immune tolerance when injected *in vivo* (285).

2. *Cell–Matrix Interactions*

The intricate interplay between lymphocytes and the intercellular matrix may constitute an additional regulatory factor in cellular activation. Thus, the interaction of the extracellular matrix protein fibronectin with VLA-5 receptors ($\alpha_5\beta_1$ integrin) on $CD4^+$ T cells induces the IL-2 gene transcription factor AP-1 (*286*), and the $\alpha_v\beta_3$ integrin (ligands are vitronectin, fibronectin, osteropontin, and collagen) has been identified as a receptor of costimulatory signals in γ/δ T cells for IL-4 production (*287*). Collagen mediates costimulation via CD26 (*288*) and VLA-3. Laminin and fibronectin costimulate via VLA-3, VLA-4, VLA-5 (only fibronectin), and VLA-6 (only laminin), respectively (*289–291*). At least two ectoenzymes may serve as costimulator receptors on T cells. Antibodies recognizing CD26 (previously THAM, Tp 103, or F7, identical with the dipeptidyl peptidase IV) and another glycosyl phospatidylinositol-anchored protein, CD73 (ecto 5′-nucleotidase), potentiate submitogenic signals provided by anti-CD2 or anti-CD3 stimulation (*292,293*). Possibly, ectoenzymes serve as cell–matrix interaction molecules that enhance activation signals via conventional pathways at the time they modify the composition of the extracellular milieu. Thus, CD26 binds collagen and provides costimulation in a anti-CD3 mAb-mediated proliferation system (*288*). As detailed above, multiple molecules expressed on the surface of lymphocytes receive signals either from bystanding APCs or from matrix molecules. It may be anticipated that these costimulatory signals may especially promote the survival or expansion of determined lymphocyte subsets.

A requirement for combined diffusible (cytokine) and nondiffusible (matrix) signals may be an important mechanism for localizing the responses of cells (*294*). The fact that certain cytokines may be produced in matrix-bound or cell surface-bound forms, in addition to the diffusible form, may contribute to the compartmentalization of cytokine responses. This is the case for the platelet-derived growth factor (*295*) and the differentiation-inhibiting activity/leukemia inhibitory factor (LIF) (*296*) that may be generated in isoforms built up by different chains (PDGF-A and PDGF-B) or forms, depending on alternate promoter use (LIF). The relevant isoforms of LIF and PDGF-B, as well as fibroblast growth factors (FGFs) (*297*) and members of the transforming growth factor β (TGF-β) family (*298*), are captured by extracellular proteoglycans that, in turn, differ in their growth factor specificity. Thus, heparan binds FGF, LIF, IL-3, granulocyte–macrophage colony-stimulating factor, and several members

of the intercrine family, whereas β-glycan and decorin bind TGF-β (*299,300*). In addition to passively adsorbing cytokines, thus limiting their diffusion, the extracellular matrix has been postulated to store information in the carbohydrate moieties of its macromolecules, which would reflect a relatively stable engram of past metabolic experiences and could influence lymphocytes via relevant lectinlike receptors (*294,301*).

3. *Cytokines*

Together with cell- or matrix-mediated signals, cytokines form part of a complex cellular signaling language in which individual factors are the equivalents of an alphabet or code (*302*). Cytokines, soluble (glyco)proteins, nonimmunoglobulin in nature, are released by living cells of the host and act nonenzymatically in picomolar to nanomolar concentrations to regulate host cell function (*294*). In physiological conditions they function as auto- or paracrine, not endocrine, factors. The temporal and spatial limitation of the production, permanence in the microenvironment, and target cell responsivity of T helper cell-derived cytokines is of special importance, because lymphokines transduce antigenic triggers—which depend on specific recognition events involving the polymorphic TCRs—into monomorphic signals that exert local immunostimulatory and proinflammatory effects. Stringent control mechanisms intervene in the response to, as well as the production and bioavailability of, cytokines. First, the production of these mediators is subject to specific regulation, in which the stimulated T cell not only has to receive triggers via the TCR, but has to integrate a variety of costimulatory and antagonistic signals from the microenvironment. Second, their effects are topographically restricted in an autocrine or paracrine fashion due to directed secretion into the intercellular space formed between interacting cells, local adsorption by cytokine receptor-bearing cells, and rapid elimination from the circulation [half-lives of 3–8 min for IL-1, IL-2, IL-6, and tumor necrosis factor (TNF), respectively (*303–305*)]. Third, the competence to respond to cytokines depends on the activation state of the target cell and responses are subjected to a postreceptive control (*306,307*). Interestingly, soluble cytokine receptors that are secreted in a regulated fashion, e.g., that for TNF, may serve as transport proteins, stabilizing the structure of the relevant mediator and preserving its activity (*308*). Thus, soluble cytokine receptors could subserve an immunoregulatory function by augmenting the half-life of cytokines.

According to the lymphokine production pattern, murine and human T helper cells may be grouped into several classes (T_{H0}, T_{H1}, T_{H2}, T_{HX}) (*309,310*) that show an uneven tissue distribution (*vide supra*). To a certain extent lymphokines produced by a specific T_H subpopulation enhance the differentiation of their producers in a positive-feedback loop, a feature that may favor the local accumulation of a specific T_H type. Thus, IL-4 induces the differentiation of T_H cells to the T_{H2} type (which secrete IL-4, IL-5, and IL-10); IFN-γ induces differentiation to T_{HX} cells (producing predominantly IFN-γ) (*16*). This may ensure that a community of neighboring T cells collectively react to their own signal to differentiate into the same direction. In addition, T_{H1} cells (which produce IL-2, IL-2, and IFN-γ) and T_{H2} cells exhibit a mutual antagonism based on their specific products, IFN-γ and IL-10, respectively (*309,311*), thus helping to increase local inhomogeneities in the distribution of both subsets. Glucocorticoids and prostaglandins of the E series (PGE), as well as other substances that increase intracellular cAMP levels, skew T_{H1} to T_{H2} responses (*27,312*).

As mentioned above, the presence of predetermined B cell populations may be correlated with certain lymphokines locally produced in the tissue, a finding that can be accommodated in the context that IL-4 favors isotype switching to $\gamma 1$ and ε; IFN-γ to $\gamma 2a$; and TGF-β, to α. IL-2 enhances the production of IgM, and IL-5 and IL-6 enhance that of IgA. Thus, the fact that T_{H2} cells (producing IL-4, IL-5, IL-6, etc.) occur more frequently in mucosal than in nonmucosal lymphoid tissues could be correlated with the selective production of secretory IgA. The mutual antagonism of T_{H1} and T_{H2} cell lymphokines on the production of IgE correlates with the elevated local presence of T_{H2} cells in IgE-mediated allergies (*313,314*).

Cytokines may also be important as chemoattractants for different lymphocyte subclasses, e.g., IL-2 and various members of the intercrine family [IL-8 and the molecules RANTES, human macrophage inflammatory protein-1α (HuMIP-1α) and -1β (HuMIP-1β), which attract memory, cytotoxic, and virgin T cells, respectively] (*315*). In addition, cytokines may regulate the expression of adhesion molecules on vascular endothelia. Resting endothelium expresses few ICAM-1 and no VCAM-1 and ELAM-1. All three molecules are rapidly induced by IL-1β and TNF. IFN-γ specifically upregulates ICAM-1, whereas IL-4 induces VCAM-1 and downregulates the expression of ICAM-1 (*316*). Thus, T cell adhesion to endothelium, the first step in T cell extravasation, may be regulated by cytokines. Similarly, TNF-α has been recently reported to induce the expression of

VCAM-1 and ICAM-1 on human neural cell lines (*317*), a phenomenon that might facilitate the attachment of pathogenic autoreactive T cells to neural cells in autoimmune diseases of the central nervous system.

As summarized above, several local factors influence the selection of a particular local lymphocyte population: (1) the antigenic composition of the tissue, (2) the antigen-presenting function of specialized cells, (3) particular features of the intercellular matrix, and (4) the presence of soluble mediators. Local selection and induction processes, together with differential extravasation pattern and lineage-determined differences, account for the inhomogeneity of lymphocyte distribution.

V. The Significance of Compartments

A. Specialized Functions

Although the immune system has been called our "mobile brain," it appears that many properties of the system reside in the function of sessile lymphoid and nonlymphoid cells. As will be recapitulated in this section, the topography of interactions between immune cells and the microenvironment contributes an additional dimension to the function of lymphocytes that are specialized in function and specific in antigen recognition.

Naive T lymphocytes and "virgin" B cells circulating in the blood patrol the body and migrate through different tissues until they are activated in an antigen-specific fashion and acquire a memory phenotype with different migratory and functional characteristics. Thus, for instance, B cells that have been stimulated to switch to IgA production after local stimulation in Peyer's patches of the gut migrate to mesenteric lymph nodes and recirculate via the thoracic duct and the blood to different mucosal tissues in order to differentiate locally into IgA-producing plasma cells (*140*). In the lymph node, lymph follicles constitute a particular meeting point between locally drained antigen, T helper cells, and T-dependent B cells, as well as specialized dendritic cells that retain antigen–MHC and antigen–antibody complexes as a substrate of immunological memory. The spleen contains a reservoir of lymphocytes and is dedicated to the purging of old and mutated cells of the erythroid, myeloid, and lymphoid lineages, thus it constitutes a terminal differentiation organ for T and B lymphocytes. Epithelium-associated T lymphocytes bearing a restricted or monomorphic repertoire specific for self-peptides may exert a senti-

nel function, detecting damage to relevant epithelial cells (keratinocytes, enterocytes, and respiratory epithelial cells), thus alarming local and systemic defenses via the production of cytokines and possibly participating in the elimination of damaged cells. Moreover, these epithelia may recapitulate the association of T cells with thymic epithelium that is important in the induction of nondeletional tolerance (*84*), as well as in the maturation of T cell precursors. The particular lymphokine secretion profile of intestinal IELs and lamina propria lymphocytes may support the production of secretory IgA and influence the behavior of local lymph follicles. The peritoneum serves as a reservoir of $CD5^+$ B cells, which may participate in repertoire selection and secrete polyreactive antibodies, involved in the first line of defense against infectious microorganisms (*vide supra*).

Thus, each compartment arranges a specialized microanatomy with a different lymphocyte typology to achieve an optimal functional output. It may be anticipated that the restriction of predetermined lymphocytes to different compartments moreover allows for the refinement of the immune function. In the same way that it is difficult to conceive a nervous system built up by mobile circulating neurons, the spatial organization of immune structures can be expected to contribute to the "intelligence" of the system.

It can be anticipated that the limitation in lymphocyte recirculation, which obviously is a requirement for the maintenance of an inhomogeneous distribution, will lead to a partial fragmentation of immunoregulatory circuits. Immunoglobulins, like hormones, are well distributed in the organism; they are absent in only a few areas that also are free of lymphocytes and thus could be involved in a ubiquitous idiotype-dependent immunoregulation. In contrast, cell contact-dependent regulatory events, e.g., the cooperation of T cells with B cells, the stimulation or silencing of specific T lymphocytes by professional or nonprofessional antigen-presenting cells, as well as different T–T interactions, will be dictated by the local distribution of lymphocytes and accessory cells. It thus becomes conceivable that a local immune reaction might become uncoupled from a contact-dependent regulatory mechanism that is operative at another site. Along the same line, immunoglobulin-mediated selection of T cell repertoires may be expected to have a high systemic impact. Thus, it has been extensively shown that antibodies specific for idiotypes, V_H isotypes, or allotypes stimulate or suppress specific T cell responses (*318*). The best evidence for the selection of T cell reactivities by antibodies has been obtained by comparing TCR reper-

toires in the absence or presence of B cells and immunoglobulins. T cell clonotypes expressed in B cell-deprived mice are different from those available in normal individuals (*319,320*). Depletion–reconstitution experiments directly support the interpretation that TCR repertoires available in normal individuals are positively selected and expanded by B cells or immunoglobulins and that this selection occurs mostly during the first 4 weeks of age in mice (*321*). Experiments aiming at the characterization of the B cells implicated in the selection of TCR repertoires have also been performed (*322,323*) and suggest that peritoneal $CD5^+$ B cells as well as activated splenic $CD5^-$ B cells from normal donors, but not from athymic nude mice, are competent. It seems therefore that the pool of immunoglobulins patrolling the organism not only provides a first line of defense against infectious agents but also establishes a common link among different lymphoid compartments (*188*).

B. Site-Dependent Tolerance

According to the specialized milieu, tolerance induction obeys different rules. This is not only true for the expression of (neo)self-antigens in the thymus or bone marrow (as opposed to the periphery), but also concerns different peripheral compartments. Apparently, the same antigen expressed in different tissues may elicit diverging types of peripheral tolerance.

In double-transgenic mice, T cells expressing a transgenic *H-2K*^{b} (MHC class I)-specific α/β TCR have been confronted with a (neo)autoantigen expressed in five different extrathymic localizations, namely, under the control of the promoters for the rat insulin promoter (beta cells of the endocrine pancreas), β-globulin (erythroid lineage), glial fibrillary acid protein (GFAP) (cells of neuroectodermal origin, i.e., brain and autonomous ganglia), albumin (hepatocytes), or 2.4-kb keratin IV (epithelial cells from hair root sheaths and tongue epithelium). In each case, a different profile of extrathymic tolerance was observed. In TCR ×GFAP *K*^{b} mice, a large proportion of $Thy\text{-}1^+$ splenocytes and lymph node cells downmodulates TCR and CD8 molecules, which are rescued by *in vitro* stimulation with K^{b+} splenocytes in the absence of IL-2. In contrast, in TCR × albumin *K*^{b} mice, which also lack clonotype-positive T cells in the periphery, the clonotype-negative $CD8^-$ cell could not be activated *in vitro*, suggesting permanent downregulation. No reduction in clonotype-positive $CD8^+$ T cells was observed in TCR × 2.4-kb keratin

IV K^b mice, although such animals are tolerant to K^{b+} skin grafts. Curiously, in this case the clonotype-positive $CD8^+$ T cells were fully responsive *in vitro* (*84*). In another series of experiments, transgenic mice expressing a K^b-specific TCR were mated with animals expressing *H-2K*^{b} under the control of the rat insulin promoter. Again, cells expressing the transgenic TCR plus CD8 persisted in the periphery, although the mice were tolerant to *H-2K*^{b} (*324*). Finally, transgenic mice carrying *H-2K*^{b} linked to β-globulin gene transcriptional control elements, which direct erythroid lineage-specific expression, exhibit a phenotype of tolerance, whereas *H-2K*^{b}-specific proliferative responses remain intact, but cytotoxic *in vitro* responses are abolished even in the presence of IL-2 (*325*).

These findings show that, depending on the site of autoantigen expression, but independent of the nature of the antigen, different self-tolerance-preserving mechanisms may guarantee postthymic nonresponsiveness of potentially autoaggressive T cells, namely, functional deletion, reversible downregulation of CD8 and the α/β TCR, selective loss of the cytotoxic function of T effector cells, or postdeletional/postanergy types of tolerance. The finding that an antigen that is expressed exclusively in the nervous system will induce immunological tolerance contradicts previous ideas on antigenic sequestration; at the same time these results argue against the existence of immunologically privileged sites. It is intriguing that the same molecule expressed in different tissues induces disparate types of tolerance. Future studies will address the respective contribution of antigenic load and tissue-specific antigen presentation to this phenomenon.

Recently it has been shown that fluids from immunologically privileged sites, for example, aqueous humor from the anterior chamber of the eye, cerebrospinal fluid from the subarachnoid space of the central nervous system, and amniotic fluid from the fetoplacental unit within the pregnant uterus, contain significant levels of transforming growth factor β2 (TGF-β2) (*326*). TGF-β is a predominantly immunosuppressive cytokine that suppresses the IL-1- (*327*), IL2-, and IL-4-dependent proliferation of T cells (*328*), inhibits the production of TNF and IL-1 as well as IL-2 mRNA accumulation (*329*), and suppresses experimental autoimmune diseases *in vivo* (*330,331*). Antigen-pulsed macrophages that are exposed *in vitro* to aqueous humor, cerebrospinal, or amniotic fluid present antigens in a deviant manner and suppress antigen-specific delayed hypersensitivity *in vivo*, an effect that can be blocked by TGF-β-specific antibodies (*326*). Thus, three immune privileged sites (eye, brain, and feto-

placental unit) are bathed in biological fluids that are endowed with immunosuppressive (at least partially TGF-β-mediated) properties. Decidual large granular lymphocytes are NK-like cells that might control trophoblast invasion into the uterus wall (*332*) and that release a TGF-β2-related suppressor factor (*333*). Recent evidence indicates that this cytokine may also be involved in the regulation of oral tolerance. Oral administration of myelin basic protein (MBP) prevents subsequent induction of experimental autoimmune encephalitis (EAE) by MBP immunization. In transfer experiments, disease prevention is mediated by $CD8^+$ T cells that are capable of producing TGF-β1 on stimulation with MBP *in vitro*. Antibodies specific for TGF-β1 abrogate the protective effect of oral tolerization to MBP in EAE and provoke an aggravation of the disease when injected into nontolerized EAE animals (*334*). Thus, TGF-β that is contained in certain biological fluids or is produced by specific lymphocytes might be an important tolerance-mediating cytokine.

An unresolved problem concerns lymphocytes that have been selected *in situ* (and are only tolerant against the set of tissue-specific and housekeeping peptides that are locally present) and thus would have a certain chance to be specific for self-antigens present in other sites. It remains an open question whether a failure of the mechanisms that hinder such cells from recirculating or the disruption of barriers between compartments may contribute to the development of autoimmune diseases. It can be speculated that the control of T cell homing might constitute a fail-safe mechanism preventing unwarranted autoaggressive responses (*59,335*). To initiate an autoimmune response, a T cell would have to fulfill a series of requirements: (1) it must express a TCR specific for the relevant autoantigen, (2) belong to a subclass of T lymphocytes with proautoimmune potential (e.g., cells capable of producing IL-2 and other cytokines), (3) respond in a productive fashion to the antigenic peptide–MHC complex while receiving a complete set of costimulatory signals (i.e., the cell must not have been exposed to previous anergizing or immunosuppressive signals), and (4) last but not least it should express the appropriate adhesion receptors that will allow for migration to the tissue containing the relevant autoantigen. If this premise is correct, then it must be concluded that a tight control of lymphocyte migration reduces the probability of autoimmune disease development at the time that it allows for the maintenance of distinct immunological compartments. In addition, blockade of the specific homing of T cells to a predetermined site might be taken advantage of in the prevention (*336*) or therapy of autoimmune diseases.

VI. Summary

The periphery of the immune system—as opposed to the central lymphoid organs—contains inhomogeneously distributed B and T cells whose phenotype, repertoire, developmental origin, and function are highly divergent. Nonconventional lymphocytes bearing a phenotype that is rare in the blood, spleen, or lymph nodes of undiseased individuals are encountered at high frequency in different localizations, e.g., α/β $TCR^+CD4^-CD8^-$ cells in the bone marrow and gut epithelium, particular invariant γ/δ $TCR^+CD4^-CD8\alpha^+CD8\beta^-$ and γ/δ $TCR^+CD4^-CD8\alpha^-CD8\beta^-$ T cells in various epithelia, or $CD5^+$ B cells in the peritoneum. The antigen receptor repertoire is different in each localization. Thus, different γ/δ TCR gene products dominate in each site, and the proportion of cells expressing transgenic and endogenous α/β TCR and immunoglobulin gene products follows a gradient, with a maximum of endogenous gene expression in the peritoneum, intermediate values in other peripheral lymphoid organs (spleen, lymph nodes), and minimum values in thymus and bone marrow. Forbidden T cells that bear self-superantigen-reactive $V\beta$ gene products are physiologically detected among α/β $TCR^+CD4^-CD8^-$ lymphocytes of the bone marrow, as well as in the gut. Violating previous ideas on self-tolerance preservation, self-peptide-specific γ/δ T cells are present among intestinal intraepithelial lymphocytes, and $CD5^+$ B cells produce low-affinity crossreactive autoantibodies in a physiological fashion. It appears that, in contrast to the bulk of T and B lymphocytes, certain γ/δ and α/β T cells found in the periphery, as well as most $CD5^+$ B cells, do not depend on the thymus or bone marrow for their development, respectively, but arise from different, nonconventional lineages.

In addition to divergent lineages that are targeted to different organs guided by a spatiotemporal sequence of tissue-specific homing receptors, local induction or selection processes may be important in the diversification of peripheral lymphocyte compartments. Selection may be exerted by local antigens, antigen-presenting cells whose function varies in each anatomical localization, cytokines, and cell–matrix interactions, thus leading to the expansion and maintenance of some clones, whereas others are diluted out or deleted. The spatial compartmentalization of lymphocytes in different microenvironments has major functional consequences and leads to a partial fragmentation of immunoregulatory circuits at the local level. Lymphocytes residing in certain antigen-exposed compartments are likely to combat tissue-specific pathogens or self-proteins. In addition, specific microenviron-

ments may be important for T and B cell cooperation, the storage of antigen and immunological memory, and the establishment of immunological tolerance to self-antigens or to the harmless non-self (e.g., alimentary compounds). The division of the immune system into different microenvironments is likely to augment the complexity of the organization of the system. Future investigation will unravel whether aberrations in the mechanisms that create and maintain immune compartments may contribute to the pathogenesis of autoimmune diseases.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Owen Williams for a critical reading of the manuscript. Moreover, we thank J. A. Gonzalo, J. M. Alonso, J. M. de Alborán, and R. de Cid for allowing us to comment on their unpublished data. This work was partially supported by CICyT,EC, and FISS.

REFERENCES

1. Pabst, R. (1992). *Immunol. Today* **13,** 119–122.
2. Möller, G. (ed.) (1988). *Immunol. Rev.* **101,** 5–189.
3. Möller, G. (ed.) (1988). *Immunol. Rev.* **104,** 1–6.
4. Möller, G. (ed.) (1989). *Immunol. Rev.* **111,** 5–183.
5. Möller, G. (ed.) (1989). *Immunol. Rev.* **109,** 5–153.
6. Möller, G. (ed.) (1991). *Immunol. Rev.* **120,** 5–191.
7. Horejsi, V. (1991). *Adv. Immunol.* **49,** 75–147.
8. Parnes, J. R. (1989). *Adv. Immunol.* **44,** 265–311.
9. Bierer, B. E., Sleckman, B. P., Ratnofsky, S. E., and Burakoff, S. J. (1989). *Annu. Rev. Immunol.* **7,** 579–599.
10. Auffray, C., Piatier-Tonneau, D., and Kroemer, G. (1991). *Trends Biotechnol.* **9,** 124–130.
11. Morita, C. T., Verma, S., Aparicio, P., Martínez-A., C., Spits, H., and Brenner, M. B. (1991). *Eur. J. Immunol.* **21,** 2999–3007.
12. Bandeira, A., Itohara, S., Bonneville, M., Burlen-Defranoux, O., Mota-Santos, T., Coutinho, A., and Tonegawa, S. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 43–47.
13. Aparicio, P., Alonso, J. M., Toribio, M. L., Gutierrez, J. C., Pezzi, L., and Martínez-A., C. (1989). *Immunol. Rev.* **111,** 5–33.
14. Mosmann, T. R., and Coffman, R. L. (1988). *Adv. Immunol.* **46,** 111–147.
15. Mosmann, T. R., Schumacher, J. H., Street, N. F., Budd, R., O'Garra, A., Fong, T. A. T., Bond, M. W., Moore, K. W. M., Sher, A., and Fiorentino, D. F. (1991). *Immunol. Rev.* **123,** 209–229.
16. Swain, S. L. (1991). *Curr. Opin. Immunol.* **3,** 304–310.
17. Thomas, M. L. (1989). *Annu. Rev. Immunol.* **7,** 339–369.
18. Crabtree, G. R. (1989). *Science* **243,** 355–361.
19. Claassen, E. (1991). *Res. Immunol.* **142,** 315–372.
20. Mackay, C. R. (1991). *Immunol. Today* **12,** 189–192.
21. Singer, P. A., and Theofilopoulos, A. N. (1990). *Immunol. Rev.* **118,** 103–127.
22. Blackman, M., Kappler, J., and Marrack, P. (1990). *Science* **248,** 1335–1341.
23. Hünig, T. (1983). *Immunol. Today* **4,** 84–87.
24. Pardoll, D., Fowlkes, B. J., Lew, A. M., Maloy, W., Weston, M., Bluestone, J. A.,

Schwartz, R. H., Coligan, J. E., and Kruisbeek, A. M. (1988). *J. Immunol.* **140,** 4091–4096.
25. Raulet, D. H. (1989). *Annu. Rev. Immunol.* **7,** 175–207.
26. Cron, R., Koning, F., Maloy, W., Pardoll, D., Coligan, J., and Bluestone, J. (1988). *J. Immunol.* **141,** 1074–1082.
27. Daynes, R. A., Araneo, B. A., Dowell, T. A., Huang, K., and Dudley, D. (1990). *J. Exp. Med.* **171,** 979–996.
28. Daynes, R. A., Dowell, T., and Araneo, B. A. (1991). *J. Exp. Med.* **174,** 1323–1333.
29. Mega, J., McGhee, J. R., and Kiyono, H. (1992). *J. Immunol.* **148,** 2030–2039.
30. Kelly, E. A. B., Cruz, E. S., Hauda, K. M., and Wassom, D. L. (1991). *J. Immunol.* **147,** 306–311.
31. Gonzalo, J. A., Moreno de Alborán, I., Alés-Martínez, J. E., Martínez-A., C., and Kroemer, G. (1992). *Eur. J. Immunol.* **22,** 1007–1011.
32. Rellahan, B. L., Jones, L. A., Kruisbeek, A. M., Fry, A. M., and Matis, L. A. (1990). *J. Exp. Med.* **172,** 1091–1100.
33. Newell, K. A., Ellenhorn, J. D. I., Bruce, D. S., and Bluestone, J. A. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 1074–1078.
34. Hölden, I., and Möller, G. (1991). *Eur. J. Immunol.* **21,** 2703–2709.
35. Schmitz, J., and Radbruch, A. (1992). *Int. Immunol.* **4,** 43–52.
36. Lefrançois, L. (1991). *Immunol. Today* **12,** 436–438.
37. Goodman, T., and Lefrancois, L. (1988). *Nature (London)* **333,** 855–858.
38. Lefrançois, L., and Goodman, T. (1989). *Science* **243,** 1716–1718.
39. Guy-Grand, D., Cerf-Bensussan, N., Malissen, B., Malassis-Seris, M., Briottet, C., and Vassalli, P. (1991). *J. Exp. Med.* **173,** 471–481.
40. Bonneville, M., Janeway, Jr., C. A., Ito, K., Hasr, W., Ishida, I., Nakanishi, N., and Tonegawa, S. (1988). *Nature (London)* **336,** 479–482.
41. Takagiki, Y., Decloux, A., Bonneville, M., and Tonegawa, S. (1989). *Nature (London)* **339,** 712–714.
42. Croitoru, K., Stead, R. H., Bienenstock, J., Fulop, G., Harnish, D. G., Shultz, L. D., Jeffery, P. K., and Ernst, P. B. (1990). *Eur. J. Immunol.* **20,** 645–650.
43. Mosley, R. L., Styre, D., and Klein, J. R. (1990). *J. Immunol.* **145,** 1369–1375.
44. Lefrançois, L., LeCorre, R., Mayo, J., Bluestone, J. A., and Goodman, T. (1990). *Cell (Cambridge, Mass.)* **63,** 333–340.
45. Carding, S. R., Kyes, S., Jenkinson, E. J., Kingston, E. J., Bottomly, K., and Hayday, A. C. (1990). *Genes Dev.* **4,** 1304–1315.
46. Guy-Grand, D., Vaden Brocke, C., Briottet, C., Malassis-Seris, M., Selz, F., and Vassalli, P. (1992). *Eur. J. Immunol.* **22,** 505–510.
47. Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. (1992). *Cell (Cambridge, Mass.)* **68,** 869–877.
48. Skinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. (1992). *Cell (Cambridge, Mass.)* **68,** 855–867.
49. Dent, A. L., Matis, L. A., Hooshmand, F., Widacki, S. M., Bluestone, J. A., and Hedrick, S. M. (1990). *Nature (London)* **343,** 714–719.
50. Barrett, T. A., Delvy, M. L., Kennedy, D. M., Lefrancois, L., Matis, L. A., Dent, A. L., Hedrick, S. M., and Bluestone, J . A. (1992). *J. Exp. Med.* **175,** 65–70.
51. Viney, J. L., and MacDonald, T. T. (1990). *Eur. J. Immunol.* **20,** 2809–2812.
52. Rocha, B., Vassalli, P., and Guy-Grand, D. (1991). *J. Exp. Med.* **173,** 483–486.
53. Mosley, R. L., Styre, D., and Klein, J. R. (1990). *Int. Immunol.* **2,** 361–368.
54. Murosaki, S., Yoshikai, Y., Ishida, A., Nakamura, T., Matsuzaki, G., Takimoto, H., Yuuki, H., and Nomoto, K. (1991). *Int. Immunol.* **3,** 1005–1013.

55. Hodes, R. J., Sharrow, S. O., and Salomon, A. (1989). *Science* **246,** 1041–1044.
56. Fry, A. M., Jones, L. A., Kruisbeek, A. M., and Matis, L. A. (1989). *Science* **246,** 1044–1046.
57. Yuuki, H., Yoskijai, Y., Kishihara, K., Iwasaka, A., Matsuzaki, G., Takimoto, H., and Nomoto, K. (1990). *Eur. J. Immunol.* **20,** 1475–1482.
58. Kroemer, G., de Cid, R., Moreno de Alboran, N., Gonzalo, J.-A., Iglesias, A., Martínez, C., and Gutierrez-Ramos, J. C. (1991). **122,** 183–204.
59. Kroemer, G., and Martínez-A., C. (1991). *Lancet* **338,** 1246–1249.
60. Whitacre, C. C., Gienapp, I. E., Orosz, C. G., and Bitar, D. M. (1991). *J. Immunol.* **147,** 2155–2163.
61. Thompson, H. S. G., and Staines, N. A. (1990). *Immunol. Today* **11,** 396–399.
62. Fujihashi, K., Taguchi, T., McGhee, J. R., Eldridge, J. H., Bruce, M. G., Green, D. R., Singh, B., and Kiyono, H. (1990). *J. Immunol.* **145,** 2010–2019.
63. Fujihashi, K., Taguchi, T., Aicher, W. K., McGhee, J. R., Bluestone, J. A., Elridge, J. H., and Kiyono, H. (1992). *J. Exp. Med.* **175,** 695–707.
64. Mosley, R. L., Whetsell, M., and Klein, J. R. (1991). *Int. Immunol.* **3,** 563–569.
65. Balk, S. P., Ebert, E. C., Blumenthal, R. L., McDermott, F. V., Wucherpfennig, K. W., Landau, S. B., and Blumberg, R. S. (1991). *Science* **253,** 1411–1415.
66. Czerkinsky, C., Andersson, G., Ferrua, B., Nordström, I., Quiding, M., Eriksson, K., Larsson, L., Hellstrand, K., and Ekre, H.-P. (1991). *Immunol. Rev.* **119,** 5–22.
67. Taguchi, T., Aicher, W. K., Fujihashi, K., Yamamoto, M., McGhee, J. R., Bluestone, J. A., and Kiyono, H. (1991). *J. Immunol.* **147,** 3736–3744.
68. Taguchi, T., McGhee, J. R., Coffman, R. L., Beagley, K. W., Elridge, J. H., Takatsu, K., and Kiyono, H. (1990). *J. Immunol.* **145,** 68–77.
69. Fujihashi, K., Kiyono, H., Aicher, W. K., Green, D. R., Singh, B., Elridge, J. H., and McGhee, J. R. (1989). *J. Immunol.* **143,** 3415–3420.
70. Mega, J., Bruce, M. G., Beagley, K. W., McGhee, J. R., Taguchi, T., Pitts, A. M., McGhee, M. L., Mucy, R. P., Elridge, J. H., Mestecky, J., and Kiyono, H. (1991). *Int. Immunol.* **3,** 793–805.
71. Asarnow, D. M., Kuziel, W. A., Bonyhadi, M., Tigelaar, R. E., Tucker, P. W., and Allison, J. P. (1988). *Cell (Cambridge, Mass.)* **55,** 837–847.
72. Havran, W., and Allison, J. P. (1988). *Nature (London)* **335,** 443–445.
73. Lafaille, J. J., DeCloux, A., Bonneville, M., Takagaki, Y., and Toneawa, S. (1989). *Cell (Cambridge, Mass.)* **59,** 859–870.
74. Haas, W., Kaufman, S., and Martínez-A., C. (1990). *Immunol. Today* **11,** 340–343.
75. Raulet, D. H., Spencer, D. M., Hsiang, Y. H., Goldman, J. P., Bix, M., Liao, N.-S., Zijlstra, M., Jaenisch, R., and Correa, I. (1991). *Immunol. Rev.* **120,** 185–204.
76. Allison, J. P., and Havran, W. L. (1991). *Annu. Rev. Immunol.* **9,** 679–705.
77. Sim, G.-K., and Augustin, A. (1990). *Cell (Cambridge, Mass.)* **61,** 397–405.
78. Havran, W. L., and Allison, J. P. (1990). *Nature (London)* **344,** 68–70.
79. Ohteki, T., Abo, T., Seki, S., Kobata, T., Yagita, H., Okumura, K., and Kumagai, K. (1991). *Eur. J. Immunol.* **21,** 1733–1740.
80. Ota, Y., Kobata, T., Seki, M., Yagita, H., Shimada, S., Huang, Y.-Y., Takagaki, Y., and Okumura, K. (1992). *Eur. J. Immunol.* **22,** 595–598.
81. Payer, E., Elbe, A., and Stingl, G. (1991). *J. Immunol.* **146,** 2536–2543.
82. Bonneville, M., Itohara, S., Krecko, E. G., Mombaerts, P., Ishida, I., Katsuki, M., Berns, A., Farr, A. G., Janeway, Jr., C. A., and Tonegawa, S. (1990). *J. Exp. Med.* **171,** 1015–1026.
83. Havran, W. L., Poenie, M., Tigelaar, R. E., Tsien, R. Y., and Allison, J. P. (1989). *J. Immunol.* **142,** 1422–1428.
84. Hämmerling, G. J., Schönrich, G., Momburg, F., Auphan, N., Malissen, M.,

Malissen, B., Schmitt-Verhulst, A.-M., and Arnold, B. (1991). *Immunol. Rev.* **122,** 47–67.

85. Happ, M. P., Kubo, R. T., Palmer, E., Born, W. K., and O'Brien, R. L. (1989). *Nature (London)* **342,** 696–698.
86. Ezquerra, A., Wilde, D. B., McConnell, T. J., Sturmhöfel, K., Valas, R. B., Shevach, E. M., and Coligan, J. E. (1992). *Eur. J. Immunol.* **22,** 491–498.
87. Kikuchi, G. E., Roberts, K., Shevach, E. M., and Coligan, J. E. (1992). *J. Immunol.* **148,** 1302–1307.
88. Wilde, D. B., Roberts, K., Sturmhöfel, K., Kikuchi, G., Coligan, J. E., and Shevach, E. M. (1992). *Eur. J. Immunol.* **22,** 483–489.
89. Seki, S., Abo, T., Ohteki, T., Sugiura, K., and Kumagni, K. (1991). *J. Immunol.* **147,** 1214–1221.
90. Theofilopoulos, A. N., Kofler, R., Singer, P. A., and Dixon, E. J. (1989). *Adv. Immunol.* **46,** 61–109.
91. Cohen, P. L., and Eisenberg, R. A. (1991). *Annu. Rev. Immunol.* **9,** 243–269.
92. Takahama, Y., Kosugi, A., and Singer, A. (1991). *J. Immunol.* **146,** 1134–1141.
93. Levitsky, H. I., Golumbek, P. T., and Pardoll, D. M. (1991). *J. Immunol.* **148,** 1113–1117.
94. Singer, P. A., McEvilly, R. J., Dixon, F. J., and Theophilopoulos, A. N. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7018.
95. Singer, P. A., Balderas, R. S., McEvilly, R. J., Bobardt, M., and Theofilopoulos, A. N. (1989). *J. Exp. Med.* **170,** 1869–1877.
96. Okuyama, R., Abo, T., Seki, S., Ohteki, T., Sugiura, K., Kusumi, A., and Kumagai, K. (1992). *J. Exp. Med.* **175,** 661–669.
97. Abo, T., Ohteki, T., Seki, S., Koyamada, N., Yoshikai, Y., Masuda, T., Rikiishi, H., and Kumagai, K. (1991). *J. Exp. Med.* **174,** 417–424.
98. Ohteki, T., Seki, S., Abo, T., and Kumagai, K. (1990). *J. Exp. Med.* **172,** 7–12.
99. Masuda, T., Ohteki, T., Abo, T., Seki, S., Nose, M., Nagura, H., and Kumagai, K. (1991). *J. Immunol.* **147,** 2907–2912.
100. Seki, S., Abo, T., Sugiura, K., Ohteki, T., Kobate, T., Yagita, H., Okamura, K., Rikiishi, H., Masuda, T., and Kumagai, K. (1991). *Cell. Immunol.* **137,** 46–53.
101. Prud'homme, G. J., Boccarro, D. C., and Luke, E. C. H. (1991). *J. Immunol.* **147,** 3314–3318.
102. Martínez-A., C., Marcos, M. A. R., M. de Alborán, I., Alsonso, J. M., de Cid, R., Kroemer, G., and Coutingo, A. (1993). *Eur. J. Immunol.* (in press).
103. Seman, M., Boudaly, S., Roger, T., Morisset, J., and Pham, G. (1990). *Eur. J. Immunol.* **20,** 1265–1272.
104. Benveniste, P., Chadwick, B. S., Miller, R. G., and Reimann, J. (1990). *J. Immunol.* **144,** 411–419.
105. Palathumpat, V., Dejbakhsh, S., Holm, B., Wang, H., Liang, O., and Strober, S. (1992). *J. Immunol.* **148,** 373–380.
106. Woodland, D. L., Happ, M. P., Gollob, K. J., and Palmer, E. (1990). *Nature (London)* **349,** 529–530.
107. Egerton, M., and Scollay, R. (1990). *Int. Immunol.* **2,** 157–164.
108. Ivars, F. (1992). *Eur. J. Immunol.* **22,** 635–639.
109. Jones, L. A., Chin, L. T., Merriam, G. R., Nelsom, L. M., and Kruisbeck, A. M. (1990). *J. Exp. Med.* **172,** 1277–1285.
110. Schwartz, R. H. (1990). *Science* **248,** 1349–1356.
111. Hämmerling, G. J., Schönrich, G., Momburg, F., Auphan, N., Malissen, M., Malissen, B., Schmitt-Verhulst, A.-M., and Arnold, B. (1991). *Immunol. Rev.* **122,** 47–67.

112. Schönrich, G., Kalinke, U., Momburg, F., Malissen, M., Schmitt-Verhulst, A.-M., Malissen, B., Hämmerling, G. J., and Arnold, B. (1991). *Cell (Cambridge, Mass.)* **65,** 293–304.
113. Gonzalo, J. A., M. de Alborán, M., and Kroemer, G. (1993). *Scand. J. Immunol.* (in press).
114. Wadsworth, S., Yui, K., and Greene, M. I. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1018–1022.
115. Porcelli, S., Brenner, M., Steinstein, J., Balk, S., Terhorst, C., and Bleicher, P. (1989). *Nature (London)* **341,** 447–449.
116. Pelkonen, J., and Palacios, R. (1990). *Int. Immunol.* **2,** 555–562.
117. Sékaly, R.-P., Croteau, G., Bowman, M., Scholl, P., Burakoff, S., and Geha, R. S. (1991). *J. Exp. Med.* **173,** 367–371.
118. Shivakumar, S., Tsokos, G. C., and Datta, S. K. (1989). *J. Immunol.* **143,** 103–112.
119. Datta, S. K. Patel, H., and Berry, D. (1987). *J. Exp. Med.* **165,** 1252–1263.
120. Sainis, K., and Datta, S. K. (1988). *J. Immunol.* **140,** 2215–2220.
121. Hayakawa, K., and Hardy, R. R. (1991). *Immunol. Rev.* **123,** 145–167.
122. Andreu-Sánchez, J. L., Moreno de Alborán, I., Marcos, M. A. R., Sánchez-Movilla, A., Martínez-A, C., and Kroemer, G. (1991). *J. Exp. Med.* **173,** 1323–1329.
123. Carroll, A. M., Hardy, R. R., and Bosma, M. J. (1989). *J. Immunol.* **143,** 1087–1093.
124. Simpson, E., Farrant, J., and Chandler, P. (1991). *Immunol. Rev.* **124,** 97–111.
125. Torbett, B. E., Picchio, G., and Mosier, D. E. (1991). *Immunol. Rev.* **124,** 139–164.
126. Smith, C. I. E., Abedi, M. R., Islam, K. B., Johansson, M. E. B., Christensson, B., and Hammarström, L. (1991). *Immunol. Rev.* **124,** 113–138.
127. de Cid, R., Kroemer, G., Moreno de Alborán, I., Gonzalo, J. A., and Martínez-A., C. (1993). Submitted for publication.
128. Gao, E.-K., Lo, D., Cheney, R., Kanagawa, O., and Sprent, J. (1988). *Nature (London)* **136,** 176–179.
129. Jenkins, M. K., Schwartz, R. H., and Pardoll, D. M. (1988). *Science* **241,** 1655–1658.
130. Bryson, J. S, Jennings, C. D., Caywood, B. E., and Kaplan, A. M. (1989). *Transplant.* **48,** 1042–1046.
131. Bryson, J. S., Caywood, B. E., and Kaplan, A. M. (1991). *J. Immunol.* **147,** 391–397.
132. Kahan, B. D. (1989). *N. Engl. J. Med.* **321,** 1725–1729.
133. Kisielow, P., Bluethmann, H., Staerz, U. D., Steinmetz, M., and von Boehmer, H. (1988). *Nature (London)* **333,** 742–745.
134. Teh, H. S., Kieselow, P., Scott, B., Kishi, H., Uematsu, Y., Blüthmann, H., and von Böhmer, H. (1988). *Nature* **335,** 229–232.
135. Rocha, B., and von Boehmer, H. (1991). *Science* **251,** 1225–1228.
136. Cuende, E., Alonso, J. M., Alés-Martínez, J. E., Kroemer, G., and Martínez-A., C. (1993). Submitted for publication.
137. von Boehmer, H. (1990). *Annu. Rev. Immunol.* **8,** 531–556.
138. Ishida, I., Verbeek, S., Bonneville, M., Itohara, S., Berns, A., and Tonegawa, S. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 3067–3071.
139. Freitas, A. A., Lembezat, M.-P., and Coutinho, A. (1989). *Int. Immunol.* **1,** 342–354.
140. Mestecky, J., and McGhee, J. R. (1987). *Adv. Immunol.* **40,** 153–245.
141. Sminia, T., van der Brugge-Gamelkoorn, G. J., and Jeurissen, S. H. M. (1989). *Crit. Rev. Immunol.* **9,** 119–150.
142. Alberola-Ila, J., Places, L., Cantrell, D. A., Vives, J., and Lozano, F. (1992). *J. Immunol.* **148,** 1287–1293.
143. Van de Velde, H., von Hoegen, I., Luo, W., Parnes, J. R., and Thielemans, K. (1991). *Nature (London)* **351,** 662–664.
144. Kantor, A. B. (1991). *Immunol. Today* **12,** 388–389.

145. Hayakawa, K., Hardy, R. R., Parks, D. R., and L. A., H. (1983). *J. Exp. Med.* **157,** 202–215.
146. Wetzel, G. D. (1989). *Eur. J. Immunol.* **19,** 1701–1708.
147. Herzenberg, L. A., Stall, A. M., Lalor, P. A., Sidman, C., Moore, W. A., Parks, D. R., and Herzenberg, L. A. (1986). *Immunol. Rev.* **93,** 81–109.
148. Waldschmidt, T. J., Kroese, F. G. M., Tygrett, L. T., Conrad, D. H., and Lynch, R. G. (1991). *Int. Immunol.* **3,** 305–315.
149. Marcos, M. A. R., Gutiérrez, J. C., Huetz, F., Martínez-A., C., and Dieterlen-Lièvre, F. (1991). *Scand. J. Immunol.* **34,** 129–135.
150. Kasaian, M. T., Ikematsu, H., and Casali, P. (1992). *J. Immunol.* **148,** 2690–2702.
151. Hayakawa, K., Hardy, R. R., and Herzenberg, L. A. (1986). *Eur. J. Immunol.* **16,** 450–456.
152. Marcos, M. A. R., Huetz, F., Pereira, P., Andreu, J. L., Martínez-A., C., and Coutinho, A. (1989). *Eur. J. Immunol.* **19,** 2031–2038.
153. Miyama-Inaba, M., Kuma, S., Inaba, K., Ogata, H., Iwai, H., Yasumizu, R., Muramatsu, S., Stainman, R. M., and Ikehara, S. (1988). *J. Exp. Med.* **168,** 811–115.
154. Andreu-Sánchez, J. L., Faro, J., Alonso, J. M., Paige, C. J., Martínez-A., C., and Marcos, M. A. R. (1990). *Eur. J. Immunol.* **20,** 1767–1773.
155. Bhat, N. M., Kantor, A. B., Bieber, M. M., Stall, A. M., Herzenberg, L. A., and Teng, N. H. T. (1992). *Int. Immunol.* **4,** 243–252.
156. Hayakawa, K., Hardy, R. R., Stall, A. M., Herzenberg, L. A., and Herzenberg, L. A. (1986). *Eur. J. Immunol.* **16,** 1313–1318.
157. Förster, I., and Rajewsky, K. (1987). *Eur. J. Immunol.* **17,** 521–528.
158. Solvason, N., Lehuen, A., and Kearney, J. F. (1991). *Int. Immunol.* **3,** 543–550.
159. Kroese, F. G. M., Butcher, E. C., Stall, A. M., Lalor, P. A., Adams, S., and Herzenberg, L. (1988). *Int. Immunol.* **1,** 75–81.
160. Marcos, M. A. R., Malenchero, E., Sundblad, A., Huetz, F., Grandien, A., Martínez-A., C., and Coutinho, A. (1992). *Ann. N.Y. Acad. Sci.* **651,** 433–442..
161. Dieterlen-Lièvre, F. (1975). *J. Embryol. Exp. Morphol.* **33,** 607–625.
162. Solvason, N., and Kearney, J. F. (1992). *J. Exp. Med.* **175,** 397–404.
163. Iacomini, J., and Imanishi-Kari, T. (1992). *Eur. J. Immunol.* **22,** 745–751.
164. Freedman, A. S., Freeman, G., Whitman, J., Segil, J., Dailey, J., Levine, H., and Nadler, H. (1989). *Eur. J. Immunol.* **19,** 849–894.
165. Ying-zi, C., Rabin, E., and Wortis, H. H. (1991). *Int. Immunol.* **3,** 467–476.
166. Andrade, L., Huetz, F., Poncet, P., Thomas-Vaslin, V., Goodhardt, M., and Coutinho, A. (1991). *Eur. J. Immunol.* **21,** 2017–2023.
167. Gu, H., Förster, I., and Rajewsky, K. (1990). *EMBO J.* **9,** 2133–2139.
168. Gu, H., Tarlinton, D., Müller, K., Rajewsky, K., and Förster, I. (1991). *J. Exp. Med.* **173,** 1357–1371.
169. Pennell, C. A., Arnold, L. W., Haughton, G., and Clarke, S. H. (1988). *J. Immunol.* **141,** 2788.
170. Förster, I., Gu, H., and Rajewsky, K. (1988). *EMBO J.* **7,** 3693–3701.
171. Litman, G. W., Berger, L., Murphy, K., Litamn, R., Hinds, K., and Erickson, B. W. (1985). *Proc. Natl. Acad. Sci. U.S.A.* **82,** 2082–2086.
172. Schroeder, H. W., Hillson, J. L., and Perlmutter, R. M. (1990). *Int. Immunol.* **2,** 41–50.
173. Carmack, C. E., Shinton, S. A., Hayakawa, K., and Hardy, R. R. (1990). *J. Exp. Med.* **172,** 371–374.
174. Reininger, L., Ollier, P., Poncet, P., Klaushik, A., and Jaton, J. C. (1987). *J. Immunol.* **138,** 316–323.

175. Pennell, C. A., Sheehan, K. M., Brodeur, P. H., and Clarke, S. H. (1989). *Eur. J. Immunol.* **19,** 2115–2121.
176. Masmoudi, H., Mota-Santos, T., Huetz, F., Coutinho, A., and Cazenave, P.-A. (1990). *Int. Immunol.* **2,** 515–522.
177. Mageed, R. A., MacKenzie, L. E., Stevenson, F. K., Yuksel, B., Shokri, F., Maziak, B. R., Jefferis, R., and Lydyard, P. M. (1991). *J. Exp. Med.* **174,** 109–113.
178. Schutte, M. E. M., Ebeling, S. B., Akkermands, K. E., Gmelig-Meyeling, F. H. J., and Logtenberg, T. (1991). *Eur. J. Immunol.* **21,** 1115–1121.
179. Kipps, T. J. (1989). *Adv. Immunol.* **47,** 117–185.
180. Perlmutter, R. M., Kearney, J. F., Chang, S. P., and Hood, L. E. (1985). *Science* **227,** 1597–1599.
181. Schroeder, J. H. W., Hiollsonn, J. L., and Perlmutter, R. M. (1987). *Science* **338,** 791–793.
182. Schroeder, H. W. J., and Wang, J. Y. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 6146–6150.
183. Casali, P., and Notkins, A. L. (1989). *Immunol. Today* **10,** 364–368.
184. Hayakawa, K., Hardy, R. R., Hands, M., Herzenberg, L. A., Steinberg, A. D., and Herzenberg, L. A. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2494–2498.
185. Hayakawa, K., Carmack, C. E., Hyman, R., and Hardy, R. R. (1990). *J. Exp. Med.* **163,** 155–161.
186. Van der Hejden, R. W. J., Bunschoten, H., Hoek, A., Van Es, J., Punter, M., Osterhaus, A. D. M. E., and Uytedehaag, F. G. C. M. (1991). *J. Immunol.* **146,** 1503–1508.
187. Harindranath, N., Goldfarb, I. S., Ikematsu, H., Burastero, S. E., Wilder, R. L., Notkins, A. L., and Casali, P. (1991). *Int. Immunol.* **3,** 865–875.
188. Pereira, P., Bandeira, A., Coutinho, A., Marcos, M. A., Toribio, M., and Martínez-A., C. (1989). *Annu. Rev. Immunol.* **7,** 209–249.
189. UytedeHaag, F., van der Heijden, R., and Osterhaus, A. (1991). *Immunol. Today* **12,** 439–442.
190. Lalor, P. A., Herzenberg, L. A., Adams, S., and Stall, A. M. (1989). *Eur. J. Immunol.* **19,** 507–513.
191. Marcos, M. A. R., Sundblad, A., Malenchere, E., and Coutinho, A. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 9944–9948.
192. O'Garra, A., Chang, R., Go, N., Hastings, R., Haughton, G., and Howard, M. (1992). *Eur. J. Immunol.* **22,** 711–717.
193. Herzenberg, L. A., Stall, A. M., Braun, J., Weaver, D., Baltimore, D., Herzenberg, L. A., and Groschedl, R. (1987). *Nature (London)* **329,** 71–73.
194. Okamoto, M., Murakami, M., Shimizo, A., Ozaki, S., Tsubata, T., Kumagai, S. I., and Honjo, T. (1992). *J. Exp. Med.* **175,** 71–79.
195. Liou, L. B., Warner, G. L., and Scott, D. W. (1992). *Int. Immunol.* **4,** 15–22.
196. Stall, A. M., Fariñas, M. C., Tarlinton, D. M., Lalor, P. A., Herzenberg, L. A., Strober, S., and Herzenberg, L. A. (1988). *Proc. Natl. Acad. Sci. U.S.A.* **85,** 7312–7316.
197. Hardy, R. R., Hayakawa, K., Shimizy, M., Yamasaki, K., and Kishimoto, T. (1987). *Science* **236,** 81–84.
198. Sidman, C. L., Schultz, L. D., Hardy, R. R., Hayakawa, K., and Herzenberg, L. A. (1986). *Science* **232,** 1423–1425.
199. de la Hera, A., Marcos, M. A. R., Toribio, M. L., Márquez, C., Gaspar, M. L., and Martínez-A., C. (1987). *J. Exp. Med.* **166,** 804–809.
200. Okada, T., Takiura, F., Tokushige, K., Nozawa, S., Kiyosawa, T., Nakauchi, H., Hirose, S., and Shirai, T. (1991). *Eur. J. Immunol.* **21,** 2743–2748.

201. Martínez-A., C., Bandeira, A., Toribio, M. L., Coutinho, A., Marcos, M. A. R., and Pereira, P. (1988). *Eur. J. Immunol.* **18,** 1895–1901.
202. Thomas-Vaslin, V., and Freitas, A. A. (1990). *Int. Immunol.* **2,** 73–81.
203. Thomas-Vaslin, V., Andrade, L., Freitas, A., and Coutinho, A. (1991). *Eur. J. Immunol.* **21,** 2239–2246.
204. Abedi, M. R., Christensson, B., Islam, K. B., Hammarström, L., and Edvard Smith, C. I. (1992). *Eur. J. Immunol.* **22,** 823–828.
205. Pernis, B. G., Chiappiano, G., Kelus, A. S., and Gell, P. G. H. (1965). *J. Exp. Med.* **122,** 853–875.
206. Tonegawa, S. (1983). *Nature (London)* **302,** 575–581.
207. Rusconi, S., and Köhler, G. (1985). *Nature (London)* 330–334.
208. Iglesias, A., Lamers, M., and Köhler, G. (1987). *Nature (London)* **330,** 482–484.
209. Muller, W., Ruther, U., Vieira, P., Hombach, J., Reth, M., and Rajewsky, K. (1989). *Eur. J. Immunol.* **19,** 923–929.
210. Nemazee, D. A. (1989). *Nature (London)* **337,** 562–566.
211. Coutinho, A., Coutinho, G., Grandien, A., Marcos, M. A. R., and Bandeira, A. (1992). *Res. Immunol.* **143,** 345–354.
212. Heinen, E., Cormann, N., and Kinet-Denoël, K. (1988). *Immunol. Today* **9,** 240–243.
213. Szakal, A. K., Kosco, M. H., and Tew, J. G. (1989). *Annu. Rev. Immunol.* **7,** 91–109.
214. Liu, Y.-L., Johnson, G. D., Gordon, J., and MacLennan, I. C. M. (1992). *Immunol. Today* **13,** 17–21.
215. Mason, D. Y., Jones, M., and Goodnow, C. C. (1992). *Int. Immunol.* **4,** 163–175.
216. Day, M. J., Tse, A. G. D., Puklavec, M., Simmonds, S. J., and Mason, D. W. (1992). *J. Exp. Med.* **175,** 655–659.
217. Jacob, J., Kelsoe, G., Rajewsky, K., and Weiss, U. (1991). *Nature (London)* **354,** 389–392.
218. Weinstein, P. D., and Cebra, J. C. (1991). *J. Immunol.* **147,** 4126–4135.
219. MacLennan, I. C. M., and Gray, D. (1986). *Immunol. Rev.* **91,** 62–85.
220. Berek, C., Berger, A., and Apel, M. (1991). *Cell* **67,** 1121–1129.
221. Tew, J. G., Phipps, R. P., and Mandel, T. E. (1980). *Immunol. Rev.* **53,** 29–58.
222. Gray, D., Kosco, M., and Stockinger, B. (1991). *Int. Immunol.* **3,** 141–148.
223. Gray, D., and Matzinger, P. (1991). *J. Exp. Med.* **174,** 969–974.
224. Pardoll, D. M., Fowlkes, B. J., Bluestone, J. A., Kruisbeek, A., Maloy, W. L., Coligan, J. E., and Schwartz, R. H. (1987). *Nature (London)* **326,** 79–82.
225. Itohara, S., Nakanishi, N., Kanagawa, O., Kubo, R., and Tonegawa, S. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86,** 5094–5098.
226. Itohara, S., Farr, A. G., Lafaille, J. J., Bonneville, M., Takagaki, Y., Haas, W., and Tonegawa, S. (1990). *Nature (London)* **343,** 754–757.
227. Lafaille, J. J., Haas, W., Coutinho, A., and Tonegawa, S. (1990). *Immunol. Today* **11,** 75–78.
228. Itohara, S., and Tonegawa, S. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87,** 7935–7938.
229. Iwashima, M., Davis, M. M., and Chien, Y.-H. (1991). *J. Exp. Med.* **174,** 293–296.
230. Pober, J. S., and Cotran, R. S. (1991). *Adv. Immunol.* **50,** 261–302.
231. Dustin, M. L., and Springer, T. A. (1991). *Annu. Rev. Immunol.* **9,** 27–66.
232. Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1992). *Immunol. Today* **13,** 106–112.
233. Dustin, M. L., and Springer, T. A. (1989). *Nature (London)* **341,** 619–624.
234. Oppenheimer-Marks, N., Davis, L. S., and Lipsky, P. E. (1990). *J. Immunol.* **145,** 140–148.

235. Miyake, K., Underhill, C. B., Lesley, J., and Kincade, P. W. (1990). *J. Exp. Med.* **172,** 69–75.
236. Elices, M. J., Osnorn, L., Takada, Y., Crouse, C., Luhowskyi, A., Hemler, M. E., and Lobb, R. R. (1990). *Cell (Cambridge, Mass.)* **60,** 577–584.
237. Graber, N., Gopal, T. V., and Wilson, D. (1990). *J. Immunol.* **145,** 819–830.
238. Shimizu, Y., Shaw, S., and Graber, N. (1991). *Nature (London)* **349,** 796–799.
239. Issekutz, T. B. (1991). *J. Immunol.* **147,** 4178–4184.
240. Picker, L. J., Kishimoto, T. K., Smith, C. V., Warnock, R. A., and Butcher, E. C. (1991). *Nature (London)* **349,** 799–802.
241. Foster, C. A., Yokozei, H., and Rappersberger, K. (1990). *J. Exp. Med.* **171,** 801–817.
242. Saltini, C., Kirby, M., Trapnell, B. C., Tamura, N., and Crystal, R. G. (1990). *J. Exp. Med.* **171,** 1123–1140.
243. Sprent, J. (1976). *Cell. Immunol.* **21,** 278–302.
244. Berg, E. L., Yoshino, T., Rott, L. S., Robinson, M. K., Warnock, A., Kishimoto, T. K., Picker, L. J., and Butcher, E. C. (1991). *J. Exp. Med.* **174,** 1461–1466.
245. Holzmann, B., McIntyre, B. W., and Weissman, I. L. (1989). *Cell* **56,** 37—46.
246. Picker, L. J., Terstappen, L. W. M. M., Rott, L. S., Streeter, P. R., Stein, H., and Butcher, E. C. (1990). **145,** 3247–3255.
247. Kilshaw, P. J., and Murant, S. J. (1991). *Eur. J. Immunol.* **21,** 2591–2597.
248. Dougherty, G. J., Lansdorp, P. M., Cooper, D. L., and Humphries, P. K. (1991). *J. Exp. Med.* **174,** 1–5.
249. Butcher, E. C. (1986). *Curr. Top. Microbiol. Immunol.* **128,** 85–122.
250. Gallatin, W. M., Weissman, I. L., and Butcher, E. C. (1983). *Nature (London)* **304,** 30–34.
251. Tedder, T. F., Penta, A. C., Levine, H. B., and Freedman, A. S. (1990). *J. Immunol.* **144,** 532–540.
252. Camerini, D., James, S. P., Stamenkovic, I., and Seed, B. (1989). *Nature (London)* **342,** 78–81.
253. Hamann, A., and Thiele, H. G. (1989). *Immunol. Rev.* **108,** 19–44.
254. Lasky, L. A., Singer, M. S., Yednock, T. A., Dowbenko, D., Fennie, C., Rodriguez, H., Nguyen, T., Stachel, S., and Rosen, S. D. (1989). *Cell (Cambridge, Mass.)* **56,** 1045–1054.
255. Reichert, R. A., Gallatin, W. M., Butcher, E. C., and Weissman, I. L. (1984). *Cell. (Cambridge, Mass.)* **38,** 89–99.
256. Samlowski, W. E., Spangrude, G. J., and Daynes, R. A. (1984). *Cell. Immunol.* **88,** 309–322.
257. Hynes, R. O., and Lander, A. D. (1992). *Cell (Cambridge, Mass.)* **68,** 303–322.
258. Hynes, R. O. (1992). *Cell (Cambridge, Mass.)* **69,** 11–25.
259. Diamond, M. S., Staunton, D. E., Marlin, S. D., and Springer, T. A. (1991). *Cell (Cambridge, Mass.)* **65,** 961–971.
260. McCarron, R. M., Racke, M., Spatz, M., and McFarlin, D. E. (1991). *J. Immunol.* **147,** 503–508.
261. Pereira, P., and Rocha, B. (1991). *Int. Immunol.* **3,** 1077–1080.
262. Forni, L. (1990). *Eur. J. Immunol.* **20,** 983–989.
263. Grandien, A., Coutinho, A., and Andersson, J. (1990). *Eur. J. Immunol.* **20,** 991–998.
264. Rocha, B. (1992). *Res. Immunol.* **143,** 299–303.
265. Speiser, D. E., Stübi, U., and Zinkernagel, R. M. (1992). *Nature (London)* **355,** 170–172.
266. Webb, S., Morris, C., and Sprent, J. (1990). *Cell (Cambridge, Mass.)* **63,** 1249–1256.
267. Jones, L. A., Chin, L. T., Longo, D. L., and Kruisbeek, A. M. (1990). *Science* **250,** 1726–1729.

268. Kawabe, Y., and Ochi, A. (1991). *Nature (London)* **349,** 245–248.
269. MacDonald, H. R., Baschieri, S., and Lees, R. K. (1991). *Eur. J. Immunol.* **21,** 1963–1966.
270. Dannecker, G., Mecheri, S., Stainano-Coico, L., and Hoffman, M. K. (1991). *J. Immunol.* **146,** 2083–2087.
271. Abromson-Leeman, S. R., and Dorf, M. E. (1991). *J. Immunol.* **147,** 1–7.
272. Forman, J., Wieties, K., and Hammer, R. E. (1991). *Immunol. Rev.* **122,** 33–47.
273. van Seventer, G. A., Shimizu, Y., and Shaw, S. (1991). *Curr. Opin. Immunol.* **3,** 294–303.
274. Steinman, R. M., and Young, J. W. (1991). *Curr. Opin. Immunol.* **3,** 361–372.
275. Metlay, J. P., Puré, E., and Steinman, R. M. (1989). *Adv. Immunol.* **47,** 45–116.
276. Janeway, C. A. J. (1992). *Immunol. Today* **13,** 11–16.
277. Bhardwaj, N., Friedman, S. M., Cole, B. C., and Nisanian, A. J. (1992). *J. Exp. Med.* **175,** 267–273.
278. Ellis, J., Chain, B. M., Davies, D. H., Ibrahim, M. A. A., Katz, D. R., Kaye, P. M., and Lightstone, E. (1991). *Eur. J. Immunol.* **21,** 2803–2809.
279. Liu, Y., and Janeway, C. A. J. (1991). *Int. Immunol.* **3,** 323–332.
280. Sornasse, T., Flamand, V., de Becker, G., Bazin, H., Tielemans, F., Thielemans, K., Urbain, J., Leo, O., and Moser, M. (1992). *J. Exp. Med.* **175,** 15–21.
281. Crocker, P. R., and Gordon, S. (1989). *J. Exp. Med.* **169,** 1330–1346.
282. Rabinowitz, S., and Gordon, S. (1991). *J. Exp. Med.* **174,** 827–836.
283. Gong, J. L., McCarthy, K. M., Telford, J., Tamatani, T., Miyasaki, M., and Schneeberger, E. E. (1992). *J. Exp. Med.* **175,** 797–807.
284. Bland, P. (1988). *Immunol. Today* **9,** 174–178.
285. Gaspari, A. A., and Katz, S. I. (1991). *J. Immunol.* **147,** 4155–4161.
286. Yamada, A., Nikaido, T., Nojima, Y., Scholssman, S. F., and Morimoto, C. (1991). *J. Immunol.* **146,** 53–56.
287. Roberts, K., Yokoyama, W. M., Kehn, P. J., and Shevach, E. M. (1991). *J. Exp. Med.* **173,** 231–240.
288. Dang, N. H., Torimoto, Y., Schlossman, S. F., and Morimoto, C. (1990). *J. Exp. Med.* **172,** 649–652.
289. Shimizu, Y., van Seventer, G. A., Horgan, K. J., and Shaw, S. (1990). *J. Immunol.* **145,** 59–67.
290. Davis, L. S., Oppenheimer-Marks, N., Bednarczyk, J. L., Mcintyre, B. W., and Lipsky, P. E. (1990). *J. Immunol.* **145,** 785–793.
291. Nojima, Y., Humphries, M. J., Mould, A. P., Komoriya, A., Yamada, K. M., Schlossman, S. F., and Morimoto, C. (1990). *J. Exp. Med.* **172,** 1185–1192.
292. Naquet, P., Vivier, I., Gorvel, F. P., Brekemans, P., Barad, M., and Bernard, A.-M. (1989). *Immunol. Rev.* **11,** 77–193.
293. Thompson, L. F., Ruedi, J. M., Glass, A., Low, M. G., and Lucas, A. H. (1989). *J. Immunol.* **140,** 1815–1821.
294. Nathan, C., and Sporn, M. (1991). *J. Cell Biol.* **113,** 981–986.
295. La Rochelle, W. J., May-Siroff, M., Robbins, K. C., and Aaronson, S. A. (1991). *Genes Dev.* **5,** 1191–1199.
296. Rathjen, P. D., Toth, S., Willis, A., Heath, J. K., and Smith, A. G. (1990). *Cell* **62,** 1105–1114.
297. Cardin, A. D., and Weintraub, H. J. (1988). *Arteriosclerosis* **9,** 21–32.
298. Yamaguchi, Y., Mann, D. M., and Ruoslahti, E. (1990). *Nature (London)* **346,** 281–284.
299. Ruoslahti, E., and Yamaguchi, Y. (1991). *Cell (Cambridge, Mass.)* **64,** 867–869.

300. Jessell, T. M., and Melton, D. A. (1992). *Cell (Cambridge, Mass.)* **68,** 257–270.
301. Rademacher, T. W., Parekh, R. B., and Dwek, R. A. (1988). *Annu. Rev. Biochem.* **57,** 785–838.
302. Sporn, M. B., and Roberts, A. B. (1988). *Nature (London)* **332,** 217–219.
303. Mier, J. W., and Gallo, R. C. (1982). *J. Immunol.* **128,** 1122–1126.
304. Beutler, B. A., Milsark, I. W., and Cerami, A. (1985). *J. Immunol.* **135,** 3972–3977.
305. Castell, J. V., Geiger, T., Gross, V., Andus, T., Walter, E., Hirano, T., Kishimoto, T., and Heinrich, P. C. (1988). *Eur. J. Biochem.* **177,** 357–361.
306. Kroemer, G., Toribio, M. L., and Martínez-A., C. (1991). *New Biologist* **3,** 219–229.
307. Kroemer, G., Andreu-Sánchez, J. L., Gonzalo, J. A., Gutierrez-Ramos, J. C., and Martínez-A., C. (1991). *Adv. Immunol.* **150,** 147–235.
308. Aderka, D., Engelmann, H., Maor, Y., Brakebusch, C., and Wallach, D. (1992). *J. Exp. Med.* **175,** 323–329.
309. Mosmann, T. R., and Coffman, R. L. (1989). *Annu. Rev. Immunol.* **7,** 145–173.
310. Parronchi, P., Macchia, D., Piccinni, M.-P., Biswas, P., Simonelli, C., Maggi, E., Ricci, M., Ansari, A. A., and Romagnani, S. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4538–4542.
311. Mosmann, T. R., and Moore, K. W. (1991). *Immunol. Today* **12,** A49-A53.
312. Phipps, R. P., Stein, S. H., and Roper, R. L. (1991). *Immunol. Today* **12,** 349–353.
313. Finkelman, F. D., Holmes, J., Katona, I. M., Urban, Jr., J. F., Beckmann, M. P., Park, L. S., Schooley, K. A., Coffman, R. L., Mosmann, T. R., and Paul, W. E. (1990). *Annu. Rev. Immunol.* **8,** 303–333.
314. Maggi, E., Biswas, P., del Prete, G., Parronchi, P., Macchia, D., Simonelli, C., Emmi, L., de Carli, M., Tiri, A., Ricci, M., and Romagnani, S. (1991). *J. Immunol.* **146,** 1169–1174.
315. Butcher, E. C. (1991). *Cell (Cambridge, Mass.)* **67,** 1033–1036.
316. Thornhill, M. H., and Haskard, D. O. (1990). *J. Immunol.* **145,** 865–872.
317. Birdsall, H. H., Lane, C., Ramser, M. N., and Anderson, D. C. (1992). *J. Immunol.* **148,** 2717–2723.
318. Eichmann, K. (1978). *Adv. Immunol.* **26,** 195–232.
319. Martínez-A., C., Pereira, P., Bernabe, R. R., Bandeira, A., Larson, E. L., Cazenave, P. A., and Coutinho, A. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4520–4525.
320. Sy, M. S., Lowy, A., HayGlass, K., Janeway, C. A., Gurish, M., Greene, M. M., and Benacerraf, B. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3846–3850.
321. Martínez-A., C., Bernabé, R. R., de la Hera, A., Pereira, P., Cazenave, P. A., and Coutinho, A. (1985). *Nature (London)* **317,** 721–725.
322. Martínez-A., C., Pereira, P., de la Hera, A., Bandeira, A., Márquez, C., and Coutinho, A. (1986). *Eur. J. Immunol.* **16,** 417–422.
323. Marcos, M. A. R., de la Hera, A., Pereira, P., Toribio, M. L., Coutinho, A., and Martínez-A., C. (1988). *Eur. J. Immunol.* **18,** 1015–1019.
324. Morahan, G., Hoffmann, M. W., and Miller, J. F. A. P. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 11421–11425.
325. Yeoman, H., and Mellor, A. L. (1992). *Int. Immunol.* **4,** 59–65.
326. Wilbanks, G. A., and Streilein, J. W. (1992). *Eur. J. Immunol.* **22,** 1031–1036.
327. Wahl, S. M., Allen, J. B., Wong, H. L., Dougherty, S. F., and Ellingsworth, L. R. (1990). *J. Immunol.* **145,** 2514–2519.
328. Ruegemer, J. J., Ho, S. N., Augustine, J. A., Schlager, J. W., Bell, M. P., McKean, D. J., and Abraham, R. T. (1990). *J. Immunol.* **144,** 1767–1776.
329. Schluesener, H. J., and Lider, O. (1989). *J. Neuroimmunol.* **24,** 249–258.

330. Fava, R. A., Olsen, N. J., Postlethwaite, A. E., Broadley, K. N., Davidson, J. M., Nanney, L. B., Lucas, C., and Townes, A. S. (1991). *J. Exp. Med.* **173,** 1121–1132.
331. Kuruvilla, A. P., Shah, R., Hochwald, G. M., Liggitt, H. D., Palladino, M. A., and Thorbecke, G. J. (1991). *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2918–2921.
332. King, A., and Loke, Y. W. (1991). *Immunol. Today* **12,** 432–435.
333. Lea, R. G., Flanders, K. C., Harley, C. B., Manuel, J., Banwatt, D., and Clark, D. A. (1992). *J. Immunol.* **148,** 778–787.
334. Miller, A., Lider, O., Roberts, A. B., Sporn, M. B., and Weiner, H. L. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89,** 421–425.
335. Kroemer, G., and Martínez-A., C. (1992). *Res. Immunol.* **143,** 335–340.
336. Yednock, T. A., Cannon, C., Fritz, L. C., Sanchez-Madrid, F., Steinman, L., and Karin, N. (1992). *Nature (London)* **356,** 63–66.

This article was accepted for publication on 23 September 1992.

ADVANCES IN IMMUNOLOGY, VOL. 53

Immunological Memory

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I. Introduction

The concept of immunological memory germinated from some of the fundamental observations on immunity. It was realized from ancient times that individuals who survived a disease seldom suffered a second infection. In fact, deliberate inoculation with material from smallpox scabs was practiced over three centuries ago in China and India, and later in Great Britain. This practice was superseded by vaccination following the observations of Jenner and Pasteur, although the basis for the immune protection was not understood. After the 1920s, it was discovered that antibody responses to an antigen could be of a primary or secondary nature, depending on whether the challenged animal was immune (*1,2*). Some types of secondary responses were also referred to as anamnestic (from the Greek anamnesis, remembrance). Later, immunological theory was invoked to explain the immune system's "memories" of previous infections. In reference to the enhanced secondary response to a preencountered antigen, Burnet and Fenner stated that "the changed capacity to respond is transmitted to cells descended from those in which it was induced" (2). Later, Burnet proposed in his clonal selection theory (3) that the secondary response might be accounted for by a greatly increased number of clonally expanded, antigen-reactive cells. The notion arose that memory might also be harbored by special memory cells (*4*). Sercarz and colleagues (*5,6*) in 1965 proposed in their X–Y–Z model of immunocyte maturation that X was the first antigen-sensitive cell, which on stimulation converted to a memory (Y) cell, and further stimulation resulted in terminal differentiation to effector plasma cells (Z). These authors also suggested other schemes for the establishment of memory (*6*). For instance "asymmetric division" could generate both memory cells and effector (plasma) cells. Another suggestion was that memory was somehow a product of the terminal Z cells, either through their persistence, or through their transformation back to a Y-type memory cell. A 1971 review on immunological memory by Celada (*7*) discussed the merits of these models and recounted many of the early observations relating to the

features and possible nature of memory. Essentially the same models exist today, testimony to the slow progress and controversial nature of immunological memory.

Irrespective of its nature, immunological memory provides for considerable protection and enables an organism to benefit from experiences with its antigenic environment. Memory is a phylogenetically conserved and fundamental feature of vertebrate and invertebrate immune systems. In human medicine, the ability to produce memory to killed or attenuated pathogens has enabled protection against numerous diseases. Indeed, the humanitarian and financial benefits that have resulted from vaccination programs are presently the most obvious fruits of immunological research.

A number of recent advances have brought the enigma of immunological memory to the closer attention of immunologists. Many of the cell surface molecules on lymphocytes have been identified by monoclonal antibodies (mAbs), which has enabled the phenotyping and subdivision of T and B cells into numerous subsets. Moreover, the life span, function, and migration properties of these different subsets are being reappraised. Which of these subsets are responsible for memory, and how is memory maintained? This review will assess current knowledge on T and B cell memory, and also the function, life span, and migration of different T and B cell subsets and the relevance of these properties to memory.

II. Longevity and Maintenance of Immunological Memory

A. Observations on the Life Span of Immunological Memory

It is generally considered that immunological memory can last for many years. There are many examples in humans of secondary immune responses to previously encountered antigens occurring 10 years or more after primary infection (*8,9*). One classic example is the outbreak of measles in the Faeroe Islands in the eighteenth and nineteenth centuries. Successive epidemics were encountered on the islands in 1781, 1846, and 1875, and each epidemic affected only those who had not been infected previously and left untouched those who had (*10*). In addition, antibody to viruses such as yellow fever are detectable 50 years after infection (*2*) and, in mice, the persistence of high antibody titers following viral infection can last for a lifetime (*11*). Interestingly, immunity to some pathogens lasts a lifetime, whereas to other pathogens it is limited. In humans, immunity after infection with mumps or chicken pox is lifelong, whereas immunity

to tuberculosis and tetanus is of shorter duration. Nevertheless, infection or immunization with most pathogens does produce long-lasting memory.

Several variables affect the time span of immunological memory. The number and timing of immunizing boosts, the use of adjuvants, the dose of antigen, the route of injection, and the age of the animal all have a bearing on the longevity of memory (*2*). The properties of different antigens, and the immune responses to them, may show great variation. For instance, viral infections are usually acute and cleared within the space of days, but can become chronic because of persistence of the infection. Some antigens are retained in the body for long periods and so provide a continual stimulus, whereas other antigens are retained more transiently. Another factor that accounts for the variability in immunity to different pathogens is the frequency of natural reinfection.

The duration of immunological memory may differ for the T and B cell systems. This was suggested by observations that, in some instances, T cell memory can be extremely short-lived (*11*), i.e., after injection of virus into mice, virus titers rose and then fell within the first week and T cell responses showed a corresponding rise and fall, but with a delay of about 3 days. This was despite the persistence of high antibody titers. Moreover, in a recent review, Zinkernagel (*11*) quoted many examples of short-lived or unremarkable T cell memory to viral infection. In contrast, other studies have shown that specific T cell memory can be long-lasting. In a study in mice, cytotoxic T lymphocytes (CTLs) specific for lymphocytic choriomeningitis virus (LCMV) were found to persist indefinitely after transfer to a secondary, noninfected host (*12*), although a recent report claims that this persistence requires the presence of LCMV, a widely replicating virus (*12a*). Memory T and B cells seem to be generated in different ways (discussed below), which probably accounts for the differences in persistence of memory found in these lymphocyte subsets.

B. Theories on How Immunological Memory is Generated and Maintained

There are two different mechanisms that might account for memory. The conventional model holds that specialized, long-lived "memory cells" harbor memory. A second model assumes that memory is critically dependent on continual stimulation by antigen, which results in the persistence of effector-type or activated clones. The necessity of antigen for the persistence of memory was a fashionable idea in the 1970s (*7,13–17*) and recent experiments have given new credence to this model (*18–21*). A third possibility is that mem-

ory is derived from both persistent antigenic restimulation and long-lived cells (22); for different antigens, the relative contribution of either mechanism of memory might vary, perhaps relating to the capacity of the antigen to be retained or reencountered. The evidence for the validity of these opposing mechanisms for persistence of memory is given below.

1. Memory by Long-Lived Clonally Expanded Cells

The enduring memory for infectious agents, lasting as long as 75 years (2), led to the simple interpretation that this memory was maintained by long-lived memory cells. The enhanced and more rapid response to reinfection was proposed to result from the increased frequency of antigen-reactive cells, which result from clonal expansion during the primary response. Using limiting dilution, many studies have shown that the frequency of antigen-specific cells increases up to 50-fold following primary immunization (23). As discussed below, the memory response results also from qualitative features of memory cells; nonetheless, the increased frequency of antigen-reactive cells does have a dramatic effect on the rapidity and efficiency of the immune response to an infectious agent (*11*).

The relatively long life span of most lymphocytes (see below) favors the notion that memory can be maintained through long-lived cells. However, the formal demonstration of long-lived, antigen-specific memory cells has been difficult. Using a technique to label dividing cells (see Section V,A), one study (*24*) showed that after the initial phase of proliferation following primary immunization, memory B cells persisted in mice for long periods of time in the absence of cell division. Also, T cell memory to *Mycobacterium tuberculosis* in mice was long lasting, even when these mice were treated with cyclophosphamide or ionizing radiation, suggesting that immunity resulted from the presence of long-lived memory T cells (25).

At what point in the immune response might long-lived memory cells be generated? Effector cells could undergo transformation to memory cells, or naive lymphocytes could take alternative differentiation pathways after antigen stimulation, either to effector cells or to memory cells. Commitment to either pathway might occur at the time of antigen priming, or at some stage before or after, and presumably would relate to microenvironmental signals. For T cells, it seems that effector cells do revert to smaller, resting memory T cells or persist as activated cells. Convincing evidence for this came from experiments in which antigen-driven effector T cell clones were transferred to adoptive hosts; they persisted for long periods *in vivo* and provided

immunological memory (*12,26*). Evidence that this long-term memory is mediated by resting long-lived T cells is as follows. Cytolytic T lymphocytes have been observed to differentiate into smaller types of cells (*27*), and T cell clones maintained *in vitro* become smaller and live for some months without dividing. The effector cell → memory cell model is also consistent with phenotypic studies (see Section III,A) that show that activated/effector T cells and memory-type T cells have a similar phenotype (i.e., $CD45RO^+$, $adhesion^{hi}$). Memory-type T cells are smaller than effector cells but slightly larger than naive T cells. Memory T cell production in the course of primary stimulation has not been demonstrated, and to my knowledge, there is no evidence for the asymmetric production of effector and memory T cells.

In contrast, memory B cells and effector B cells appear to develop by alternative differentiation pathways (*28*). Antibody-forming plasma cells produced after antigen challenge are terminally differentiated, whereas small IgG^+ memory B cells still retain the capacity to differentiate to plasma cells at a later date (discussed in Section IV). One study suggested that the commitment by B cells either to primary antibody-forming cell clones or to memory B cells occurred even before encounter with antigen (*28*).

2. *Maintenance of Memory by Constant Antigenic Restimulation*

The dependence of memory on persistent antigenic stimulation is an old idea (*13–16*) that never achieved wide acceptance, until some recent experiments strongly suggested its relevance (*18–21*). In the early studies, memory responses were found to decay quite rapidly following adoptive transfer of immune cells to syngeneic recipients (*13*). In more recent studies, memory B cells transferred to adoptive hosts in the absence of antigen decayed over a period of 12 weeks (*19,21*), whereas those transferred with antigen survived indefinitely (*19*). In these studies, strenuous efforts were made to ensure that antigen was not transferred along with lymphocytes. Gray (*29*) suggests that in the early studies in which a small element of long-term memory was observed, antigen might well have been transferred with the cells. A dependence on antigen appears to be necessary also for T cell memory. When primed T cells were transferred to adoptive hosts, both T helper responses and T cytotoxic responses decayed rapidly in the absence of antigen (*18*). These results favored a model whereby antigen-reactive cells must be restimulated constantly to ensure the survival of memory.

Antigen restimulation of primed cells could be accounted for in

several ways. Follicular dendritic cells (FDCs) within primary follicles and germinal centers are one of the main antigen-trapping mechanisms of the lymphoid system (*30*) and these cells also serve as a long-term depot for antigen (17). Antigen–antibody complexes are deposited on FDCs via Fc or C3 receptors. Antigen can be retained on FDCs in its native form for at least 1 year (*17*), and constantly restimulate antigen-specific B cells and possibly T cells. If popliteal lymph nodes which contain retained antigen are removed from mice, there is a marked decrease in serum antibody levels (*17*). High antibody titers in serum can last for the lifetime of the individual following viral infection (*2,9,11*), which suggests that plasma cells are continually produced. Influenza-specific antibody-producing cells can be found in the lungs and spleens of mice up to 18 months after a primary influenza infection (*31*).

The sparse number of T cells in germinal centers indicates that maintenance of any form of T cell memory by constant restimulation is not from antigen stored on FDCs. It is possible that antigen from the FDC is transferred to B cells, and is then processed and presented to T cells (*32*). It has also been suggested that once an immune response has occurred, long-lasting T cell memory is less critical than long-lasting B cell memory. However, the requirement for T cell as well as B cell memory was demonstrated by the classic studies of Mitchison (*33*). Poor antihapten responses developed if the carrier used for the secondary stimulus differed from that used for the primary stimulus.

Mechanisms other than stored antigen on FDCs could provide the constant stimulus for B or T cell clones. One example is the capacity of many viruses to persist in the host despite an active immune response, for example, Epstein–Barr virus (EBV) and human immunodeficiency virus (HIV). Hence, for at least some viral infections, the potential exists for constant restimulation. Also, certain viruses may integrate into the host genome and reemerge after a period of quiescence. In one study, natural infection with influenza virus was found to protect individuals for about 5 to 6 years, whereas immunization with an inactivated whole virus vaccine provided protection for only 1 year (*34*). It is generally recognized that live, attenuated viruses usually induce long-lasting immunity, whereas inactivated virus or synthetic peptides are generally poorer immunogens. The tuberculin reaction in man, often used to exemplify the longevity of T cell memory, sometimes wanes in individuals receiving antimycobacterial drugs (*35*). A recent study in mice using LCMV (a widely replicating, persisting virus) and a recombinant vaccinia virus with LCMV glycoprotein (a poorly replicating virus) showed that immunization of mice

with the former virus induced long lasting memory, whereas the latter induced only poor memory unless very high doses were used for immunization (*12a*).

Another possibility is that cross-reactive stimulation by other antigens maintains memory (*20*). Following priming with one antigen, a secondary-type immune response may result after challenge with a structurally related antigen. This effect has been referred to as "original antigenic sin" (*36,37*). Memory cells are more readily triggered by antigen than by naive cells (see below), so that lower affinity interactions or cross-reactive antigens might be adequate to restimulate memory cells. There is considerable evidence for cross-reactivity by T cell specificities. T cell clones specific for a given antigen, in the context of the self major histocompatability complex (MHC), can also recognize alloantigen (*38*), or peptide in association with various other MHC alleles (*39*). Malaria-specific T cell responses can be obtained from individuals who have no history of exposure to malaria (*40,41*). The T cells responsible for this natural "immunity" to malaria expressed the CD45RO memory phenotype (*41,42*), and malaria-specific T cell clones derived from such individuals showed a degree of cross-reactivity with other common pathogens, such as tetanus (*42*). Studies by Zinkernagel (*11*) and co-workers and others (*8*) demonstrated that priming with one serotype of virus may lead to immunity to other serotypes.

There are claims that B cells at least can be primed and restimulated by antiidiotypes. Theoretically, therefore, the idiotypic network could maintain immunological memory (*43*), although this seems dubious.

3. *Maintenance of Memory by Short-Lived and Long-Lived Cells*

Until recently, immunological memory was viewed in a bipartisan fashion; the traditionalists argued for long-lived memory cells, whereas the heretics stressed the need for the persistence of antigen. A case can be made for the operation of both processes. Celada and others noted in the early experiments (*7*) that following adoptive transfer of primed cells to syngeneic recipients, B cell memory decayed in a nonuniform fashion. Two phases were observed. In the first phase, decay was extremely rapid ($t^{1/2}$ = 15 days), whereas in the second phase, further decay took considerably longer ($t^{1/2}$ = 100 days). The rapid early decline in memory could be due to death of activated cells, which failed to be restimulated through lack of persistent antigen. The slow decline thereafter might be attributable to the gradual attrition of longer-lived memory cells. The evidence for the operation of a long-lived and a short-lived component of memory will

emerge in the following paragraphs. A pertinent issue relevant to memory life span, the life span of particular lymphocyte subsets, will be discussed below, following a description of memory function and precursor–product relationships among T and B cell subsets.

III. The T Cell Immune System and Immunological Memory

To understand how memory might be carried by T cell or B cells, it is necessary to distinguish phenotypically and functionally distinct subsets and to know how these subsets relate to each other. Unfortunately, precursor–product relationships within the T and B cell systems are poorly understood, particularly with regard to the development of memory cells. As discussed already, memory may result by different mechanisms, and so memory might be manifest by a number of cell types in a differentiation chain.

A. Phenotypic Identification of Memory T Cells Using CD45 and Other Markers

CD45 is an alternatively spliced molecule that is expressed in one form or another on all leukocytes (*44*). Alternative splicing of certain exons of CD45 yields different isoforms. The framework region of CD45 contains a tyrosine phosphatase in its cytoplasmic region, and on T cells probably interacts with the T cell receptor (TCR) in signal transduction (*45*). The ligands for the different CD45 isoforms are not fully elucidated, although it appears that certain isoforms may interact with other molecules within the plasma membrane (*46,47*) (see below). One isoform, CD45RO, appears to serve as a ligand for the B cell adhesion molecule CD22 (*48*), although the significance of this interaction is not clear.

Experiments and discussion on T cell memory over the past 5 years have been dominated by the notion that isoforms of CD45 identify naive and memory T cells. The initial studies in humans found that T cells could be divided into relatively distinct reciprocal populations based on expression of two isoforms of CD45, CD45RO (p180) and CD45RA (p220/205) (*49–51*). T cells that expressed CD45RA generally expressed low levels of cell adhesion molecules. In contrast, the reciprocal population ($CD45RO^+$) expressed increased levels of adhesion molecules, such as CD2, CD11a/CD18 (LFA-1), CD58 (LFA-3), CD44 (Pgp-1), CD54 (ICAM-1), and $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\beta 1$ (CD29) integrins (Table I). At first these two populations of T cells were proposed to represent functionally distinct "helper–inducer" and "suppressor–inducer" lineages (52), but then later it was sug-

TABLE I
FUNCTIONAL AND PHENOTYPIC PROPERTIES OF CD45RO$^+$, CD45RA$^+$, AND EFFECTOR/ACTIVATED T CELLS[a]

Feature	Effector/Activated	CD45RO	CD45RA
In vitro recall responses	+++	+++	+/−
Cell size	Large	Medium to small	Small
Expression of			
CD44, VLA-4,5,6, LFA-1, CD2, LFA-3, CD29	+++	+++	++
L-Selectin	−	+/−	+++
CD27	−	+/−	+
Fas	++	+/−	−
IL-2R, MHCII, ICAM-1	++	+/−	−
CD44 splice variants	+	−	−
CD26, CD69	++	+/−	−
Production of			
IL-2	+++	+++	+++
IL-4, IFN-γ, IL-3	+++	+	−

[a] See text for details and individual references. Some additional phenotypes for CD45RO$^+$ T cells are listed in Table II. For the sake of simplicity, some of the information listed is a generalization.

gested that these two subsets were maturation steps, representing naive (CD45RA) and memory (CD45RO) T cells (*49,53,54*). CD45RO$^+$ but not CD45RA$^+$ T cells responded to recall antigens *in vitro* (*49,54,55*). Activation of CD45RA$^+$ T cells with antigen or mitogens resulted in a phenotypic change to CD45RO$^+$, as well as increased expression of adhesion molecules, MHC class II, and IL-2 receptors (*49,53,54*). The phenotype of activated T cells resembled the CD45RO$^+$ "memory" phenotype, except that CD45RO$^+$ T cells were smaller than activated T cells, although larger than CD45RA$^+$ T cells (*49*). It was assumed that CD45RO$^+$ memory T cells developed from activated or effector T cells and were long-lived.

Studies in mice also supported the idea that memory T cells could be distinguished phenotypically. The first studies employed CD44 (Pgp-1), a ubiquitous molecule that functions in cell adhesion (*56*). Murine CD4 and CD8 T cells could be subdivided into CD44^{hi} and CD44^{lo} subsets. CD44^{hi} T cells were found to be antigen-activated/effector cells, or their progeny (*23*), and the increased frequency of antigen-reactive cells that occurs after immunization resided in the CD44^{hi} subset (*23,57*). Many of these cells were small and noncycling, thus fitting the profile of a memory T cell. Subsequently, mAbs to the CD45RB isoform were used to distinguish functionally distinct

subsets of murine T cells (*58–60*), and it appeared that the pattern of CD45 isoform expression in the mouse was similar to that in the human. In the rat, a monoclonal antibody to the CD45RC isoform was used to distinguish functionally distinct subsets of T cells (*61*), although a comparison between the functional data for the rat and the human revealed inconsistencies (*62*). Perhaps one reason for this was that different isoforms were used to distinguish subpopulations of T cells in the different species.

A strong argument against the assignment of CD45RO for memory and CD45RA for naive T cells has emerged following studies from three groups (*63–65*). In one, $CD4^+$ $CD45RC^-$ T cells were adoptively transferred to athymic recipients. After some months, these T cells converted to $CD45RC^+$ (*63*). Although the expression of the RC isoform on T cells in rats may not equate with T cell memory in the same way as does RA/RO expression on human T cells, this study suggested that T cells do interchange CD45 isoforms, and functional properties. In addition, studies in humans showed that T cell lines could reexpress the CD45RA isoform after a period of time in culture (*64,65*). It is noteworthy that the CD45RO isoform is expressed on cortical thymocytes, indicating that T cells convert from RO^+ to RA^+ during their development in the thymus. Moreover, one of the main proponents of the "one-way conversion" model (*49*) has recently concluded that CD45 isoforms are indeed interchangeable, based on the cell kinetics of $CD45RA^+$ and $CD45RO^+$ T cells (*66*) (see below). In sheep, we observed a small response by $CD45RA^+$ cells to a recall antigen *in vitro* compared with an unseen antigen (*67*), suggesting that some form of memory existed in this population. A study in mice also concluded that a component of T cell memory existed in the $CD45RA^+$ subset (*68*). It is therefore likely that CD45RO marks activated/effector T cells and their progeny, and these cells may then revert to $CD45RA^+$ resting T cells at a subsequent stage. Thus CD45RO expression may correlate more closely with cell activation or division, rather than with memory (*22*). $CD45RA^+$ T cells might contribute to memory responses, most likely through their increased frequency rather than through hyperresponsiveness (as pertains for $CD45RO^+$ T cells; see below). Perhaps CD45RA-type memory T cells are more important for pathogens or antigens that are encountered only rarely, since $CD45RO^+$ T cells might disappear through lack of restimulation. For a given antigen, the existence of both types of memory T cells may vary, and there may be continual interchange of CD45 isoforms on a T cell, depending on its recent experiences. Most studies have found memory al-

most exclusively within the CD45RO$^+$ subset. Accordingly, in this review CD45RO$^+$ T cells will be referred to as memory-type, despite the likelihood that a small component of memory resides in the CD45RA$^+$ subset.

In conclusion, CD45RO$^+$ T cells appear to be an intermediate stage between effector/activated T cells and resting CD45RA$^+$ T cells. Observations on T cell clones *in vitro* support this notion. Some T cell clones can be "rested" for periods of up to 2 months (*26*), even without exogenous IL-2, during which time they remain dormant but retain the capacity to proliferate if restimulated with antigen. Examination of the life span and cell cycle characteristics of circulating CD45RO T cells in humans and mice (Section V,A) suggests that they are analogous to dormant T cell clones. If CD45RO$^+$ T cells are not eventually restimulated, then they probably die off, or transform to CD45RA$^+$ T cells. It should be stressed that this scheme is an oversimplification, since T cell clones show considerable heterogeneity with regard to lymphokine secretion, surface phenotype, and dependence on exogenous interleukins (*69*). The differentiation of naive T cells to different types of effector cells is probably influenced by the milieu at the time of stimulation. For instance, different types of effector T helper cells (T_{H1}/T_{H2}) develop and secrete distinct sets of lymphokines. The uncertain lineage relationship of such T cell clones is discussed elsewhere (*69*).

B. Functional Differences Between Naive, Effector, and Memory T Cells

The functional heterogeneity of peripheral T cell subsets has a bearing on memory function. First, naive T cells, memory T cells, and various populations of effector T cells produce different types and amounts of lymphokines (Table I) (*69,70*). Studies from one laboratory showed that effector T helper cells generated after primary immunization were functionally distinct from those that arose from memory T helpers following a secondary challenge. "Primary effectors" secreted more IL-2 and IL-3 but less IL-4 than did "memory effectors" (*71*). These two effector populations also provided different helper activity to B cells, the former providing help for IgM and IgG_1 production and the latter providing help for all isotypes. This functional difference between primary effector T cells and secondary effector T cells resembles the situation for B cells, wherein primary effectors are IgM plasma cells and secondary effectors are IgG and IgA plasma cells.

The second functional difference between naive and memory-type

T cells is their sensitivity to antigen stimulation. Thus memory-type ($CD45RO^+$) T cells respond very well to antigen, whereas naive-type T cells respond less well. For instance, although naive and memory-type T cells express similar levels of TCRs on the cell surface, memory-type T cells can be stimulated with far less anti-CD3 mAbs (*72,73*) or anti-CD2 mAbs (*72*). Moreover, naive T cells do not respond as well as memory T cells to superantigens (*74*). Despite the apparent hyporesponsiveness of naive T cells, both naive and memory T cells respond well to mitogens such as phytohemagglutinin (PHA) (*72–74*). These studies led to the assumption that memory T cells can respond to lower amounts of antigen or can be triggered by lower affinity TCR interactions.

1. The TCR and Signal Transduction in Naive and Memory T Cells

The hyperresponsiveness of memory-type T cells is an important mechanism for the memory response. The functional differences between naive and memory-type T cells can be explained in two ways: (1) signal-transducing molecules such as CD45 function differently on the two cell types, and (2) costimulatory requirements for the two cell types differ. A variety of molecules participate in signal transduction, including the TCR/CD3 complex, CD4, and CD8, CD2, and CD45, and a number of other adhesion/activation molecules (*45,75,76*). The TCR, CD4, and CD8 are associated with the $p56^{lck}$ tyrosine kinase. Signal transduction by these antigen receptors or their coreceptors is significantly enhanced when two or more components are physically associated on the surface of the T cell (*75*). For instance, association of CD4 with the CD3/TCR complex results in a 100-fold reduction in the level of ligand required for T cell activation (*75*). CD45 appears to function in a similar way, by physically associating with the TCR/CD3 complex and transducing a signal to the T cell (*75*). The importance of CD45 in signal transduction was underscored by observations that mutant T cell lines that lack CD45 were rendered much less responsive to stimulation with antigen (*77*) and were defective in signal transduction through the TCR (*45*). It is likely that CD45 regulates the state of tyrosine phosphorylation of $p56^{lck}$ (*75*), and possibly other substrates.

The association of the TCR/CD3 complex and various other signal-transducing molecules is different on naive and memory-type T cells. This has been demonstrated by cocapping studies in both mice (*46*) and humans (*47*). On naive T cells, high-molecular-weight isoforms of CD45 migrate in the cell membrane independently of the TCR, whereas on memory-type T cells, the lower molecular weight iso-

forms of CD45 associate with the TCR complex or other coreceptors. Hence, it is possible that the external domains of the CD45 isoforms serve as ligands for other surface molecules such as CD4 or CD8. Interestingly, the majority of developing thymocytes express the CD45RO isoform, and Janeway (75) has speculated that associations between CD45RO and CD4 and the TCR may render these thymocytes hyperresponsive during positive or negative selection.

2. *Accessory Signals for Naive and Memory T Cells*

Molecules other than the TCR complex or CD45 assist in signal transduction. Many of these molecules serve as adhesion molecules, for instance, CD2, LFA-3, LFA-1, CD44, and CD29. Costimulating signals from these molecules appear to be critically important for naive T cell activation. One study (74) showed that particular combinations of costimuli, such as certain interleukins, or anti-CD28 or CD44 mAbs, rendered naive T cell stimulation as strong as that of memory T cells. It would thus appear that naive T cells have much more stringent requirements for activation. This relates to the cellular requirements for naive and memory T cell activation; "professional" antigen-presenting cells (APCs) such as dendritic cells or macrophages are required to activate naive T cells, whereas memory T cells can be activated by other types of APCs, such as B cells or $CD8^+$ cells (78). The stringent activation requirements of naive T cells may be a critical factor for peripheral tolerance (Section VII).

C. A Scheme for T Cell Memory

Figure 1 depicts a scheme that summarizes the likely maturation of T cells into effector cells and memory-type T cells after antigen stimulation. Newly produced naive T cells ($CD45RA^+$) are assumed to be functionally meek, and recognition of antigen with sufficient affinity and the right cosignals leads to activation and transformation to blast/effector cells with a distinct surface phenotype (Table I). These cells are clonally expanded, and in the absence of further stimulation they slowly adopt the appearance of small $CD45RO^+$ T cells. Such cells have phenotypic properties similar to blast/effector cells (Table I) but are intermediate in their transition back to the CD45RA phenotype. The expression of CD45RA denotes a truly resting state for T cells and a long life span (discussed below). Immunological memory by T cells is probably mediated both by the persistance of functionally potent $CD45RO^+$ effectors or by the smaller $CD45RO^+$ memory-type T cells, and by an increased frequency of long-lived, resting $CD45RA^+$ T cells. Moreover, transition of $CD45RO^+$ T cells to

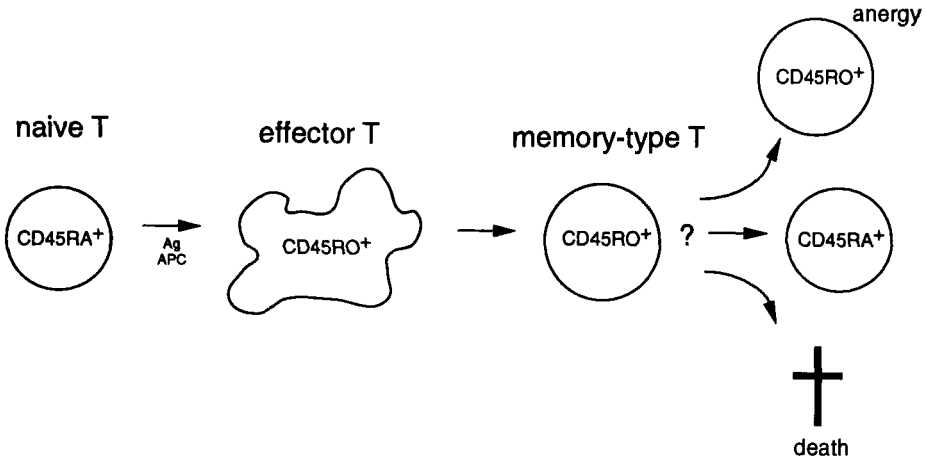

FIG. 1. Schematic representation of T cell differentiation following antigen stimulation. A more complete description of the phenotypes for each of the subsets is given in Table I. The most uncertain aspect of the model is the differentiation of memory-type T cells ($CD45RO^+$) to $CD45RA^+$ T cells, and the signals that are involved for this pathway. The model assumes that some memory-type T cells also die or become anergic (see Section VI). The scheme is an oversimplification, and cell death or anergy may also operate on cells other than memory-type T cells. It is likely that T cell memory is mediated by subsets in addition to memory-type T cells.

$CD45RA^+$ may be only one alternative. A proportion probably dies or enters into a state of anergy (discussed below). The signals that influence these differentiation steps are presently unknown.

IV. The B Cell Immune System and Immunological Memory

B cells and T cells harbor and manifest immunological memory in different ways. This relates to basic differences between the B cell and T cell immune systems: the B cell antigen receptor undergoes somatic mutation whereas the TCR does not. B cells are produced throughout life, whereas T cells are produced mostly during fetal and early life, and the life span of B and T cells is different. Effector B cells (plasma cells) produce antibody without target interaction, are terminally differentiated, and can be long-lived, in contrast to effector T cells. Also, the pathways that lead to the development of effector and memory cells appear to be fundamentally different for T cells and B cells.

Primary immunization with antigen induces low antibody titers,

mostly of the IgM type. A secondary challenge with the same antigen yields a quicker response, in which the antibodies are of other isotypes and of higher affinity. Thus the primary response serves to tool up the system, so that subsequent responses are qualitatively and quantitatively different. This results from the production and persistence of memory B cells expressing high-affinity receptors, particularly of the IgG isotype. A key site where the B cell responses to antigen occur is germinal centers, which develop in lymph nodes and spleen after antigen challenge. Germinal centers provide the microenvironment necessary for the differentiation of B cells into memory cells, for the switch in immunoglobulin isotypes on B cells, and for the selection of higher affinity mutants arising from somatic mutation of immunoglobulin variable region genes.

A. Germinal Centers and Their Role in Memory

The germinal center reaction is a complex interplay between stromal elements, cytokines, and B cells at various stages of differentiation. The germinal center is a particularly important site for the generation of memory B cells. In an experiment in which germinal center formation was inhibited by complement depletion, secondary antibody responses were severely reduced (*79*). Moreover, germinal center cells from primed animals, identified by their enhanced ability to bind peanut agglutinin (PNA), could transfer memory to naive recipients (*80*). The germinal center serves also as a major depot for antigen, which can be retained on FDCs for many months (discussed above). Germinal centers also provide a microenvironment conducive to the hypermutation process that operates on immunoglobulin variable region genes after antigen stimulation. Development of germinal centers appears to be T cell dependent, since athymic rodents lack germinal centers. The intricacies of the germinal center reaction will not be dealt with here, but rather their role in the development of memory, since informative reviews on germinal centers have been published recently (*81–85*).

What cells give rise to germinal centers? Although there is a degree of uncertainty about this (*83,86–88*), most of the evidence suggests that germinal centers can arise either from memory-type B cells or from recently primed naive B cells. Germinal center formation after primary challenge probably occurs after naive B cells are activated by antigen outside of follicles, probably in T cell areas where there is the necessary T cell help and antigen-presenting cells. This is suggested by the fact that during the early stage of the primary response, antigen-specific B cells proliferate in the T cell zone (*81,89*). These cells must

migrate to follicles to initiate the germinal center reaction. The use of anti-IgD to determine the nature of germinal center precursor cells has yielded conflicting results (*83,86,90*); however, it is likely that $\mu^+\delta^+$ naive B cells stimulated by antigen in T cell zones transform to $\mu^+\delta^-$ (*91*). Studies with the J11D marker, which discriminates between functional subsets of B cells (see below), indicated that J11D^{lo} (memory) B cells but not J11D^{hi} (naive) B cells generated germinal centers following transfer to adoptive hosts (*88*). When memory and naive B cells were transferred to recipients that had antigen already localized on FDCs, the memory cells responded whereas the naive cells did not (*92*).

Once in the germinal center, B cells are subjected to two important processes—the hypermutation and selection of high-affinity B cell clones and the generation of memory B cells. These processes occur in distinct compartments of the germinal center. These compartments can be distinguished, based on the phenotypes of B cells and the type of FDC in each region (*81*). By histological staining there is a dark zone of densely packed cells and a light zone of loosely packed cells. Overlying these is the follicular mantle, comprising small, resting, sIg$^+$ recirculating B cells. A scheme of events proposed to account for the division, movement, and differentiation of B cells within these different regions (*81*) will be recounted here in brief. Primary B cell blasts localize initially in the dark zone; these cells are sIg$^-$, rapidly dividing, and have been termed centroblasts. These cells give rise to progeny termed centrocytes that are nondividing, sIg$^+$, and migrate to the light zone of the germinal center. The centrocytes represent a labile population of cells that is continually replenished by the rapidly proliferating centroblasts. As discussed below, the process of hypermutation of immunoglobulin variable region genes probably occurs at the centroblast stage or earlier, and not at the centrocyte stage (*81,93*). It has been proposed that antigen on FDCs within the light zone selects those centrocytes that have accumulated appropriate somatic mutations in their immunoglobulin variable region genes, such that their immunoglobulin has a high affinity for antigen. The interaction of centrocytes with antigen localized on FDCs may be necessary to prevent cells from entering a default program of cell death through apoptosis (*94*), and in this regard, the *bcl-2* gene product appears to play an important role (Section V,B).

Different signals appear to drive germinal center cells along different pathways of differentiation, resulting, for instance, in the production of plasmablasts or small memory B cells. Activation of centrocytes with anti-CD40 antibody induces these cells to stop cycling and ac-

quire the physical and phenotypic properties of small B cells (*94*). Anti-CD40 also induces bcl-2, which most likely protects centrocytes from apoptosis. Another pathway of differentiation is induced by a combination of signals, including IL-1 and interaction of B cell CD23 with its ligand. This combination of signals turns centrocytes into cells resembling plasmablasts—they express cytoplasmic immunoglobulin and bcl-2, and have developed endoplasmic reticulum (*95*). IL-2 is yet another signal that is involved in centrocyte differentiation. IL-2-stimulated cells also differentiate to plasmablasts, but these cells are distinct from CD23/IL-1-derived plasmablasts in that they show little expression of bcl-2 (*81*). These signals may occur within different microenvironments of the light zone of germinal centers. FDCs in the apical region of the light zone express high levels of CD23, whereas those in the basal region are essentially $CD23^-$ (*81*).

B. Affinity Maturation

The affinity of immunoglobulin for an antigen increases during the course of an immune response (reviewed in Ref. *96*) and, moreover, secondary responses typically produce antibodies with an affinity higher than that seen in the primary response. Two processes account for this. The first is the clonal selection of antigen-reactive cells by antigen (*3*). As the concentration of antigen decreases during the course of the response, only B cells with high-affinity receptors are selected and expanded. The second and perhaps more important process is somatic hypermutation, which operates on immunoglobulin variable region genes and which introduces point mutations mainly into the complementarity-determining regions of the immunoglobulin combining site. The frequency of these mutations is extraordinarily high after antigen stimulation, on the order of 10^{-3} per base pair per generation. As discussed above, germinal centers are the key site where somatic mutation occurs (*82,97*) and a model proposed to explain the selection of high-affinity mutants within germinal centers (*92*) holds that when centrocytes come out of cell cycle and reexpress somatically mutated surface Ig, they must successfully compete for antigen bound as immune complexes on FDCs. In this way, only those clones that have a higher affinity for antigen than that of the preexisting antibody on FDCs will be selected. The mechanism of somatic mutation is unknown, but it is T cell dependent. Once the memory B cell population has been generated, further division of B cells outside of germinal centers probably no longer involves somatic mutation (*98*), although this has not been established definitively.

Antibodies produced early in the primary response are free of muta-

tions; the hypermutation process operates on those cells that will eventually give rise to memory B cells (*99,100*). Thus the following events most likely occur when naive B cells are stimulated with a T-dependent antigen. Some B cells are driven to form antibody-secreting cells, and the immunoglobulin genes of these cells are free of mutations. In parallel, other B cells are driven into the memory pathway; these cells switch their class of immunoglobulin and incorporate mutations in their V region genes. This occurs within the confines of the germinal center, and at some stage small resting memory B cells are produced. After a second stimulation by antigen, memory B cells are driven to antibody secretion, resulting in the production of high-affinity antibody. It is uncertain whether the hypermutation process is reactivated during secondary and subsequent responses, since one study showed that it was (*101*), while other studies concluded it was not (*98*).

C. Phenotype of Memory B Cells

A degree of uncertainty surrounds the true nature of memory B cells, just as it does for memory T cells. A variety of surface markers is differentially expressed on various subsets of B cells, but it appears that expression of any one surface molecule is not definitive for the distinction of memory B cells. Moreover, it could be that memory is a function of more than one B cell subset (*92*) and might be maintained by more than one mechanism. The best hallmark of a memory B cell may well be a somatically mutated immunoglobulin gene, since germinal center formation and affinity maturation appear to be consistent features of secondary responses. B cells are continually produced by the bone marrow, and a source of confusion centers on the fact that small proportions of these that enter the peripheral pool are apparently selected by antigen (*93,102*). Recognition of antigen at this stage is part of a developmental process and does not lead to class switching, somatic mutation, or antibody secretion. Because antigen shapes the peripheral repertoire, it is possible that a component of memory, albeit short-lived, results from this selection process.

The phenotype of memory B cells has been assessed in a number of ways. In some experiments, B cells were separated into subsets based on certain markers, which were assessed for their capacity to transfer memory to adoptive hosts or for the presence of somatic mutations within immunoglobulin variable region genes. Recently, memory B cells have also been identified by their ability to bind fluorescently labeled antigen with high affinity.

1. *Immunoglobulin Isotypes*

The majority of peripheral B cells in the mouse coexpress IgM and IgD on the cell surface ($\mu^{\delta}\delta^{+}$). Following an immune response to a T-dependent antigen, most antigen-stimulated B cells switch from the expression of IgM and IgD to other isotypes (*103,104*), and such B cells usually constitute only a minor fraction of the total B cell pool. By adoptive transfer, a number of studies have shown that B cell memory was carried predominantly by the δ^{-} B cell subset (*105,106*). More recently, the fluorochrome phycoerythrin (PE) has been used as an antigen. PE-binding memory B cells could be identified in spleens from PE-primed mice; these cells produced strong IgG_1 anti-PE responses *in vitro*, and phenotypically were almost always $\mu^{-}\delta^{-}$ (*24,104*). These PE-binding memory B cells also expressed lower levels of immunoglobulin light chain compared with naive B cells. Similar studies using a fluorescently labeled antigen (*98*) also showed that antigen-binding memory B cells were IgG^{+} and had incorporated somatic mutations in their immunoglobulin variable region genes. In a series of experiments (*102,107*), B cells from normal mice were separated into $\mu^{+}\delta^{+}$ and $\mu^{-}\delta^{-}$ populations and the immunoglobulin heavy chain variable region genes were analyzed. In most cases, the immunoglobulin genes of $\mu^{-}\delta^{-}$ B cells had incorporated somatic mutations. Nevertheless, very small numbers of $\mu^{+}\delta^{+}$ B cells also contained variable region genes with somatic mutations (*102*). Moreover, studies by another group reported that memory B cells could be recruited from both the δ^{-} and the δ^{+} pool (*108*). Most likely, the vast majority of memory B cells are $\mu^{-}\delta^{-}$, although some might be $\mu^{+}\delta^{+}$ or $\mu^{+}\delta^{-}$.

2. *Other Surface Markers*

A marker that has been used extensively to distinguish subsets of T and B cells is the heat-stable antigen (HSA), recognized by the mAb J11D (*109*). Most naive B cells express high levels of HSA, whereas memory B cells express low levels (*28,88,110,111*). This was shown by separating murine B cells into $J11D^{hi}$ and $J11D^{lo}$ subsets and transferring them to syngeneic hosts: antigen-specific $J11D^{lo}$ B cells transferred a memory response, whereas $J11D^{hi}$ cells did not (*28,88,110,111*). Somatic mutations were found only in the immunoglobulin variable region genes of $J11D^{lo}$ B cells (*28*). Also, SCID mice, which do not normally develop germinal centers, formed germinal centers after transfer of $J11D^{lo}$ B cells but not $J11D^{hi}$ B cells (*88*).

Other surface markers that appear to distinguish functional subsets of B cells are the same as those that are differentially expressed on T

cells. This is because the same molecule may serve an activation or an adhesion function on both cell types, which correlates with naive and memory status. Thus CD44 is reported to be up-regulated on memory B cells (*112*). Other studies found that germinal center cells could transfer memory to adoptive hosts (these cells are L-selectin$^-$, PNA$^+$). As discussed above, germinal center cells might harbor precursor cells that differentiate to memory B cells, and at some point these cells revert to PNA$^-$ and reexpress L-selectin (*113*).

D. Functional Properties of Memory B Cells

Like memory T cells, memory B cells appear to be functionally more potent than naive B cells. Memory B cells proliferate in response to lower amounts of antigen and require fewer carrier-primed T helper cells (*114*). Consequently, they can be stimulated more easily by cross-reactive antigens (*115*). However, memory B cells may be less responsive to polyclonal activation with mitogens than are naive B cells. There is also evidence that primary and secondary B cell responses have different requirements for lymphokines such as IL-6 (*116*). As discussed above, it appears that naive B cells must be activated in the extrafollicular areas of lymphoid tissue where there is the appropriate T cell help. In contrast, memory B cells can respond to antigen localized on FDCs within germinal centers (*93*).

E. Schemes for the Generation of Memory B Cells

As evident from the above discussion, the identity and precursor–product relationships of naive and memory B cells remain uncertain. There is general agreement that newly produced, naive B cells are $\mu^+\delta^+$ and have no somatic mutations (although some mammals may use somatic mutation to diversify the primary repertoire) (*117*). At some point, the B cell lineage diverges into cells that are destined to produce antibody and cells that survive as memory cells. The discussion to date has assumed that commitment to either of these pathways occurs after antigen challenge, and is influenced by microenvironmental signals. A scheme depicting this possibility is shown in Fig. 2A. However, another possibility, promoted by Linton and Klinman (*28,115*), is that precursor B cells exist that are precommitted to give rise to memory cells, or primary antibody-forming cells, at a stage before being stimulated by antigen (see Fig. 2B). Linton and Klinman identified two precursor cell subpopulations in the spleen cells of nonimmune mice: a J11D^{hi} subpopulation that, on T cell-dependent antigenic stimulation, gave rise to primary antibody-forming cells, but not secondary B cells. In contrast, J11D^{lo} B cells failed to generate

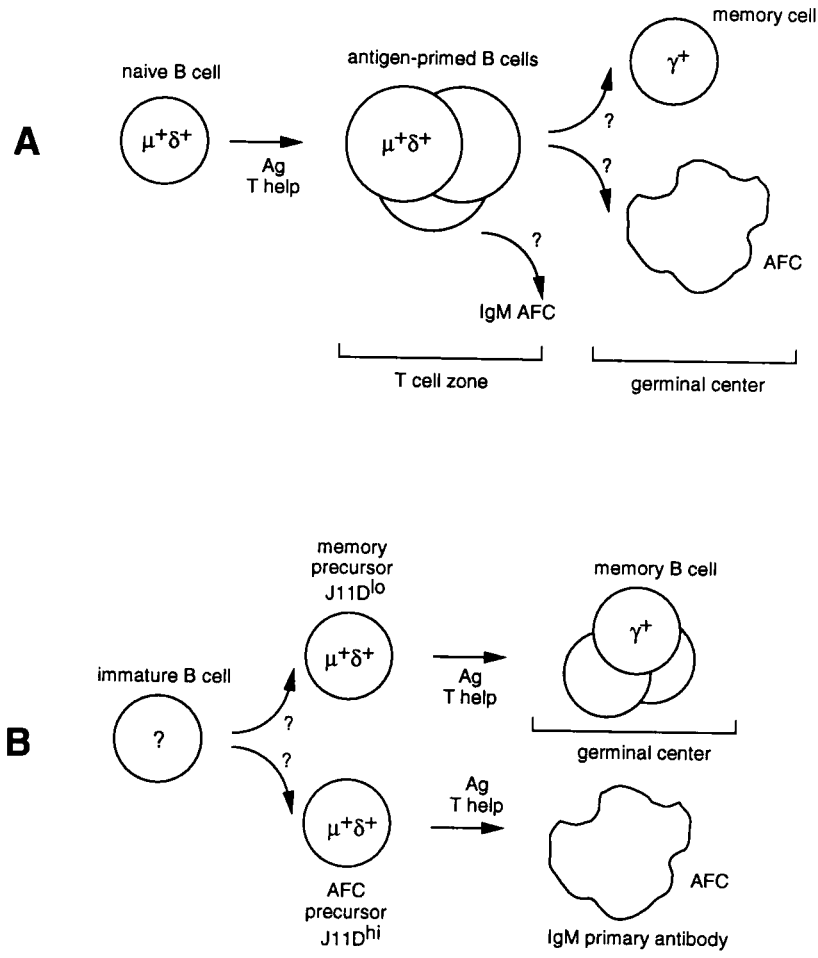

FIG. 2. Alternative models for the development of memory B cells. The fundamental difference between the two models is the point at which commitment to the memory lineage or antibody-forming cell (AFC) lineage occurs. Model A assumes that commitment occurs after antigen stimulation, whereas model B assumes that the memory B lineage and the primary AFC lineage are established at a point before antigen encounter. These diagrams are oversimplified, purely to illustrate the basic differences between the two models.

antibody-forming cells but gave rise to memory-type B cells (*28,115*). In addition, only the J11D^{lo} population was enriched for cells that produced germinal centers (*88*) and that accumulated somatic mutations in their immunoglobulin variable region genes (*28*). It is un-

known what determines the commitment of immature B cells to the memory lineage in this model. It should be stressed, however, that strong evidence also exists for the contrary model, depicted in Fig. 2B. A recent study (*117a*) assessed the clonal origin of antigen specific germinal center cells and foci of primary APCs situated outside of the lymphoid follicles. Similar sequences within CDR3 established a clonal relatedness between germinal center B cells and primary APCs, indicating the two populations were derived from a single B cell activated by antigen. Determining which model, A or B in Fig. 2, most closely resembles the true situation will require a more complete knowledge of B cell precursor–product relationships. The contribution of persistent antigenic stimulation to B cell memory has been omitted from the schemes in Fig. 2, in view of the uncertainty as to if, and at which point, persistent antigen operates to maintain memory.

In summary, therefore, memory B cells are generated following T-dependent antigen activation, mostly or exclusively in germinal centers, and that such cells have somatically mutated immunoglobulin mainly of the IgG type. The persistence of other cell types and their contribution to memory remain speculative.

V. The Life Span of T and B Cells and Relevance to Memory

An understanding of T and B cell life span is essential for discriminating between the different models for memory persistence. Life span can be considered in two different ways, the most straightforward being the intermitotic period of the cell. However, a clone of dividing cells can be considered to have a life span, by virtue of its survival and persistence. Here, life span will refer to intermitotic period. [More detailed considerations of lymphocyte life span are discussed elsewhere (*118,118a*).]

A. Intermitotic Life Span of T and B Cells

The immune system continuously produces and loses cells, but maintains a steady state. Peripheral lymphocytes are capable of self-renewal and the division rate of some cells may relate to the need to maintain or control the number of cells in the periphery. Primary lymphoid organs, the thymus and the bone marrow, produce large numbers of T and B cells, hence one has to distinguish between cell division that occurs during the primary generation of lymphocytes, and that which occurs within the peripheral pool.

Not surprisingly, studies over the years on the intermitotic life span of lymphocytes have yielded widely disparate results. The consensus

view now favors a relatively long life span for most T cells and a somewhat shorter life span for B cells. One of the first indications for this came from studies with patients that had been exposed to ionizing radiations for therapeutic reasons (*119,120*). Radiation produces acentric chromosomal lesions in lymphocytes; such lesions results in cell death during mitosis. Hence, persistence of such cells over time gives a measure of lymphocyte turnover. One such study showed an average lymphocyte life span of 530 days (*119*) and another around 3 years (*120*). The significance of life span to immunological memory was not lost on these authors. Buckton *et al.* (*120*) found a small percentage of lymphocytes with dicentric lesions for as long as 10 years after radiotherapy, and surmised that the lymphocyte mediated immunological memory through its potential for long-term survival *in vivo*.

Lymphocyte life span has been studied in rodents using DNA precursors to label dividing cells. Early studies using tritiated thymidine showed that T cells in the thoracic duct lymph of mice had an average life span of 4–6 months, whereas B cells had a shorter life span, ~5–7 weeks (*121*). Later studies using the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) found that B cells in rats had an average life span of about 4 weeks (*122*). B cell life span is complicated by the fact that the bone marrow produces B cells continuously throughout life. This production is substantial, sufficient to replace the peripheral B cell pool every 4–5 days (*123*). However, the majority of these newly produced B cells have a life span of only 3–4 days; to join the peripheral pool as longer lived cells, they need to be selected, possibly by antigen (*93,102*). A study in mice found that more than two-thirds of splenic B cells in the adult mouse had a life span of several weeks or months, whereas a more rapid turnover took place in young (4-week-old) mice (*124*). It was concluded that the peripheral B cell pool, once having been built up early in life, is only slowly renewed.

Not all studies have found a long life span for peripheral lymphocytes. Drugs that selectively kill cycling cells, such as hydroxyurea or radioactive strontium, led to a depletion of 50% of peripheral lymphocytes within 3 days of treatment (reviewed in Ref. *125*). Lymphocyte life spans were also studied by transferring cells into adoptive hosts and monitoring their disappearance; B cells were found to disappear progressively, with only 10–40% persisting for >7 days after transfer (*126*). In addition, adult thymectomy in mice led to a large reduction of naive T cell numbers (*70*), suggesting that these cells had a short life span. Thus, the conclusion to be drawn from the studies on cell persistence and the cell arrest was that most peripheral lymphocytes had a very short life span (*125*). In addition, one group using BrdU found that B cells and T cells in mice had a high turnover rate, with ~30–40%

renewal every 48 hr (*127*). Most of the B cell turnover was accounted for by production of B cells within the bone marrow, whereas T cell turnover resulted from cell division in the periphery.

The reason for the discrepancy in estimates of lymphocyte life span between groups is unclear. The majority of studies, including our own (*67*), have demonstrated an average life span for T cells of the order of months or years, and a life span for B cells of the order of several weeks or months. However, the peripheral population of T and B cells is heterogeneous with respect to life span, and distinguishing minor populations of long-lived cells is difficult. Referring to the life span as an average can be misleading.

The central question is whether immunological memory resides within the population of long-lived lymphocytes, or in a small population that is turning over? In one of the first studies to address this issue (*67*), the life span of $CD45R^-$ T cells (memory phenotype) was compared with that of $CD45R^+$ T cells, using sheep and BrdU labeling. A sizeable proportion of $CD45R^-$ memory-type T cells had a high turnover rate, whereas the $CD45R^+$ (naive) T cells had a very slow turnover rate. This result has been confirmed by recent studies in humans. The technique of monitoring the disappearance of cells with dicentric lesions after radiotherapy was used in conjunction with the naive and memory T cell markers CD45RA and CD45RO. $CD45RO^+$ T cells with dicentric chromosomal lesions disappeared much faster than did similarly affected $CD45RA^+$ T cells, indicating that $CD45RO^+$ T cells had a relatively short life span whereas CD45RA T cells had a long life span (*66*). Also, murine T cells expressing the memory phenotype ($CD45RB^-$, $CD44^{hi}$) possessed features of activated cells and appeared to be maintained in the G_1 stage of the cell cycle, although they were not actively cycling (*128*). When T and B cells from normal mice were transferred to SCID mice, many retained a naive phenotype and persisted for long periods, apparently without cell division (*129*). So, in conclusion, it appears that most if not all memory phenotype T cells have a short to medium life span (weeks or months), whereas naive T cells have a long life span (months to years). Assessing the life span of these two populations is complicated by the possibility that there may be interconversion between memory-type T cells (CD45RO) and naive-type T cells (Fig. 1).

A careful study of the life span of memory B cells in mice by Schittek and Rajewsky (*24*) concluded that memory B cells were a long-lived population. They used the fluorochrome PE as an antigen and as a means of detecting antigen-specific B cells, and BrdU to label dividing cells. Using a number of experimental regimens, they found that at 9–10 weeks or at 20 weeks after priming, PE-specific memory B cells

were mostly nondividing cells (life span > 7 weeks). Gray (29) contends that the small amount of cell division observed by Schittek and Rajewsky in the memory B cell population at 20 weeks after priming is evidence that there was in fact an ongoing stimulation of these memory B cells. Currently, it is difficult to reconcile the two sets of apparently conflicting experimental findings, i.e., the long life span of most memory B cells and the apparent need for a continued presence of antigen to maintain B cell memory. An explanation for this paradox might be a requirement by memory B cells for persistent antigenic stimulation, without concomitant cell division, although this seems unlikely.

B. *bcl-2*, Fas, AND IMMUNOLOGICAL MEMORY

The *bcl-2* oncogene was discovered as a result of its translocation to the immunoglobulin heavy chain locus in certain B cell lymphomas (reviewed in Ref. *130*). The *bcl-2* gene encodes for a 26-kDa protein (bcl-2) that associates with the inner membrane of the mitochondrion; expression of *bcl-2* enhances the survival of many cell types, including both B and T lymphocytes, apparently by preventing programmed cell death (apoptosis) (*131–133*). Transgenic mice expressing a *bcl-2* gene subjugated to an immunoglobulin enhancer contain a large excess of B cells with enhanced survival capacity. T cells from the same mice showed prolonged survival under adverse conditions, indicating that such cells were more resistant to cell death compared with normal T cells (*133*). The *bcl-2* gene appears, therefore, to be part of the machinery that is involved with signals that regulate the survival and longevity of cells. The role of *bcl-2* in B cell memory was assessed by comparing the ability of antigen-primed B cells from normal mice and *bcl-2* transgenic mice to transfer memory to adoptive hosts (*21*). The half-life of transferred memory B cells from normal mice was remarkably short, in agreement with other studies (*13,14,19,134*) (see Section II,A), whereas memory B cells from *bcl-2* transgenic mice persisted much longer (*21*). It was suggested that antigen-induced reactivation of bcl-2 was necessary for the maintenance of B cell memory (*21*). Within the B lineage, the bcl-2 product is normally expressed in pre-B cells, is quiescent in resting B cells, and is expressed again in activated B cells (*135*).

The Fas antigen/APO-1 is another molecule that plays a role in apoptosis. This molecule belongs to a family of cell surface proteins that includes TNF receptor, CD40, and nerve growth factor receptor (*136*). Within the lymphoid system, Fas antigen/APO-1 is restricted in its expression to activated T and B cells, as well as to cells that harbor memory such as $CD45RO^+$ T cells and $sIgD^-$ B cells (*137*). The regulation of the bcl-2, Fas/APO-1, and other apoptosis-related molecules

seems therefore to be important for immunological memory, by promoting the survival of appropriately selected antigen-reactive cells.

VI. The Migration and Localization of Naive and Memory Cells

A. The Importance of Lymphocyte Migration to Immunological Memory

Lymphocyte migration is a fundamental feature of the immune system that permits the repertoire of immune specificities to be very large, and consequently for each immune specificity to exist at a very low frequency. Another important function of the lymphatic system is to disseminate antigen-experienced memory cells to all regions of the body, following an immune response in a single location. Thus lymphoid and lymphatic systems are organized so as to collect antigen draining from sites of challenge, to capture and store that antigen, to respond to it, and to liberate antigen-experienced cells to the circulation via the lymph. Lymphoid tissue is placed throughout the body in a highly strategic manner, so as to maximize the capture of antigens penetrating the body's surface. The importance of the lymphatic system for the dissemination of memory was demonstrated by removing the lymphocytes emigrating from an antigen-challenged lymph node of a sheep, through an indwelling cannula in the efferent lymphatic duct. Systemic memory was thereby abrogated (*138*). Also, memory can be transferred from one animal to another with thoracic duct lymphocytes (15,134,139). These studies led to the widely held belief that memory was contained within a long-lived population of cells that recirculated from blood to lymph nodes. This concept has been reappraised because of recent findings on the distribution and migration of phenotypically defined subsets of T and B cells.

B. The Migration Pattern of Naive T Cells, Effector T Cells, and Memory T Cells

1. Basic Migration of Naive, Effector, and Memory T Cells

Lymphocytes recirculate mostly by crossing high endothelial venules (HEVs) of secondary lymphoid tissue. A small number also leave the blood in peripheral vascular beds by crossing normal or inflamed endothelium, whereupon they drain to lymph nodes via the afferent lymph. Lymphocytes leave lymph nodes through the efferent lymph ducts and eventually drain to the thoracic duct and then back to the blood. A large number of lymphocytes also leave the blood by crossing endothelium within the spleen; however, these lymphocytes migrate directly back into the blood.

Over the past few years, a great deal of evidence has emerged to indicate that naive, effector, and memory cells migrate preferentially through different tissues, and this results from the differential expression of certain adhesion molecules on lymphocytes and on endothelium (*22,51,140*). An analysis of lymph cells draining from normal skin in sheep revealed that the T cells were entirely $CD45RO^+$, $adhesion^{hi}$, i.e., memory type. In contrast, the T cells within efferent lymph, the majority of which derive from the blood via HEVs, were mostly of the naive phenotype (*51,67*). It appears that a proportion of circulating $CD45RO^+$ T cells are capable of crossing lymph node HEVs (*141*). The recirculating cells in the thoracic duct lymph of mice were also found to be mostly the naive type (J. Sprent, personal communication). The above observations has led to a revised model of naive and memory T cell homing (*22,51*), which holds that naive T cells traffic mostly through lymphoid tissue by crossing HEVs, whereas memory-type T cells traffic preferentially through tissues and inflammatory sites and reenter the lymph stream by way of the afferent lymph. The pattern of naive or memory T cell traffic through the spleen has not been reported.

A wealth of evidence supports the above model. Naive T cells newly emerged from the thymus express L-selectin ("the lymph node homing receptor") and immediately recirculate by crossing HEVs (*142*). In the sheep fetus, which is free of antigen, there is extensive recirculation of naive T cells through lymph nodes and Peyer's patches from an early stage of gestation (*143*). In humans, naive-type (CD45RA) T cells are rarely seen in nonlymphoid tissues, such as the skin, the lung, and the lamina propria of the gut. On the other hand, $CD45RO^+$ effector/memory T cells are the predominant cell type within inflammatory lesions (*144*) and at epithelial surfaces.

Before the advent of surface markers for T cell subsets, memory was assessed by functional criteria. One early study showed that T cell memory preferentially resided in peripheral tissues rather than in lymph nodes (*145*), and this conclusion has been arrived at again in recent experiments (*146*). Studies in rats in the early 1970s (*147*) analyzed the function and migratory properties of short-lived and long-lived lymphocytes. These studies showed that the cells that migrated to inflammatory lesions and provided protection against an immunizing antigen were short-lived cells; long-lived cells migrated mostly from blood to lymph nodes. The authors concluded that the localization of "committed" lymphocytes to inflammatory sites enabled the host to focus its cellular defenses at sites of bacterial challenge. In another study, the recirculating pool of lymphocytes in rats was depleted by chronic drainage of the thoracic duct (*148*). This resulted in a

severe depression of primary antibody responses but normal secondary responses.

It has been suggested that the migration patterns observed for $CD45RO^+$ and RA^+ T cells in sheep and other species were those of effector T cells and resting T cells, respectively. As outlined above, effector T and memory-type cells are closely related cell types. However $CD45RO^+$ T cells are physically small and comprise a large proportion of the peripheral pool in old individuals. The memory-type T cells in sheep afferent lymph were small cells, only fractionally larger than naive-type T cells (*149*). However, many of these cells were recently divided (*67*), but in this respect they were probably not so different from memory-type T cells from other tissues. In humans, a high proportion of T cells that adhered to cultured endothelium *in vitro* were $CD45RO^+$ and recently activated (*150*), and so it is possible that the most activated portion of $CD45RO^+$ T cells binds to normal endothelium. When T cells were activated *in vitro* and injected into syngeneic recipients, they were found to migrate mostly to the gut and the spleen and not to lymph nodes (*151*) (see also below).

The recirculation of large numbers of naive T cells through lymphoid organs serves to increase the likelihood of these cells encountering their cognate antigen. Naive or resting T cells seem to have very stringent activation requirements, probably best achieved in lymphoid tissue. However, antigen-primed effector T cells and memory-type T cells serve their function best at sites of likely antigenic encounter, such as epithelial surfaces, enabling an immediate response to recall antigens. Epithelial surfaces are not designed for massive lymphocyte recirculation, so that the surveillance of these tissues is best served by memory-type T cells, which can provide protection against previously encountered, and possibly frequently encountered, antigen.

2. *Tissue Tropic Subsets of Effector Cells and Memory Cells*

A proportion of circulating lymphocytes home preferentially to certain tissues. This preferential homing was demonstrated originally for the gut (*151–156*) and the skin (*157,158*), but may also exist for the lung (*159*), inflamed synovium (*160*), and other tissues. Studies in both rodents and sheep showed that lymphocytes from the gut migrated preferentially back to the gut, whereas cells draining from the skin or from lymph nodes likewise migrated preferentially back to the skin or lymph nodes (*154,156–158*). These *in vivo* studies were supported by the Stamper Woodruff frozen section assay, which showed that lymphoblasts from mesenteric lymph nodes bound to gut-associated HEVs much better than to HEVs in peripheral lymph nodes (*161*). Certain lymphoma cell lines likewise showed a similar

tissue selectivity (*156,161*). The concept of tissue-specific migration by lymphocytes was also supported by very convincing evidence from another field, that of tumor metastasis. Some tumors metastasize in a highly tissue-specific manner, presumably because they express surface structures that enable them to bind to endothelium in particular tissues. Indeed, many of the cell adhesion molecules of epithelia and other cell types are also expressed on leukocytes.

An association between tissue-specific migration of lymphocytes and immunological memory was proposed several years ago (*162*), and firm evidence for this association has emerged over the last years (*22,51,140,163–165*). The early experiments showed that T cell blasts were the subset that migrated selectively to tissues such as the gut (*152,153,156,166,167*). As for small T cells, some reports found no tissue bias (*166,167*) whereas others did (*154,156*). It now appears that the T cells that exhibit a tissue tropism are either recently stimulated cells or smaller memory-type T cells (*163,165*). In the sheep, a T cell subset that migrated preferentially back to the gut had a memory phenotype, but was phenotypically distinct from memory T cells draining from the skin (*163*). This finding was supported by the fact that the gut-specific pathway was not observed until after birth (*168*). In humans, tissue-specific subsets of T cells have been identified by their expression of particular adhesion molecules. A skin-tropic subset expresses a molecule termed cutaneous lymphocyte-associated (CLA) molecule. CLA^+ T cells are almost always of memory phenotype (*169*). Moreover, a population of gut-associated T cells in humans that expressed a novel α-integrin in association with $\beta 7$ (recognized by mAb HML-1) was also of memory phenotype (*169*). A population of gut-associated lymphocytes in mice expresses $\alpha 4\beta 7$, suggesting that $\beta 7$ expression somehow confers a gut-homing tendency. A summary of these memory-type T cell subsets and their homing preferences is outlined in Table II.

Because $\alpha\beta$ T cells that home specifically to tissues such as the gut or the skin are antigen experienced, then presumably tissue tropisms are learned during a primary immune response, possibly under the influence of particular cytokines. This imprinting presumably occurs in organized lymphoid tissue such as lymph nodes or Peyer's patches, since this is where primary immune responses usually develop. Presumably gut-associated and skin-associated lymph nodes provide different environments for T cell activation. Studies with the skin-homing molecule CLA suggest that it is preferentially induced on memory T cells in skin-associated lymph nodes, possibly by the cytokine TGF-β (*170*). Another possibility is that memory-type T cells learn a tissue preference at a subsequent stage, possibly as a

TABLE II
TISSUE-SPECIFIC MIGRATION PATTERNS BY SUBSETS OF NAIVE AND MEMORY $\alpha\beta$ T CELLS[a]

Lymphocyte Subset	Predominant Migration Pattern	L-Selectin Expression	Molecular Interaction for Homing	
			Receptor	Ligand
Naive T ($CD45RA^+$)	Lymph nodes	+++	L-Selectin	PNAd
Memory T ($CD45RO^+$), ubiquitous type	Numerous tissues, inflammatory lesions	+/−	$\alpha4\beta1$ LFA-1	VCAM-1 ICAM-1
Memory T ($CD45RO^+$), tissue-tropic subsets				
CLA^+	Skin	+/−	CLA	E-Selectin
αMLA$\beta7$	Gut	−	$\alpha\chi\beta7$	?
$\alpha4\beta7$	Gut	−	$\alpha4\beta7$	MadCAM-1
?	Joints	?		?
?	Lung	?		?

[a] Information is a summary of findings from studies in humans, mice, and sheep. Activated/effector T cells may show properties similar to those listed for memory T cells.

consequence of further stimulation in a particular tissue. It is likely that only a proportion of effector and memory T cells migrate in a tissue-selective way and the majority probably show a nonspecific distribution. $\alpha\beta$ T cells appear to differ from $\gamma\delta$ T cells in that $\gamma\delta$ T cells colonize distinct epithelia during fetal ontogeny as part of a developmental program, rather than as a result of an antigenic experience (*171*).

In conclusion, it appears that naive T cells migrate in a random fashion through lymphoid tissue. Following antigen stimulation, some lymphoblasts up-regulate tissue-tropic adhesion molecules, which endows them with tissue-tropic homing capacity. Populations of tissue-homing effector or memory T cells probably represent a rationalization of immune resources, to enable cells to migrate to the tissues where they are most likely to reencounter their priming antigen (*22*). In a sense, tissue-specific homing is a mechanism for achieving a type of memory that is focused, and probably evolved in

response to the tissue-specific localization shown by many pathogens. However, lymphocytes that show certain tissue preferences are still capable of migrating to other tissues.

C. Migration of Naive B Cells, Memory B Cells, and Plasma Cells

The homing pattern of naive and memory B cells has become more difficult to access because the phenotype of these two populations is less well defined than that of naive and memory T cells. Nearly all recirculating B cells are $\mu^+\delta^+$, and these cells migrate from blood to lymphoid tissue and to lymph. Naive B cells in prenatal animals are $\mu^+\delta^+$ and adopt this pathway. These cells express L-selectin and readily cross lymph node HEVs. These cells also migrate well through Peyer's patches and the spleen (*139*), although there has been the widespread belief that B cells recirculated more sluggishly than T cells (*139*). The primary follicles of spleen and lymph nodes are composed almost exclusively of the $\mu^+\delta^+$ recirculating B cells.

The migration of naive and memory B cells has been inferred from results of transfer experiments. Strober and Dilley (*15,134*) found that naive B cells could not be recovered from the thoracic duct lymph of an intermediate recipient after transfer. In contrast, B cells from primed donors did recirculate, as evidenced by the large secondary response after transfer of thoracic duct lymph to adoptive hosts. Observations on the life span of naive and memory B cells by Strober and Dilley (*15*) led to the general belief that naive cells were a short-lived, nonrecirculating population.

The experiments by Gray (*19*) and others (*21*), which showed that B cell memory transferred to adoptive hosts decayed rapidly, used thoracic duct lymph as a source of memory B cells. They concluded that B cell memory was strictly dependent on antigen for its persistence, although another possibility could be that the types of memory B cells isolated from the thoracic duct were not representative of the memory pool. Earlier experiments had suggested that the cells responsible for B cell memory were underrepresented in the recirculating pool (*148*), which led to the notion that although memory was disseminated throughout the body by the lymphatic system, a large fraction of memory depended on sessile cells (*139*). In summary, the recirculating pool of B cells is composed mostly of naive B cells and a population of memory B cells of uncertain nature.

During a secondary response, the majority of antibody produced comes from plasma cells in the bone marrow (*172*). These cells leave lymph nodes and migrate to the bone marrow via the lymphatics and

peripheral blood. The most obvious example of tissue-selective homing by B cells is the association of IgA plasma cells with mucosal tissues (*162*). In contrast, blast cells or plasma cells in lymph nodes or the spleen are usually of the IgM or IgG type.

D. Adhesion Molecules Mediating the Migration of Naive and Memory Cells

The most straightforward hypothesis to account for the different homing patterns of naive and memory cells, whether of T or B phenotype, is the differential expression of adhesion molecules on these two cell types, and the restricted expression of endothelial ligands to particular types of tissue. This concept, although valid, is an over simplification, since the adhesion and transendothelial migration of lymphocytes is very much a multistep process (*173–176*). Moreover, the quantitative expression of lymphocyte adhesion molecules is not the only factor determining the homing pattern of cells, since the state of activation of a cell can also influence the affinity of certain adhesion molecules, particularly integrins (*173–176*).

The adhesion molecules responsible for lymphocyte migration have been discussed at length in recent reviews (*51,140,175,177,178*) and will be mentioned here only briefly. The adhesion–ligand pairs that direct the differential migration of naive, memory, and effector/blast cells are listed in Table II. The high expression of L-selectin (the "lymph node homing receptor", LAM-1, MEL-14) on naive T cells and the lower expression on memory T cells are consistent with the preferential migration of naive T cells through lymph nodes via HEVs (*67*), although expression of L-selectin is not necessarily indicative of a lymph node homing capability. L-Selectin expression on T cells appears to differ slightly between species, since L-selectin in humans is expressed by about 50% of memory-type T cells (*169,179*), whereas in mice and sheep it is expressed by fewer memory T cells (*163,180*). These observations have been corroborated by functional data. In mice, L-selectin-positive and -negative subsets of $CD4^+$ T cells from long-term, antigen-primed mice were evaluated for specific memory responses. Such responses were found almost exclusively within the L-selectin-negative subset of $CD4^+$ T cells (*146,180*), and, moreover, recall responses to various antigens were readily observed when T cells were isolated from tissues such as peritoneal cavity and lung, but not from lymph nodes (*145,146*). Most B cells in blood or lymph are L-selectin-positive, including IgG^+ B cells, whereas B cell blasts in germinal centers are L-selectin-negative.

Memory-type T cells bind to cytokine-activated endothelium better than do naive T cells, most likely through the $\alpha 4\beta 1$–VCAM-1 interaction (*150,181–183*). This might explain in part the preferential accumulation of memory T cells at sites of inflammation (*144,183*). Induction of VCAM-1 on lymph node HEVs might also promote the entry of memory T cells into antigen-challenged lymph nodes (*141*). In addition, memory-type T cells express higher levels of certain adhesion molecules that play an accessory role in transendothelial migration, such as LFA-1 and CD44.

Tissue-tropic T cells express distinct surface molecules that direct their specific localization and migration patterns (Table II). Studies in mice suggest that the $\alpha 4$ integrin functions as an adhesion molecule for Peyer's patch HEVs (*184,185*). $\alpha 4$ in mice can couple with either a $\beta 1$ chain (LPAM-2, VLA-4) or with a $\beta 7$ chain (LPAM-1) (*184*). Gut-tropic T cells in humans express a novel α-integrin coupled with $\beta 7$ (recognized by the HML-1/Ber ACT8 mAbs) (*169*). CLA on the skin-tropic subset of memory T cells in humans is a ligand for E-selectin, which is expressed predominantly on endothelium within inflamed skin (*165*).

VII. Immunological Memory, Tolerance, and Autoimmunity

A. Memory and Self-Tolerance

The ways in which self-tolerance is established, maintained, and broken remain one of the paramount enigmas of contemporary immunology (for reviews, see Refs. *186–188*). Self-reactive T cells are deleted as they mature in the thymus (*189*); however, peripheral mechanisms must also operate, including further clonal deletion or anergy. Unresponsiveness may result because the concentration of some self antigens is too low for the antigens to be seen by the immune system, or the receptors of self-reactive clones may have too low an affinity to trigger a resting T cell. Immune responses usually require an interaction by a number of cell types: an immunoreactive B cell or cytotoxic T cell may be unresponsive due to a lack of cognate T helper signals (*190*). Alternatively, suppressor mechanisms might negate autoreactive T or B cells (*191*). In many ways, immunological tolerance is the antithesis of immunological memory, one subduing and the other enhancing the immune response. For many antigens, foreign and self, the immune system must decide between a tolerance response or an effective immune response.

The point at which naive cells are triggered by antigen is probably

also a critical point for susceptibility to tolerance. This is because a clonally expanded, functionally potent population of autoreactive cells may be uncontrollable. Accordingly, newly generated memory B cells experience a "window" when they are particularly susceptible to tolerance induction. Before antigenic stimulation, precursors of memory B cells were found to be relatively resistant to tolerance induction whereas newly emerging memory B cells were highly susceptible (*115,192*). Newly emerging hapten-specific secondary B cells could be inactivated by the presence of hapten on a carrier not recognized by available T helper cells. This inactivation was less specific than the tolerance induction of immature neonatal or bone marrow B cells, because inactivation could be accomplished by cross-reactive determinants. The bcl-2 protein seems to play an important role at critical stages of B cell activation (see Section IV,A).

It has been suggested that tolerance should be much more stringent in the T cell system than in the B cell system, because the hypermutation of the B cell receptor facilitates the chance emergence of autoreactive cells. This may be controlled by the absence of appropriate T helper signals. Several recent reports suggest that the pathways to T cell memory and peripheral T cell tolerance are in some way related (*193,194*). Naive and memory T cells show different suseptibility to tolerance induction, similar to their differential requirements for activation (see Section III,B). Thus B cells, which can act as APCs for memory-type but not naive T cells (*78*), can also render naive T cells tolerant, but not memory-type T cells (*194a*). In another interesting set of experiments, mice were exposed to superantigens such as Mls or staphylococcal enterotoxin B (SEB) *in vivo*, whereupon antigen-specific T cells underwent rapid proliferation, typical of a normal immune response. However, a state of tolerance to these superantigens ensued. One study concluded that this unresponsiveness was due to deletion of reactive cells, possibly as a result of "exhaustion" or "terminal differentiation" of these cells (*193*). The idea of exhaustion was first invoked in the 1960s to explain forms of immune unresponsiveness to antigens that could elicit strong responses (*6,195*). Other studies concluded that the unresponsive state was due to anergy, as well as deletion (*194,196*), since antigen-specific cells were present but were nonresponsive *in vitro*. These anergized T cells bore the memory phenotype, and this anergy could be partially reversed by the addition of certain interleukins (*194*). This led Lee and Vitetta (*194*) to suggest that memory T cells were critically dependent on the appropriate costimulatory signals from APCs, and have in fact more stringent activation requirements than naive T cells. This may prevent autoimmune disease developing

from low-affinity binding of memory T cells to self antigen. The signals that drive antigen-reactive T cells to normal effector cells and memory cells, or anergic cells, are unknown.

One possibility for peripheral tolerance is that lymphocytes newly produced from the thymus or bone marrow are deleted, or rendered anergic, by reacting with self antigen in the absence of a second signal. The migration patterns of naive and memory T cells have a bearing on this concept, because virtually all naive T cells recirculate through lymphoid tissue and not other tissues (see above). It seems unlikely that dendritic cells could pick up all the available self antigens and present them to naive T cells as a means of tolerance. Accordingly, naive T cells with receptors for self antigen are normally present in the blood, and are neither deleted, anergized, nor triggered by antigen; hence the idea that some forms of peripheral tolerance occur through ignorance on the part of T cells. Double-transgenic mice that express the LCMV glycoprotein in the beta islet cells of the pancreas, as well as a TCR specific for LCMV, showed no signs of T cell reactivity against the islet cells (*197*). However, infection of these transgenic mice with LCMV abolished the tolerance to the "self" LCMV antigen, resulting in T cell-mediated diabetes. The authors concluded that LCMV glycoprotein-expressing beta islet cells were unable to elicit a response, but could become the target of the response if induced by other means. It is possible that self-reactive peripheral T cells were not deleted because they never saw self antigen, and when they were activated with the appropriate secondary signals and transformed to effector and memory cells, they were then able to migrate to the appropriate tissue and mediate a functional and destructive response against self.

A thorny issue is the role of suppressor T cells in peripheral tolerance. It has been argued that suppressor T cells behave like any other T cells and manifest effector function as well as memory (*191*). As yet, the phenotype and functional characteristics of suppressor T cells have not been well characterized, which has cast doubt on the relevance and even the existence of these T cells.

B. Memory and Autoimmunity

The mechanisms that drive immunological memory are probably responsible, if not for the development then at least for the persistence of autoimmune disease. A necessary aspect of autoimmune diseases is the presence in high amounts of circulating autoantibodies against self antigens (reviewed in Refs. *186* and *187*), resulting from a breakdown of self-tolerance, although autoantibodies may exist also in some healthy individuals. In autoimmune disease, it appears that

the body's immune system responds to particular self-antigens in a fashion akin to that of normal responses. The initial breakdown of tolerance presumably starts with a primary response, and thereafter it develops into an autonomous reaction with all the features of a secondary response. Analysis of anti-DNA antibodies from autoimmune mice showed that they were from a clonally expanded population and had accumulated somatic mutations, in a fashion similar to that seen in secondary responses to normal antigens (*198*). In addition, autoantibodies in humans are usually of the IgG isotype, indicative of a secondary immune response. Affinity selection of autoreactive memory B cells presumably occurs in germinal centers, suggesting that autoantigen deposits there.

A link between autoimmunity and memory is suggested from the studies with the protooncogene *bcl-2* (see Section V,B). Transgenic mice that overexpress the *bcl-2* gene in lymphoid cells develop an autoimmune condition similar to systemic lupus erythematosus (*199*), possibly because self-reactive B cell clones that overexpress *bcl-2* are less susceptible to the tolerogenic signals that normally induce programmed cell death. The *bcl-2* gene also appears to play a role in B cell memory, probably by saving memory B cells from programmed cell death, and thus extending their life span. In some instances, inappropriate expression of bcl-2 protein may protect autoreactive B cells from tolerance and endow them with memory capability. The apoptosis-related molecule Fas (see Section V,B), which is expressed on memory-type T and B cells (*137*), also plays a role in autoimmunity, since *lpr* mice with systemic lupus erythematosus-like autoimmune disease have a defect in Fas expression (*200*).

In comparison with B cells, the role of T cells in the pathogenesis of autoimmune disease is less clear. Inappropriate helper signals from $CD4^+$ T cells to B cells or to cytotoxic T cells are probably one of the critical elements in the breakdown of self-tolerance (*187*). The overwhelming accumulation of memory-type T cells in autoimmune lesions (*187*) suggests that the autoimmune T cell response is probably driven in the same way as in a normal secondary response to foreign antigen, but with the difference that the provoking antigen is never eliminated. Autoimmunity is therefore an enduring process, although there are relapses and remissions.

Can the hyperresponsiveness of memory or effector T cells be blamed for autoimmunity? The immune system must tread a fine line by providing strong and effective immunity against foreign antigens, but remain unreactive against self. An important feature of memory-type T cells is that they are functionally potent, and require less of an activation signal through the TCR for stimulation (see Section III,B).

It follows then that lower affinity interactions, or lower amounts of antigen, could stimulate memory-type T cells yet fail to stimulate naive-type T cells. In certain instances, self-reactive T cells can be activated to produce autoimmunity by hyperimmunization (*197,201*). Perhaps in these cases functionally potent effector or memory clones, once-established, are capable of responding to self antigen. This raises an interesting consideration. If naive and memory-type T cells do have different thresholds of activation, then which threshold operates during negative selection of thymocytes? Evidence to date suggests that the process of clonal deletion is more sensitive than naive T cell activation (*75,202*). Interestingly, thymocytes express the RO isoform of CD45, which may render their TCR complex more sensitive to triggering by self antigen (*75*).

VIII. Concluding Remarks

The central paradigm for immunological memory over the past 20 years has been that memory depends on long-lived, recirculating cells. This paradigm is in the process of shifting toward a model whereby persistent antigenic stimulation plays an important role. There is now little doubt that at least a component of memory results from persistent antigenic stimulation, and future experiments should determine whether memory is entirely dependent on this mechanism. Most likely, immunological memory will prove to be multifaceted, relying on a number of mechanisms for its function, just as immunological tolerance relies on several mechanisms.

Differentiation pathways to effector cells, or to cells that harbor memory, are different for the T and B cell systems. For T cells, the evidence indicates that effectors transform to smaller $CD45RO^+$ T cells. These T cells still seem to resemble blast/effector cells with respect to phenotype and functional criteria. Perhaps the most obvious difference is that they are physically smaller. The most uncertain and controversial aspect of this scheme is whether $CD45RO^+$ T cells then revert to $CD45RA^+$. If they do, then they probably harbor memory in a manner different than that of $CD45RO^+$ T cells. A convincing demonstration of long-lived T cell memory by resting $CD45RA^+$ T cells is necessary, and toward this end, transgenic mice with defined TCRs should prove useful. Notions on T cell memory have taken a tortuous path over the last 5 years. The concept of long-lived memory T cells led to the search for their identity. Functional studies showed the phenotype to be $CD45RO^+$, $CD44^{hi}$, etc. However, recent studies now show that these cells are probably short-lived and

capable of converting to the naive phenotype. To refer to either population as memory T cells is probably misleading (although this was done here for want of a better terminology).

B cells, in contrast to T cells, diverge into either terminally differentiated effector (plasma) cells or memory cells. One of the questions that needs to be clarified is whether commitment to either pathway occurs before or after antigen priming, and what signals influence this commitment. Another paradox is the apparent need for antigen for maintenance of memory, even though memory B cells are mostly a nondividing population. It is possible that memory may exist at different levels in the B cell system, as it does in the T cell system.

Immunological memory is interconnected and often inseparable from other fields of immunological research, such as peripheral tolerance, lymphocyte homing, lymphocyte differentiation, vaccine development, transplantation, and autoimmunity. Controversy and uncertainty surround many aspects concerning the precursor–product relationships by subsets of T and B cells, their functional behavior, and their life span. As these uncertainties are resolved, a more complete understanding of immunological memory, which clearly is of the highest priority, will reach realization.

Acknowledgments

I would like to thank Birgit Schittek for helpful suggestions, and those collegues who shared unpublished work. The Basel Institute for Immunology was founded and is supported by F. Hoffman-La Roche Ltd., Basel, Switzerland.

References

1. Glenny, A. T., and Sudmersen, H. J. (1921). Notes on the production of immunity to diphtheria toxin. *J. Hyg.* **20**, 176–220.
2. Burnet, F. M., and Fenner, F. (1949). The production of antibodies. MacMillan and Co., London.
3. Burnet, M. (1959). The clonal selection theory of acquired immunity. Cambridge University Press, Cambridge, Mass.
4. Nossal, G. J. V. (1962). Cellular genetics of immune responses. *Adv. Immunol.* **2**, 163–204.
5. Sercarz, E., and Coons, A. H. (1962). The exhaustion of specific antibody producing capacity during a secondary response. *In* "Mechanisms of Immunological Tolerance" (M. Hasek, A. Lengerova, and M. Vojtiskova, eds.), pp. 73–83. Czechoslovak Academy of Sciences Press, Prague.
6. Byers, V. S., and Sercarz, E. E. (1968). The X-Y-Z scheme of immunocyte maturation. IV. The exhaustion of memory cells. *J. Exp. Med.* **127**, 307–325.
7. Celada, F. (1971). The cellular basis of immunologic memory. *Proc. Allergy* **15**, 223–267.
8. Couch, R. B., and Kasel, J. A. (1983). Immunity to influenza in man. *Annu. Rev. Microbiol.* **37**, 529–549.

9. Ada, G. L. (1989). Vaccines. *In* "Fundamental Immunology" (W. E. Paul, ed.), pp. 985–1032. Raven Press, New York.
10. Panum, P. L. (1847). *Virchow's Arch. Pathol. Anat. Physiol.* **1,** 492.
11. Zinkernagel, R. M. (1990). Antiviral T-cell memory? *Curr. Top. Microbiol. Immunol.* **159,** 65–77.
12. Jamieson, B. D., and Ahmed, R. (1989). T cell memory. Long-term persistence of virus-specific cytotoxic T cells. *J. Exp. Med.* **169,** 1993–2005.
12a. Oehen, S., Waldner, H., Kündig, M., Hengartner, H., and Zinkernagel, R. M. (1992). Antivirally protective cytotoxic T cell memory to lymphocytic choriomeningitis virus is governed by persisting antigen. *J. Exp. Med.* **176,** 1273–1281.
13. Celada, F. (1967). Quantitative studies of the adoptive immunological memory in mice. I. Linear transmission of cellular memory. *J. Exp. Med.* **125,** 199–211.
14. Feldbush, T. L. (1973). Antigen modulation of the immune response. The decline of immunological memory in the absence of continuing antigenic stimulation. *Cell. Immunol.* **8,** 435–444.
15. Strober, S., and Dilley, J. (1973). Maturation of B lymphocytes in the rat. I. Migration pattern, tissue distribution, and turnover rate of unprimed and primed B lymphocytes involved in the adoptive antidinitrophenyl response. *J. Exp. Med.* **138,** 1331–1344.
16. Askonas, B. A., and Williamson, A. R. (1972). Factors affecting the propagation of a B cell clone forming antibody to the 2,4-dinitrophenyl group. *Eur. J. Immunol.* **2,** 487–493.
17. Tew, J. G., Phipps, R. P., and Mandel, T. E. (1980). The maintenance and regulation of the humoral immune response: Persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol. Rev.* **53,** 175–201.
18. Gray, D., and Matzinger, P. (1991). T cell memory is short-lived in the absence of antigen. *J. Exp. Med.* **174,** 969–974.
19. Gray, D., and Skarvall, H. (1988). B-cell memory is short-lived in the absence of antigen. *Nature (London)* **336,** 70–73.
20. Beverley, P. C. L. (1990). Is T-cell memory maintained by crossreactive stimulation? *Immunol. Today* **11,** 203–205.
21. Nunez, G., Hockenbery, D., McDonnell, T. J., Sorensen, C. M., and Korsmeyer, S. J. (1991). Bcl-2 maintains B cell memory. *Nature (London)* **353,** 71–73.
22. Mackay, C. R. (1991). T-cell memory: The connection between function, phenotype and migration pathways. *Immunol. Today* **12,** 189–192.
23. Cerottini, J.-C., and MacDonald, H. R. (1989). The cellular basis of T-cell memory. *Annu. Rev. Immunol.* **7,** 77–89.
24. Schittek, B., and Rajewsky, K. (1990). Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. *Nature (London)* **346,** 749–751.
25. Orme, I. M. (1988). Characteristics and specificity of acquired immunologic memory to *Mycobacterium tuberculosis* infection. *J. Immunol.* **140,** 3589–3593.
26. Cheever, M. A., Thompson, D. B., Klarnet, J. P., and Greenberg, P. D. (1986). Antigen-driven long term-cultured T cells proliferate *in vivo,* distribute widely, mediate specific tumor therapy, and persist long-term as functional memory T cells. *J. Exp. Med.* **163,** 1100–1112.
27. Engers, H. D., and MacDonald, H. R. (1976). Generation of cytotoxic T lymphocytes *in vitro*. *In* "Contemporary Topics in Immunobiology" (W. Weigle, ed.), pp. 145–190. Plenum, New York.
28. Linton, P. L., Decker, D. J., and Klinman, N. R. (1989). Primary antibody-forming

cells and secondary B cells are generated from separate precursor cell subpopulations. *Cell (Cambridge, Mass.)* **59,** 1049–1059.
29. Gray, D. (1992). Immunological memory. *Annu. Rev. Immunol.* (in press).
30. Nossal, G. J. V., Abbot, A. P., Mitchell, J., and Lummus, Z. (1968). Antigens in immunity. XV. Ultrastructural features of antigen capture in primary and secondary follicles. *J. Exp. Med.* **127,** 277.
31. Jones, P. D., and Ada, G. L. (1987). Persistence of influenza virus-specific antibody-secreting cells and B-cell memory after primary murine influenza virus infection. *Cell. Immunol.* **109,** 53–64.
32. Gray, D., Kosco, M., and Stockinger, B. (1991). Novel pathways of antigen presentation for the maintenance of memory. *Int. Immunol.* **3,** 141–8.
33. Mitchison, N. A. (1971). The carrier effect in the secondary response to hapten-protein conjugates. V. Use of antilymphocyte serum to deplete animals of helper cells. *Eur. J. Immunol.* **1,** 68–75.
34. Hoskins, T. W., Davies, J. K., Smith, A. J., Miller, C. L., and Allchin, A. (1979). Assessment of inactivated influenza-A vaccine after three outbreaks of influenza A at Christ's hospital. *Lancet* **1,** 33–35.
35. Robinson, A., Meyer, M., and Middlebrook, G. (1955). *N. Engl. J. Med.* **252,** 983.
36. Fazekas de St. Groth, S., and Webster, R. G. (1966). Disquisitions on original antigenic sin. I. Evidence in man. *J. Exp. Med.* **124,** 331–361.
37. Fish, S., Zenowich, E., Fleming, M., and Manser, T. (1989). Molecular analysis of original antigenic sin. I. Clonal selection, somatic mutation, and isotype switching during a memory B cell response. *J. Exp. Med.* **170,** 1191–209.
38. Ashwell, J. D., and Schwartz, R. H. (1986). T-cell recognition of antigen and the Ia molecule as a ternary complex. *Nature (London)* **320,** 176–9.
39. Panina-Bordignon, P., Tan, A., Termijtelen, A., Demotz, S., Corradin, G., and Lanzavecchia, A. (1989). Universally immunogenic T cell epitopes: Promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* **19,** 2237–2242.
40. Good, M. F. (1991). The implications for malaria vaccine programs if memory T cells from non-exposed humans can respond to malaria antigens. *Curr. Opin. Immunol.* **3,** 496–502.
41. Jones, K. R., Hickling, J. K., Targett, G. A., and Playfair, J. H. (1990). Polyclonal *in vitro* proliferative responses from nonimmune donors to *Plasmodium falciparum* malaria antigens require UCHL1$^+$ (memory) T cells. *Eur. J. Immunol.* **20,** 307–315.
42. Currier, J., Sattabongkot, J., and Good, M. F. (1992). Natural tolerance to malaria: Evidence implicating immunological cross-reactivity in the maitenance of TCR$\alpha\beta$+ malaria-specific responses from non-exposed donors. *Int. Immunol.* **4,** 985–994.
43. Parisi, G. (1990). A simple model for the immune network. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 429–433.
44. Thomas, M. L. (1989). The leukocyte common antigen family. *Annu. Rev. Immunol.* **7,** 339–369.
45. Koretzky, G. A., Picus, J., Thomas, M. L., and Weiss, A. (1990). Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature (London)* **346,** 66–68.
46. Dianzani, U., Luqman, M., Rojo, J., Yagi, J., Baron, J. L., Woods, A., Janeway, C. A., and Bottomly, K. (1990). Molecular associations on the T cell surface correlate with immunological memory. *Eur. J. Immunol.* **20,** 2249–2257.
47. Dianzani, U., Redoglia, V., Malavasi, F., Bragardo, M., Pileri, A., Janeway, C. A.,

and Bottomly, K. (1992). Isoform-specific associations of CD45 wtih accessory molecules in human T lymphocytes. *Eur. J. Immunol.* **22,** 365–371.

48. Stamenkovic, I., Sgroi, D., Aruffo, A., Sy, M. S., and Anderson, T. (1992). The B lymphocyte adhesion molecule CD22 interacts with the leukocyte common antigen CD45RO on T cells and α2-6-sialyl-transferase, CD75, on B cells. *Cell (Cambridge, Mass.)* **66,** 1133–1144.
49. Beverley, P. C. L. (1990). Human T-cell memory. *Curr. Top. Microbiol. Immunol.* **159,** 111–122.
50. Sanders, M. E., Makgoba, M. W., and Shaw, S. (1988). Human naive and memory T cells: Reinterpretation of helper-inducer and suppressor-inducer subsets. *Immunol. Today* **9,** 195–199.
51. Mackay, C. R. (1992). Migration pathways and immunologic memory among T lymphocytes. *Semin. Immunol.* **4,** 51–58.
52. Morimoto, C., Letvin, N. L., Boyd, A. W., Hagan, M., Brown, H. M., Kornacki, M. M., and Schlossman, S. F. (1985). The isolation and characterization of the human helper inducer T cell subset. *J. Immunol.* **134,** 1508.
53. Tedder, T. F., Cooper, M. D., and Clement, L. T. (1985). Human lymphocyte differentiation antigens HB-10 and HB11. II. Differential production of B cell growth and differentiation factors by distinct helper T cell subpopulations. *J. Immunol.* **134,** 2989–2994.
54. Sanders, M. E., Makgoba, M. W., Sharrow, S. O., Stephany, D., Springer, T. A., Young, H. A., and Shaw, S. (1988). Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. *J. Immunol.* **140,** 1401–1407.
55. Smith, S. H., Brown, M. H., Rowe, D., Callard, R. E., and Beverley, P. C. L. (1986). Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. *Immunology* **58,** 63–70.
56. Haynes, B. F., Telen, M. J., Hale, L. P., and Denning, S. M. (1989). CD44-a molecule involved in leukocyte adherence and T-cell activation. *Immunol. Today* **10,** 423–428.
57. Budd, R. C., Cerottini, J.-C., Horvath, C., Bron, C., Pedrazzini, T., Howe, R. C., and MacDonald, H. R. (1987). Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J. Immunol.* **138,** 3120–31299.
58. Bottomly, K., Luqman, M., Greenbaum, L., Carding, S., West, J., Pasqualini, T., and Murphy, D. B. (1989). A monoclonal antibody to murine CD45R distinguishes CD4 T cell populations that produce different cytokines. *Eur. J. Immunol.* **19,** 617–623.
59. Birkeland, M. L., Johnson, P., Trowbridge, I. S., and Puré, E. (1989). Changes in CD45 isoform expression accompany antigen-induced murine T-cell activation. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6734–6738.
60. Lee, W. T., and Vitetta, E. S. (1990). Limiting dilution analysis of CD45Rhi and CD45Rlo T cells: Further evidence that CD45Rlo cells are memory cells. *Cell. Immunol.* **130,** 459–71.
61. Powrie, F., and Mason, D. (1989). The MRC OX-22− CD4+ T cells that help B cells in secondary immune responses derive from naive precursors with the MRC OX-22+ CD4+ phenotype. *J. Exp. Med.* **169,** 653–662.
62. Bell, E. B. (1992). Function of CD4 T cell subsets *in vivo:* Expression of CD45R isoforms. *Semin. Immunol.* **4,** 43–50.
63. Bell, E. B., and Sparshott, S. M. (1990). Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature (London)* **348,** 163–166.

64. Rothstein, D. M., Yamada, A., Schlossman, S. F., and Morimoto, C. (1991). Cyclic regulation of CD45 isoform expression in a long term human CD4+ CD45RA+ T cell line. *J. Immunol.* **146,** 1175–1183.
65. Warren, H. S., and Skipsey, L. J. (1991). Loss of activation-induced CD45RO with maintenance of CD45RA expression during prolonged culture of T cells and NK cells. *Immunology* **74,** 78–85.
66. Michie, C. A., McLean, A., Alcock, C., and Beverley, P. C. L. (1992). Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature (London)* **360,** 264–265.
67. Mackay, C. R., Marston, W. L., and Dudler, L. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* **171,** 801–817.
68. Mitchison, N. A. (1992). Specialization, tolerance, memory, competition, latency, and strife among T cells. *Annu. Rev. Immunol.* **10,** 1–12.
69. Street, N. E., and Mosmann, T. R. (1991). Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J.* **5,** 171–177.
70. Swain, S. L., Bradley, L. M., Croft, M., Tonkonogy, S., Atkins, G., Weinberg, A. D., Duncan, D. D., Hedrick, S. M., Dutton, R. W., and Huston, G. (1991). Helper T-cell subsets: Phenotype, function and the role of lymphokines in regulating their development. *Immunol. Rev.* **123,** 115–144.
71. Bradley, L. M., Duncan, D. D., Yoshimoto, K., and Swain, S. L. (1992). Memory effectors: A potent, IL-4 secreting helper T cell population that develops *in vivo* after restimulation with antigen. Submitted.
72. Sanders, M. E., Makgoba, M. W., June, C. H., Young, H. A., and Shaw, S. (1989). Enhanced responsiveness of human memory T cells to CD2 and CD3 receptor-mediated activation. *Eur. J. Immunol.* **19,** 803–808.
73. Byrne, J. A., Butler, J. L., and Cooper, M. D. (1988). Differential activation requirements for virgin and memory T cells. *J. Immunol.* **141,** 3249–257.
74. Horgan, K. J., Van Seventer, G. A., Shimizu, Y., and Shaw, S. (1990). Hyporesponsiveness of "naive" (CD45RA+) human T cells to multiple receptor-mediated stimuli but augmentation of responses by co-stimuli. *Eur. J. Immunol.* **20,** 1111–1118.
75. Janeway, C. A. (1992). The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu. Rev. Immunol.* **10,** 645–674.
76. Springer, T. A. (1990). Adhesion receptors of the immune system. *Nature (London)* **346,** 425–434.
77. Pingel, J. R., and Thomas, M. L. (1989). Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell (Cambridge, Mass.)* **58,** 1055–1062.
78. Ronchese, F., and Hausmann, B. (1992). B lymphocytes *in vivo* fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. *J. Exp. Med.* (in press).
79. Klaus, G. G. B., and Humphrey, J. H. (1977). The generation of memory cells. I. The role of C3 in the generation of B memory cells. *Immunology* **33,** 31–40.
80. Coico, R. F., Bhogal, B. S., and Thorbecke, G. J. (1983). Relationship of germinal centers in lymphoid tissue to immunologic memory. VI. Transfer of B cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. *J. Immunol.* **131,** 2254–2257.
81. Liu, Y. J., Johnson, G. D., Gordon, J., and MacLennan, I. C. M. (1992). Germinal centres in T-cell-dependent antibody responses. *Immunol. Today* **13,** 17–21.

82. Berek, C. (1992). The development of B cells and the B-cell repertoire in the microenvironment of the germinal center. *Immunol. Rev.* **126,** 5–19.
83. Tsiagbe, V. K., Linton, P. J., and Thorbecke, G. J. (1992). The path of memory B-cell development. *Immunol. Rev.* **126,** 113–41.
84. Leanderson, T., Kallberg, E., and Gray, D. (1992). Expansion, selection and mutation of antigen-specific B cells in germinal centers. *Immunol. Rev.* **126,** 47–61.
85. Kosco, M. H., and Gray, D. (1992). Signals involved in germinal center reactions. *Immunol. Rev.* **126,** 63–76.
86. Nieuwenhuis, P., Kroese, F. G., Opstelten, D., and Seijen, H. G. (1992). *De novo* germinal center formation. *Immunol. Rev.* **126,** 77–98.
87. Seijen, H. G., Bun, J. C. A. M., Wubbena, A. S., and Loehlefink, K. G. L. (1988). The germinal center precursor cell is surface IgM and IgD positive. *Adv. Exp. Med. Biol.* **237,** 233.
88. Linton, P. J., Lo, D., Lai, L., Thorbecke, G. J., and Klinman, N. R. (1992). Among naive precursor cell subpopulations only progenitors of memory B cells originate germinal centers. *Eur. J. Immunol.* **22,** 1293–1297.
89. MacLennan, I. C. M., Liu, Y. J., and Johnson, G. D. (1992). Maturation and dispersal of B-cell clones during T cell-dependent antibody responses. *Immunol. Rev.* **126,** 143–161.
90. Vonderheide, R. H., and Hunt, S. V. (1991). Comparison of IgD+ and IgD− thoracic duct B lymphocytes as germinal center precursor cells in the rat. *Int. Immunol.* **3,** 1273–1281.
91. Kraal, G., Weissman, I. L., and Butcher, E. C. (1982). Germinal centre B cells: Antigen specificity and changes in heavy chain class expression. *Nature (London)* **298,** 377–379.
92. MacLennan, I. C. M., Liu, Y. J., Oldfield, S., Zhang, J., and Lane, P. J. (1990). The evolution of B-cell clones. *Curr. Top. Microbiol. Immunol.* **159,** 37–63.
93. MacLennan, I. C. M., and Gray, D. (1986). Antigen-driven selection of virgin and memory B cells. *Immunol. Rev.* **91,** 61–85.
94. Liu, Y. J., Mason, D. Y., Johnson, G. D., Abbot, S., Gregory, C. D., Hardie, D. L., Gordon, J., and MacLennan, I. C. M. (1991). Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. *Eur. J. Immunol.* **21,** 1905–1910.
95. Liu, Y. J., Cairns, J. A., Holder, M. J., Abbot, S. D., Jansen, K. U., Bonnefoy, J. Y., Gordon, J., and MacLennan, I. C. M. (1991). Recombinant 25-kDa CD23 and interleukin-1-alpha promote the survival of germinal center B cells: Evidence for bifurcation in the development of centrocytes rescued from apoptosis. *Eur. J. Immunol.* **21,** 1107–14.
96. Nossal, G. J. V. (1992). The molecular and cellular basis of affinity maturation in the antibody response. *Cell (Cambridge, Mass.)* **68,** 1–2.
97. Jacob, J., Kelsoe, G., Rajewsky, K., and Weiss, U. (1991). Intraclonal generation of antibody mutants in germinal centres. *Nature (London)* **354,** 389–392.
98. McHeyzer-Williams, M. G., Nossal, G. J. V., and Lalor, P. A. (1991). Molecular characterization of single memory B cells. *Nature (London)* **350,** 502–505.
99. Siekevitz, M., Kocks, C., Rajewsky, K., and Dildrop, R. (1987). Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. *Cell (Cambridge, Mass.)* **48,** 757–770.
100. Weiss, U., Zoebelein, R., and Rajewsky, K. (1992). Accumulation of somatic mutants in the B cell compartment after primary immunization with a T cell-dependent antigen. *Eur. J. Immunol.* **22,** 511–517.

101. Rada, C., Gupta, S. K., Gherardi, E., and Milstein, C. (1991). Mutation and selection during the secondary response to 2-phenyloxazolone. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 5508–5512.

102. Gu, H., Tarlinton, D., Muller, W., Rajewsky, K., and Forster, I. (1991). Most peripheral B cells in mice are ligand selected. *J. Exp. Med.* **173,** 1357–1371.

103. Coffman, R. L., and Cohn, M. (1977). The class of surface immunoglobulin on virgin and memory B lymphocytes. *J. Immunol.* **118,** 1806–1815.

104. Hayakawa, K., Ishii, R., Yamasaki, K., Kishimoto, T., and Hardy, R. R. (1987). Isolation of high-affinity memory B cells: Phycoerythrin as a probe for antigen-binding cells. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 1379–1383.

105. Herzenberg, L. A., Black, S. J., Tokuhisa, T., and Herzenberg, L. A. (1980). Memory B cells at successive stages of differentiation. Affinity maturation and the role of IgD receptors. *J. Exp. Med.* **151,** 1071–1087.

106. Colle, J.-H., Le Moal, M. A., and Truffa-Bachi, P. (1990). Immunological memory. *Crit. Rev. Immunol.* **10,** 259–288.

107. Schittek, B., and Rajewsky, K. (1992). Natural occurrence and origin of somatically mutated memory B cells in mice. *J. Exp. Med.* **176,** 427–438.

108. Zan-Bar, I., Strober, S., and Vitetta, E. S. (1979). The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. IV. Role of IgD-bearing cells in the propagation of immunologic memory. *J. Immunol.* **123,** 925–930.

109. Bruce, J., Symington, F. W., McKearn, T. J., and Sprent, J. (1981). A monoclonal antibody discriminating between subsets of T and B cells. *J. Immunol.* **127,** 2496–2501.

110. Klinman, N. R., and Linton, P. J. (1990). The generation of B-cell memory: A working hypothesis. *Curr. Top. Microbiol. Immunol.* **159,** 19–35.

111. Yin, X. M., Lee, W. T., and Vitetta, E. S. (1991). Changes in expression of J11d on murine B cells during activation and generation of memory. *Cell. Immunol.* **137,** 448–460.

112. Camp, R. L., Kraus, T. A., Birkeland, M. L., and Pure, E. (1991). High levels of CD44 expression distinguish virgin from antigen-primed B cells. *J. Exp. Med.* **173,** 763–776.

113. Kraal, G., Weissman, I. L., and Butcher, E. C. (1988). Memory B cells express a phenotype consistent with migratory competence after secondary but not short-term primary immunization. *Cell. Immunol.* **115,** 78–87.

114. Yefenof, E., Sanders, V. M., Uhr, J. W., and Vitetta, E. S. (1986). *In vitro* activation of murine antigen-specific memory B cells by a T-dependent antigen. *J. Immunol.* **137,** 85–90.

115. Linton, P. J., and Klinman, N. R. (1992). The generation of memory B cells. *Semin. Immunol.* **4,** 3–9.

116. Hilbert, D. M., Cancro, M. P., Scherle, P. A., Nordan, R. P., Van Snick, J., Gerhard, W., and Rudikoff, S. (1989). T cell derived IL-6 is differentially required for antigen-specific antibody secretion by primary and secondary B cells. *J. Immunol.* **143,** 4019–4024.

117. Reynaud, C. A., Mackay, C. R., Muller, R. G., and Weill, J. C. (1991). Somatic generation of diversity in a mammalian primary lymphoid organ: The sheep ileal Peyer's patches. *Cell (Cambridge, Mass.)* **64,** 995–1005.

117a. Jacob, J., and Kelsoe, G. (1992). *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J. Exp. Med.* **176,** 679–687.

118. Sprent, J. (1993). Lifespans of naive, effector and memory lymphocytes. *Curr. Opin. Immunol.* (in press).
118a. Immunol. Today **114,** No. 1 (1993).
119. Norman, A., Sasaki, M. S., Ottoman, R. E., and Fingerhut, A. G. (1965). Lymphocyte lifetime in women. *Science* **147,** 745.
120. Buckton, K. E., Court Brown, W. M., and Smith, P. G. (1967). Lymphocyte survival in men treated with X-rays for ankylosing spondylitis. *Nature (London)* **214,** 470–473.
121. Sprent, J., and Basten, A. (1973). Circulating T and B lymphocytes of the mouse. II. Lifespan. *Cell. Immunol.* **7,** 40–59.
122. Gray, D. (1988). Population kinetics of rat peripheral B cells. *J. Exp. Med.* **167,** 805–816.
123. Osmond, D. G. (1986). Population dynamics of bone marrow B lymphocytes. *Immunol. Rev.* **93,** 103–124.
124. Forster, I., and Rajewsky, K. (1990). The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4781–4784.
125. Freitas, A. A., Rocha, B., and Coutinho, A. A. (1986). Lymphocyte population kinetics in the mouse. *Immunol. Rev.* **91,** 5–37.
126. Freitas, A. A., Rocha, B., and Coutinho, A. A. (1986). Life span of B lymphocytes: The experimental basis for conflicting results. *J. Immunol.* **136,** 470–476.
127. Rocha, B., Penit, C., Baron, C., Vasseur, F., Dautigny, N., and Freitas, A. A. (1990). Accumulation of bromodeoxyuridine-labeled cells in central and peripheral lymphoid organs: Minimal estimates of production and turnover rates of mature lymphocytes. *Eur. J. Immunol.* **20,** 1697–1708.
128. Stout, R. D., and Suttles, J. (1992). T cells bearing the CD44hi "memory" phenotype display characteristics of activated cells in G1 stage of cell cycle. *Cell. Immunol.* **141,** 433–443.
129. Sprent, J., Schaefer, M., Hurd, M., Surh, C. D., and Ron, Y. (1991). Mature murine B and T cells transferred to SCID mice can survive indefinitely and many maintain a virgin phenotype. *J. Exp. Med.* **17,** 717–728.
130. Korsmeyer, S. J. (1992). Bcl-2: A repressor of lymphocyte death. *Immunol. Today* **13,** 285–288.
131. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature (London)* **348,** 334–336.
132. Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S. J. (1991). bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell (Cambridge, Mass.)* **67,** 879–888.
133. Strasser, A., Harris, A. W., and Cory, S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell (Cambridge, Mass.)* **67,** 889–899.
134. Strober, S., and Dilley, J. (1973). Biological characteristics of T and B memory lymphocytes in the rat. *J. Exp. Med.* **137,** 1275–1292.
135. Strasser, A., Harris, A. W., Vaux, D. L., Webb, E., Bath, M. L., Adams, J. M., and Cory, S. (1990). Abnormalities of the immune system induced by dysregulated bcl-2 expression in transgenic mice. *Curr. Top. Microbiol. Immunol.* **166,** 175–181.
136. Itoh, N., Yonehara, S., Ishii, A., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for the cell surface antigen Fas can mediate apoptosis. *Cell (Cambridge, Mass.)* **66,** 233–241.

137. Miyawaki, T., Uehara, T., Nibu, R., Tsuji, T., Yachi, A., Yonehara, S., and Taniguchi, N. (1992). Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. *J. Immunol.* **149,** 3753–3758.
138. Hall, J. G., Morris, B., Moreno, G. D., and Bessis, M. (1967). The ultrastructure and function of the cells in lymph following antigenic stimulation. *J. Exp. Med.* **125,** 91–110.
139. Ford, W. L. (1975). Lymphocyte migration and immune responses. *Prog. Allergy* **19,** 1–59.
140. Picker, L. J. (1992). Mechanisms of lymphocyte homing. *Curr. Opin. Immunol.* **4,** 277–286.
141. Mackay, C. R., Marston, W. L., and Dudler, L. (1992). Altered patterns of T cell migration through lymph nodes and skin following antigen challenge. *Eur. J. Immunol.* **22,** 2205–2210.
142. Reichert, R. A., Gallatin, W. M., Butcher, E. C., and Weissman, I. L. (1984). A homing receptor-bearing cortical thymocyte subset: Implications for thymus cell migration and the nature of cortisone-resistant thymocytes. *Cell (Cambridge, Mass.)* **38,** 89–99.
143. Pearson, L. D., Simpson Morgan, M. W., and Morris, B. (1976). Lymphopoiesis and lymphocyte recirculation in the sheep fetus. *J. Exp. Med.* **143,** 167–186.
144. Pitzalis, C., Kingsley, G., Haskard, D., and Panayi, G. (1988). The preferential accumulation of helper-inducer T lymphocytes in inflammatory lesions: Evidence for regulation by selective endothelial and homotypic adhesion. *Eur. J. Immunol.* **18,** 1397–1404.
145. Rosenstreich, D. L., Blake, J. T., and Rosenthal, A. S. (1971). The peritoneal exudate lymphocyte. I. Differences in antigen responsiveness between peritoneal exudate and lymph node lymphocytes from immunized guinea pigs. *J. Exp. Med.* **134,** 1170–1186.
146. Forsthuber, T., Soares, L. R., Miller, A., Sercarz, E. E., and Lehmann, P. V. (1992). *In vivo* memory T cells do not express the lymph node homing receptor MEL-14 (abstr.). *World Congr. of Immunol., Budapest, Hungary.*
147. McGregor, D. D., Koster, F. T., and Mackaness, G. B. (1970). The mediator of cellular immunity. I. The life-span and circulation dynamics of the immunologically committed lymphocyte. *J. Exp. Med.* **133,** 389–399.
148. McGregor, D. D., and Gowans, J. L. (1963). The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct. *J. Exp. Med.* **117,** 303–320.
149. Mackay, C. R., Kimpton, W. G., Brandon, M. R., and Cahill, R. N. (1988). Lymphocyte subsets show marked differences in their distribution between blood and the afferent and efferent lymph of peripheral lymph nodes. *J. Exp. Med.* **167,** 1755–1765.
150. Masuyama, J.-I., Berman, J. S., Cruikshank, W. W., Morimoto, C., and Center, D. M. (1992). Evidence for recent as well as long term activation of T cells migrating through endothelial cell monolayers in vitro. *J. Immunol.* **148,** 1367–11374.
151. Sprent, J. (1976). Fate of H2-activated T lymphocytes in syngeneic hosts. I. Fate in lymphoid tissues and intestines traced with ^{3}H-thymidine, ^{125}I-deoxyuridine and ^{51}chromium. *Cell. Immunol.* **21,** 278–302.
152. Griscelli, C., Vassali, P., and McCluskey, R. T. (1969). The distribution of large dividing lymph node cells in syngeneic recipient rats after intravenous injection. *J. Exp. Med.* **130,** 1427–1451.
153. Hall, J. G., Hopkins, J., and Orlans, E. (1977). Studies on the lymphocytes of sheep

III: Destination of lymph-borne immunoblasts in relation to their tissue of origin. *Eur. J. Immunol.* **7,** 30–37.

154. Cahill, R. N. P., Poskitt, D. C., Frost, D. C., and Trnka, Z. (1977). Two distinct pools of recirculating T lymphocytes: Migratory characteristics of nodal and intestinal T lymphocytes. *J. Exp. Med.* **145,** 420–428.
155. Scollay, R., Hopkins, J., and Hall, J. G. (1976). Possible role of surface Ig in non-random recirculation of small lymphocytes. *Nature (London)* **260,** 528–530.
156. Butcher, E. C., Scollay, R. G., and Weissman, I. L. (1980). Organ specificity of lymphocyte migration: Mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. *Eur. J. Immunol.* **10,** 556–61.
157. Chin, W., and Hay, J. B. (1980). A comparison of lymphocyte migration through intestinal lymph nodes, subcutaneous lymph nodes, and chronic inflammatory sites of sheep. *Gastroenterology* **79,** 1231–1242.
158. Abernethy, N. J., and Hay, J. B. (1988). Lymphocyte migration through skin and skin lesions. *In* "Migration and Homing of Lymphoid Cells" (A. J. Husband, ed.), pp. 113–134. CRC Press, Boca Raton, Florida.
159. McDermott, M. R., and Bienenstock, J. (1979). Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory and genital tissues. *J. Immunol.* **122,** 1892–1898.
160. Jalkanen, S., Steere, A. C., Fox, R. I., and Butcher, E. C. (1986). A distinct endothelial cell recognition system that controls lymphocyte traffic into inflamed synovium. *Science* **233,** 556–558.
161. Berg, E. L., Goldstein, L. A., Jutila, M. A., Nakache, M., Picker, L. J., Streeter, P. R., Wu, N. W., Zhou, D., and Butcher, E. C. (1989). Homing receptors and vascular addressins: Cell adhesion molecules that direct lymphocyte traffic. *Immunol. Rev.* **108,** 5–18.
162. Yednock, T. A., and Rosen, S. D. (1989). Lymphocyte homing. *Adv. Immunol.* **44,** 313–378.
163. Mackay, C. R., Marston, W. L., Dudler, L., Spertini, O., Tedder, T. F., and Hein, W. R. (1992). Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. *Eur. J. Immunol.* **22,** 887–895.
164. Mackay, C. R. (1991). Lymphocyte homing. Skin-seeking memory T cells. *Nature (London)* **349,** 737–738.
165. Picker, L. J., Kishimoto, T. K., Smith, C. W., Warnock, R. A., and Butcher, E. C. (1991). ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature (London)* **349,** 796–769.
166. Freitas, A. A., Rose, M., and Rocha, B. (1980). Random recirculation of small T lymphocytes from thoracic duct lymph in the mouse. *Cell. Immunol.* **56,** 29–39.
167. Freitas, A. A., Rose, M. L., and Parrott, D. M. (1977). Murine mesenteric and peripheral lymph nodes: a common pool of small T cells. *Nature (London)* **270,** 731–733.
168. Cahill, R. N. P., Poskitt, D. C., Hay, J. B., Heron, I., and Trnka, Z. (1979). The migration of lymphocytes in the fetal lamb. *Eur. J. Immunol.* **9,** 251–253.
169. Picker, L. J., Terstappen, L. W., Rott, L. S., Streeter, P. R., Stein, H., and Butcher, E. C. (1990). Differential expression of homing-associated adhesion molecules by T cell subsets in man. *J. Immunol.* **145,** 3247–3255.
170. Picker, L. J., Treer, J. R., Ferguson-Darnell, B., Collins, P. A., Bergstresser, P. R., and Terstappen, L. W. (1992). Control of lymphocyte recirculation in man. II. Differential regulation of the cutaneous lymphocyte-associated antigen (CLA), a tissue-selective homing receptor for skin-homing T cells. *J. Immunol.* (in press).

171. Allison, J. P., and Havran, W. L. (1991). The immunobiology of T cells with invariant γδ antigen receptors. *Annu. Rev. Immunol.* **9**, 679–705.
172. Tew, J. G., DiLosa, R. M., Burton, G. F., Kosco, M. H., Kupp, L. I., Masuda, A., and Szakal, A. K. (1992). Germinal centers and antibody production in bone marrow. *Immunol. Rev.* **126**, 99–112.
173. Lawrence, M. B., and Springer, T. A. (1991). Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell (Cambridge, Mass.)* **65**, 859–873.
174. von Andrian, U. H., Chambers, J. D., McEvoy, L. M., Bargatze, R. F., Arfors, K. E., and Butcher, E. C. (1991). Two-step model of leukocyte-endothelial cell interaction in inflammation: Distinct roles for LECAM-1 and the leukocyte beta 2 integrins *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7538–7542.
175. Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1992). Lymphocyte interactions with endothelial cells. *Immunol. Today* **13**, 106–112.
176. Butcher, E. C. (1992). Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. *Cell (Cambridge, Mass.)* **67**, 1033–1036.
177. Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1991). Lymphocyte interactions with endothelial cells. *Immunol. Today* **13**, 106–112.
178. Berg, E. L., Picker, L. J., Robinson, M. K., Streeter, P. R., and Butcher, E. C. (1991). Vascular addressins: Tissue selective endothelial cell adhesion molecules for lymphocyte homing. *In* "Cellular and Molecular Mechanisms of Inflammation. Vascular Adhesion Molecules" (C. G. Cochrane and M. A. Gimbrone, eds.), pp. 111–129. Academic Press, New York.
179. Tedder, T. F., Matsuyama, T., Rothstein, D., Schlossman, S. F., and Morimoto, C. (1990). Human antigen-specific memory T cells express the homing receptor (LAM-1) necessary for lymphocyte recirculation. *Eur. J. Immunol.* **20**, 1351–1355.
180. Bradley, L. M., Atkins, G. G., and Swain, S. L. (1992). Long-term CD4+ memory T cells from the spleen lack MEL-14, the lymph node homing receptor. *J. Immunol.* **148**, 324–331.
181. Shimizu, Y., Shaw, S., Graber, N., Gopal, T. V., Horgan, K. J., Van Seventer, G. A., and Newman, W. (1991). Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. *Nature (London)* **349**, 799–802.
182. Shimizu, Y., Van Seventer, G. A., Horgan, K. J., and Shaw, S. (1990). Regulated expression and binding of three VLA (beta 1) integrin receptors on T cells. *Nature (London)* **345**, 250–253.
183. Damle, N. K., and Doyle, L. V. (1990). Ability of human T lymphocytes to adhere to vascular endothelial cells and to augment endothelial permeability to macromolecules is linked to their state of post-thymic maturation. *J. Immunol.* **144**, 1233–1240.
184. Holzmann, B., McIntyre, B. W., and Weissman, I. L. (1989). Identification of a murine Peyer's patch-specific lymphocyte homing receptor as an integrin molecule with an alpha chain homologous to human VLA-4 alpha. *Cell (Cambridge, Mass.)* **56**, 37–46.
185. Hu, M. C.-T., Crowe, D. T., Weissman, I. L., and Holzmann, B. (1992). Cloning and expression of mouse integrin βp (β7): A functional role in Peyer's patch-specific lymphocyte homing. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8254–8258.
186. Nossal, G. J. V. (1992). Autoimmunity and self tolerance. *In* "The Autoimmune Diseases" (N. R. Rose and I. R. Mackay, eds.), pp. 27–46. Academic Press, San Diego.
187. Mackay, I. R., and Rose, N. R. (1992). Autoimmunity: Horizons. *In* "The Autoim-

mune Diseases" (N. R. Rose and I. R. Mackay, eds.), pp. 409–430. Academic Press, San Diego.

188. Schwartz, R. H. (1989). Acquisition of immunologic self-tolerance. *Cell (Cambridge, Mass.)* **57,** 1073–1081.

189. von Boehmer, H. (1988). The developmental biology of T lymphocytes. *Annu. Rev. Immunol.* **6,** 309–326.

190. Guerder, S., and Matzinger, P. (1992). A fail-safe mechanism for maintaining self-tolerance. *J. Exp. Med.* **176,** 553–564.

191. Adelstein, S., Pritchard-Briscoe, H., Loblay, R. H., and Basten, A. (1990). Suppressor T-cell memory. *Curr. Top. Microbiol. Immunol.* **159,** 123–138.

192. Linton, P. J., Rudie, A., and Klinman, N. R. (1991). Tolerance susceptibility of newly generating memory B cells. *J. Immunol.* **146,** 4099–4104.

193. Webb, S., Morris, C., and Sprent, J. (1990). Extrathymic tolerance of mature T cells: Clonal elimination as a consequence of immunity. *Cell (Cambridge, Mass.)* **63,** 1249–1256.

194. Lee, W. T., and Vitetta, E. S. (1992). Memory T cells are anergic to the superantigen staphylococcal enterotoxin B. *J. Exp. Med.* **176,** 575–579.

194a. Fuchs, E. J., and Matzinger, P. (1992). B cells turn off virgin but not memory T cells. *Science* **258,** 1156–1159.

195. Sterzl, J., and Silverstein, A. M. (1967). Developmental aspects of immunity. *Adv. Immunol.* **6,** 337–459.

196. Rammensee, H. G., Kroschewski, R., and Frangoulis, B. (1989). Clonal anergy induced in mature V beta 6+ T lymphocytes on immunizing Mls-1b mice with Mls-1a expressing cells. *Nature (London)* **339,** 541–554.

197. Ohashi, P. S., Oehen, S., Buerki, K., Pircher, H., Ohashi, C. T., Odermatt, B., Malissen, B., Zinkernagel, R. M., and Hengartner, H. (1991). Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell (Cambridge, Mass.)* **65,** 305–317.

198. Schlomchik, M. J., Marshak-Rothstein, A., Wolfowicz, C. B., Rothstein, T. L., and Weigert, M. G. (1987). The role of clonal selection and somatic mutation in autoimmunity. *Nature (London)* **328,** 805–811.

199. Strasser, A., Whittingham, S., Vaux, D. L., Bath, M. L., Adams, J. M., Cory, S., and Harris, A. W. (1991). Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 8661–8665.

200. Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (London)* **356,** 314–317.

201. Gammon, G., and Sercarz, E. (1989). How some T cells escape tolerance induction. *Nature (London)* **342,** 183–185.

202. Pircher, H., Rohrer, U. H., Moskophidis, D., Zinkernagel, R. M., and Hengartner, H. (1991). Lower receptor avidity required for thymic clonal deletion than for effector T-cell function. *Nature (London)* **351,** 482–485.

This article was accepted for publication on 19 October 1992.

ADVANCES IN IMMUNOLOGY, VOL. 53

Recognition of Bacterial Endotoxins by Receptor-Dependent Mechanisms

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I. Introduction

It is now generally agreed that the clinical problems associated with gram-negative sepsis occur as a result of the host response to endotoxin (lipopolysaccharide; LPS[1]), a component of the outer membrane of all gram-negative bacteria (Rietschel *et al.*, 1985). Typical symptoms of septic shock are fever, hypotension, diffuse intravascular coagulation (DIC), and metabolic derangements. These changes are seen clinically regardless of the type of infecting gram-negative organism and are reproduced in animal models by injections of purified LPS. Monocytes/macrophages (MOs) play a central role in mediating these changes (Beutler and Cerami, 1986; Tracey *et al.*, 1987; Ulevitch *et al.*, 1989; Johnston, 1991). MOs respond to picomolar concentrations of LPS by releasing mediators that act as autocrine and paracrine signals (Nathan, 1987). These mediators include cytokines such as the interleukins (IL-1, IL-6, and IL-8) and tumor necrosis factor (TNF; cachectin), lipid mediators derived from arachidonic acid, and toxic oxygen radicals. Rapid progress is being made in defining how these mediators produce the biologic responses to LPS. One of the best examples of this progress is found in studies of

[1] Abbreviations: ASD–LPS, LPS with covalently attached sulfosuccinimidyl-2-(*p*-azidosalicyamino)-1,3′-dithiopropionate; bmMO, bone marrow-derived macrophage; BPI, bactericidal/permeability-increasing protein; CETP, cholesteryl ester transfer protein; E-LPS, erythrocytes coated with LPS; GPI, glycan phosphatidylinositol; HKLM, heat-killed *Listeria monocytogenes;* HKSA, heat-killed *Staphylococcus aureus;* IL, interleukin; KDO, 2-keto-3-deoxyoctulosonate, i.e., 3-deoxy-D-mannooctulosonate; LBP, LPS-binding protein; LPS, lipopolysaccharide or endotoxin; MO, monocyte/macrophage; PEM, peritoneal exudate MO; PG, peptidoglycan; PHA, phytohemagglutinin; PI-PLC, phosphatidylinositol-specific phospholipase C; PMA, phorbol 12-myristate B-acetate; RBC, red blood cell; RSLA, lipid A isolated from *Rhodopseudomonas sphaeroides; SASD, sulfosuccinimidyl-2-(p*-azidosalicamino)-1,3′-dithiopropionate; 70Z/3–RSV cells, 70Z/3 cells transfected with the pRc/RSV vector only; 70Z/3–hCD14 or 70Z/3–rCD14 cells, 70Z/3 cells transfected with human (h) or rabbit (r) CD14 cDNA, respectively; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

the role of TNF in mediating endotoxin effects. The release of TNF into blood within 30–60 minutes following intravenous injection of LPS in animals (Beutler *et al.*, 1985a,b; Tracey *et al.*, 1987; Mathison *et al.*, 1988) and humans (Michie *et al.*, 1988) is one of the most rapid of the cytokine responses. The importance of TNF as a mediator of LPS action has been established in animal models using anti-TNF antibodies (Beutler *et al.*, 1985a,b; Tracey *et al.*, 1987) and has been supported by studies in humans (Michie *et al.*, 1988; Tracey, 1991).

A crucial part of the response to infection with gram-negative organisms involves host recognition of LPS; herein also lies the paradox of this host/pathogen interaction that has been most aptly described by Lewis Thomas (1974): "The gram-negative bacteria . . . display lipopolysaccharide . . . in their walls and these macromolecules are read by our tissues as the very worst of bad news. When we sense lipopolysaccharide we are likely to turn on every defense at our disposal; . . . Cells believe that it signifies the presence of gram-negative bacteria, and they will stop at nothing to avoid this threat."

The importance of recognizing LPS in responding to gram-negative infection is uniquely illustrated by the LPS-resistant mouse strain, C3H/HeJ, refractory to the toxic effects of LPS but hypersensitive to gram-negative infections (Vas *et al.*, 1973). Thus, the inability to respond to LPS compromises host defenses against gram-negative sepsis whereas an uncontrolled response to LPS results in shock, DIC, and multiorgan failure.

One of the pivotal and as yet unanswered questions about how LPS acts is the mechanism whereby MOs recognize LPS and generate transmembrane signals. In this review we will consider recent progress in identifying the cellular molecules that function as LPS receptors. This review will focus on data obtained with cells of monocytic origin, but will consider findings derived from studies of other cell types for illustration of specific points. In general, we will limit the review to work published since 1986 and we will not discuss investigations of mechanisms of transmembrane signaling that initiate lipid A-dependent cell stimulation.

II. Structure of Bacterial Lipopolysaccharide

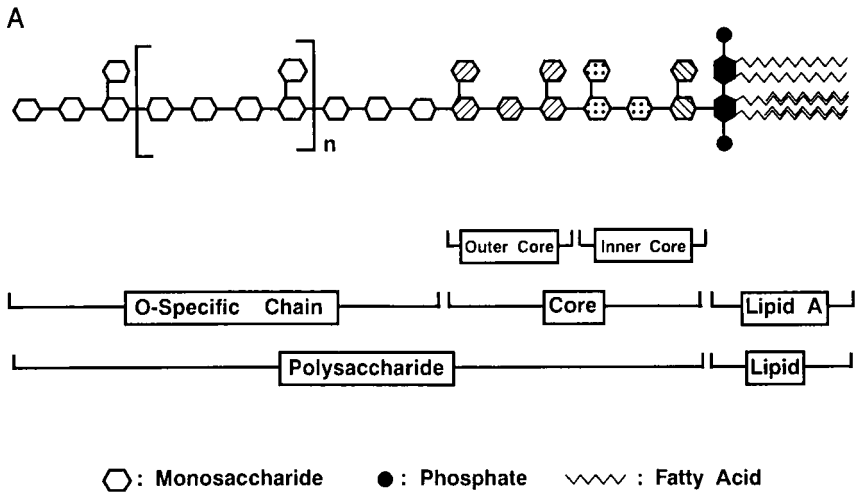
LPS is a structural component of the outer membrane of all gram-negative bacteria. To understand how LPS stimulates cells, one first must consider the structure of LPS (reviewed in Rietschel *et al.*, 1985; Raetz, 1990). LPS consists of two chemically dissimilar do-

mains: (1) the hydrophilic, polysaccharide core and O-antigen structures and (2) a hydrophobic region known as lipid A. Figure 1 shows a schematic structure for LPS from *Escherichia coli* (Fig. 1A) and the detailed chemical structure of lipid A (Fig. 1B). The O-specific chain consists of a complex, repeating oligosaccharide of up to five repeating sugar residues. There is a great deal of structural diversity when O-specific chain structures are compared within different gram-negative bacteria. In contrast, the structural variation within the core region is low. For example, only one core structure has been defined for all of the *Salmonella* serotypes and six different core structures have been characterized for more than 100 different serotypes of *E. coli* (Jansson *et al.*, 1981; K. Jann and B. Jann, 1984).

The structure of lipid A found in diverse gram-negative organisms is highly conserved, and lipid A is not found elsewhere in nature. Virtually all LPS-induced biologic responses are lipid A dependent (Rietschel *et al.*, 1982; Kotani *et al.*, 1983). The most compelling evidence supporting the concept that lipid A is the biologically active moiety of LPS derives from studies with synthetic lipid A. This product has full endotoxic activity (Takada and Kotani, 1989). Thus recognition of the lipid A of LPS by cells must be the initial step in LPS-induced cellular responses.

The lipid A from enterobacteria such as *E. coli* or *Salmonella* consists of a β-1′,6-linked D-glucosamine disaccharide phosphorylated in positions 1 and 4′, and in intact LPS the 2-keto-3-deoxyoctulosonate (KDO) is carried at the hydroxyl in position 6′. A common feature of lipid A is the presence of up to 4 mol of (*R*)-3-hydroxytetradecanoic acids in a symmetrical distribution linked to the two glucosamine residues of the backbone. Each glucosamine is substituted by one ester and one amide-linked fatty acid. Some of the 3-hydroxyl groups are additionally 3-O-acylated by tetradecanoic acid. It is these acyl-linked fatty acids that are subject to hydrolysis by an enzyme recently isolated and characterized by Munford and Hall (1989). Many of the steps of lipid A biosynthesis in bacteria have been elucidated by the work of Raetz and co-workers (Raetz, 1990). In particular, these studies have led to the identification and isolation of biosynthetic intermediates with interesting endotoxic properties.

Lipid A from nonenterobacterial organisms can be separated into two groups. One type of lipid A possesses a structure that is quite distinct from that described above and does not express typical endotoxic activities (reviewed in Mayer, 1984); the other type is from organisms such as *Pseudomonas aeruginosa*, *Fusobacterium nucleatum*, *Chromobacterium violaceum*, *Vibrio cholera*, and *Bordetella*



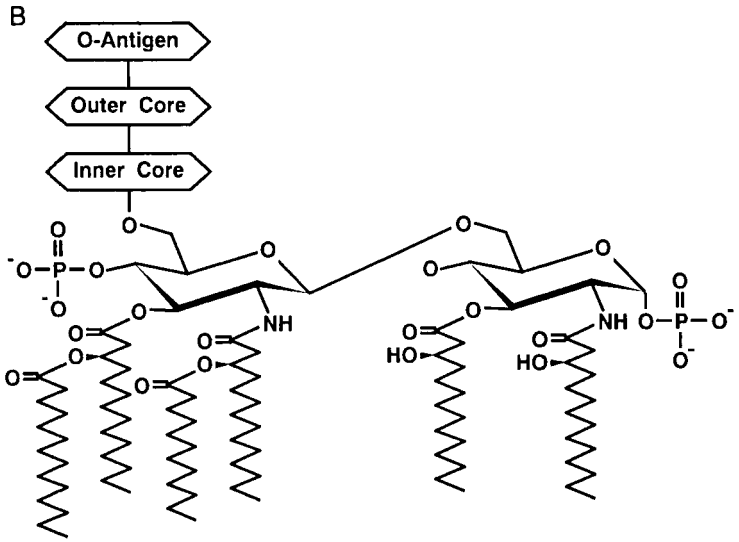


FIG. 1. (A) Schematic structure of *Escherichia coli* lipopolysaccharide. (B) Chemical structure of lipid A.

pertussis and displays endotoxic activity and a lipid A architecture similar to that determined for enterobacteria. Thus, there are structural features of lipid A that are important for biologic activity, although the exact chemical composition of an "active site" for lipid A has not been defined. Little is known about the three-dimensional structure of LPS or lipid A dispersions in water or the preferred conformation for biologic activity.

III. Recognition of Lipid A by Receptor-Mediated Mechanisms

Consideration has been given to two distinct mechanisms that explain how LPS stimulates cells via lipid A-dependent mechanisms: (1) Lipid A-dependent cell activation occurs via a specific membrane receptor that has a lipid A-binding site and initiates transmembrane signaling following receptor occupancy, or (2) via the "nonspecific" dissolution of LPS within the lipid phase of the membrane, resulting in a sufficient change in membrane structure to initiate transmembrane signaling.

The potency and the specificity of cellular responses to LPS suggest that a specific membrane receptor is involved. LPS is active at concentrations that are equal to or below those observed with protein agonists such as hormones or cytokines. For example, recent reports show LPS-induced changes in MO phenotype with as few as 10 molecules of LPS per cell (Pabst *et al.*, 1982; Mathison *et al.*, 1990). Unfortunately, classical methods using radiolabeled ligand to demonstrate specific, saturable binding of lipid A have failed to yield unambiguous evidence for a membrane receptor recognizing lipid A. Nevertheless, a number of experimental observations recently put forth firmly establish the existence of a specific LPS receptor that binds lipid A and initiates cell stimulation. These include the following observations:

1. Partial structures of lipid A prepared by organic synthesis or enzymatic deacylation of natural lipid A act as LPS antagonists, suggesting that there are specific membrane structures, most likely proteins, that recognize lipid A.
2. Application of chemical crosslinking strategies or radioactive lipid A binding to nitrocellulose-immobilized membrane proteins has led to the identification by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of proteins that are candidates for an LPS/lipid A receptor.
3. MO responses to LPS are regulated by adaptation or homol-

ogous desensitization, a mechanism that involves agonist/receptor interactions (Hausdorff *et al.*, 1990).

4. A specific protein, CD14, present on the plasma membranes of all cells of the monocyte/macrophage lineage, has recently been unambiguously shown to be a receptor for complexes of LPS and the plasma protein known as lipopolysaccharide-binding protein (LBP).

Importantly, CD14 and LPS–LBP complexes are important in mediating cellular recognition and responses to lipid A under physiologic conditions. Details of points 1–4 will be reviewed in the following sections of this article.

IV. Lipid A Structure–Function Relationships: Identification of Lipid A Antagonists

The use of nontoxic LPS or partial lipid A structures derived from natural sources or organic synthesis has provided evidence for the existence of a specific lipid A receptor. These substances can block the action of bioactive LPS/lipid A and in this regard act as if they are receptor antagonists.

Observations of Mayer and colleagues (Mayer, 1984) and others (Strittmatter *et al.*, 1983) demonstrating that LPS (or its lipid A) prepared from *Rhodopseudomonas sphaeroides* is not toxic in animal models of lethal endotoxemia suggested the utility of nontoxic forms of lipid A as antagonists. This has proved to be the case. The diphosphoryl lipid A isolated from *R. sphaeroides* LPS (RSLA) does not stimulate TNF production by RAW264.7 cells; a 10- to 100-fold excess of RSLA competes with active LPS preparation in TNF induction, suggesting an antagonism at the level of a lipid A receptor (Takayama *et al.*, 1989). Studies with the LPS-sensitive murine pre-B cell line have added additional support to the concept that RSLA acts as a receptor antagonist. LPS induces the synthesis of immunoglobulin light chains by stimulating the transcription of this gene in 70Z/3 cells. A 100-fold excess of RSLA blocks LPS-induced gene expression in these cells without changing the same responses to interferon-γ (IFN-γ) (Kirkland *et al.*, 1991).

Recently it was shown that the RSLA can act as an endotoxin antagonist *in vivo*, preventing the rapid rise in serum TNF induced by LPS injection (Quereshi *et al.*, 1991). The structural basis for the lack of toxicity or potential receptor antagonism of RSLA has not been defined. Examination of the fatty acid composition of the lipid A reveals three structural features not found in toxic lipid A from enteric

strains: (1) shorter O-linked hydroxy fatty acids, (2) an unsaturated fatty acyl chain, and (3) an N-linked β-ketomyristate. Comparative modeling of the structures of toxic and nontoxic lipid A types might reveal information to help in the design of more potent receptor antagonists.

Studies of lipid A biosynthesis in bacteria unable to synthesize 3-deoxy-D-mannooctulosonic acid have resulted in identification of a lipid A partial structure known as lipid IVa (reviewed in Raetz, 1990). Lipid IVa does not have agonist activity when used to stimulate human cells, although it does induce TNF release from RAW264.7 cells, and synthetic lipid IVa causes lethality in galactosamine-sensitized mice. Of interest are the recent data of Kovach *et al.* (1990) showing that lipid IVa is a potent antagonist of lipid A-dependent TNF production by human monocytes measured in freshly collected whole blood *ex vivo*. A 10- to 100-fold excess of lipid IVa (weight/weight) prevented a lipid A-dependent increase in TNF mRNA, suggesting that lipid IVa blocks an early step in LPS/MO interaction. In contrast, at least 100,000-fold higher concentrations of lipid IVa versus LPS were required to elicit a TNF response, indicating limited agonist activity. Lipid IVa and complete lipid A differ only by the presence of additional fatty acids esterified on the hydroxyl fatty acids linked to the disaccharide background. Thus, these acyloxyacyl chains appear to play some role in triggering the lipid A receptor following receptor occupancy in MOs from certain species. However, the antagonist activity of lipid IVa suggests that the acyloxyacyl chains are not required for binding to the lipid A receptor.

Organic syntheses have yielded a number of different partial lipid A structures with both agonist/antagonist activity. A recent study by Loppnow *et al.* (1989) reported on dose–response characteristics of a series of natural and synthetic lipid A partial structures for the induction and release of IL-1 from human monocytes. These studies also demonstrated the importance of the acyloxyacyl chains for agonist activity. Most importantly, a synthetic partial lipid A structure that is analogous (compound 406) to the naturally occurring lipid IVa was a potent antagonist for lipid A-mediated IL-1 release. Of interest are data showing that cells can be pretreated with a high concentration of compound 406 for 1 hour, washed, and when recultured with lipid A the cells do not synthesize IL-1. In contrast, pretreatment with compound 406 did not change the IL-1 response induced by heat-killed gram-positive bacteria or phytohemagglutinin (PHA).

The previous data must be interpreted cautiously in the absence of formal proof that these substances act as receptor antagonists. Lack-

ing are proper binding isotherms that provide evidence that the LPS agonist and the inhibitory substances bind to the same site. However, the use of partial lipid A structures will undoubtedly contribute to our understanding of how LPS acts.

V. Identification of Membrane Proteins That Interact with Lipid A

The difficulty in demonstrating specific, saturable binding of LPS to MOs has made biochemical strategies used to identify other membrane receptors difficult to implement. Currently the approaches that promise to yield the most information about the identity of a lipid A receptor involve the use of photochemical crosslinking strategies or ligand blotting assays using high specific activity radioactive LPS or lipid A. In many isolates of naturally occurring LPS there are primary amino groups present as a result of substitutions with ethanolamine or amino sugars. Although the content of amino groups is not stoichiometric with respect to lipid A, their presence permits covalent substitution with groups that facilitate radioiodination and photochemical crosslinking (Wollenweber and Morrison, 1985). LPS derivatives incorporating sulfosuccinimidyl-2-(*p*-azidosalicyamino)-1,3′-dithiopropionate (SASD) have been used by several groups to identify membrane proteins that are candidates for lipid A receptors.

A series of papers have been published by Morrison and colleagues using an adduct of SASD and LPS isolated from *E. coli* O111:B4 (Lei and Morrison, 1988a,b; Roeder *et al.*, 1989; Lei *et al.*, 1991) to probe cells for membrane proteins that recognize lipid A/LPS. Crosslinking of ASD–O111:B4 LPS was studied using two-dimensional polyacrylamide gel electrophoresis (2D PAGE) to analyze the characteristics of proteins that are radiolabeled following photolysis. The experiments performed by Morrison and co-workers typically used 50 μg/ml of ASD–O111:B4 LPS. An 80-kDa LPS-binding protein with an approximate p*I* of 6.5 was identified on a variety of LPS-responsive cells, including splenic B lymphocytes, T lymphocytes, and macrophages. The 80-kDa protein was also found on a variety of cell lines, including the pre-B cell line 70Z/3 and the T cell lines YAC-1 and EL-4. Interestingly, the 80-kDa protein was also determined to be present on splenocytes from C3H/HeJ mice that are hyporesponsive to LPS (Flebbe *et al.*, 1990). In contrast ASD–O111:B4 did not label an 80-kDa protein in either human erythrocytes or the undifferentiated murine cell line So2/O. An estimate of 5–10,000 LPS-binding sites per murine splenocyte was made in the initial studies. One possible pitfall in this work is the use of

LPS concentrations that exceed that required to stimulate a biologic response. For example, 70Z/3 cells typically require 0.1–1 μg/ml LPS for a maximum biologic response and MO responses to LPS are noted at LPS concentrations <1 ng/ml. Nevertheless, studies with ASD–LPS have revealed new information about membrane proteins that bind to lipid A.

Crosslinking of ASD–O111:B4 LPS to the 80-kDa protein was blocked with the parent O111:B4 LPS (not derivatized with SASD), with LPS from *Salmonella minnesota* (wild type) and its lipid A, but not with an SASD adduct of human IgG. Crosslinking of ASD–O111:B4 LPS also occurred at 4°C in the presence of 10 m*M* sodium azide. Membrane localization of the 80-kDa protein was established by isolation of radiolabeled splenocyte membranes and by the use of the detergent octylglucoside to release radiolabeled membrane proteins. However, a rigorous characterization of the subcellular localization of the 80-kDa protein has not been reported (Ooi *et al.*, 1987; Lei and Morrison, 1988a,b; Roeder *et al.*, 1989).

A survey of the reactivity of mononuclear cells from a variety of species with ASD–O111:B4 LPS revealed that the 80-kDa protein was detected in all species that demonstrate endotoxin susceptibility and was not found in the cells of chickens and frogs, species that are resistant to the endotoxic effects of LPS (Roeder *et al.*, 1989). These data have been interpreted to reflect the conservation of the 80-kDa protein in species that demonstrate sensitivity to LPS. However, these same investigators have also reported that an 80-kDa protein was radiolabeled by ASD–O111:B4 LPS on rat trophoblast cells derived from placental tissue of midgestation rats (Hunt *et al.*, 1989). These cells are not sensitive to LPS and no explanation was provided to account for the observed crosslinking.

Morrison and colleagues (Bright *et al.*, 1990; Chen *et al.*, 1990) described two hamster monoclonal antibodies (mAbs) prepared by immunizing with partially purified 80-kDa protein. Both antibodies are IgM. These antibodies recognize the 80-kDa protein in enzyme-linked immunosorbent assays (ELISAs) performed with partially purified 80-kDa protein and bind to glutaraldehyde-fixed 70Z/3 cells. Addition of O111:B4 LPS to 70Z/3 cells competes with antibody binding, suggesting that both LPS and the anti-80-kDa antibodies bind to a common site. The binding of one antibody, 3D7, to partially purified 80-kDa protein is prevented by pretreatment of the antigen with 1.0 m*M* sodium periodate, whereas the binding of the other monoclonal antibody, 5D3, is prevented by pretreatment with 0.1 μg/ml proteinase K.

The 5D3 monoclonal antibody has been shown to stimulate tumor cell cytotoxicity of murine bone marrow-derived macrophages (bmMOs) (Chen *et al.*, 1990). Addition of 5D3 to bmMOs induces tumor cell killing that is also enhanced by coaddition of IFN-γ. Similar levels of tumor cell cytotoxicity were induced with 1–10 μg/ml of LPS and LPS-induced killing was also enhanced with coaddition of IFN-γ. These data indicate that 5D3 may act as an LPS mimetic. Additional studies examined the binding of 5D3 to bmMOs derived from LPS-normoresponsive mice (C3H/HeN) and LPS-hyporesponsive mice (C3H/HeJ). Equivalent binding of 5D3 was observed in bmMOs from either strain. However, 5D3 was unable to induce tumor cell cytotoxicity when added to IFN-γ-treated bmMOs from C3H/HeJ mice, although these cells were induced by heat-killed *Listeria monocytogenes* (HKLM) to kill target cells. These findings also support the contention that 5D3 acts as an LPS mimetic. In the same experiments, bmMOs from C3H/HeN mice were induced to kill tumor cells by LPS, 5D3, or HKLM.

Interestingly, the 5D3 mAb also has been shown to protect D-galactosamine-sensitized mice against the toxic effects of both LPS and tumor necrosis factor (TNF). In contrast, LPS-hyporesponsive mice sensitized with D-galactosamine were not protected by 5D3 pretreatment. Administration of 5D3 80 minutes prior to LPS challenge afforded protection, whereas injection of the mAb 20 minutes prior to challenge did not protect.

As suggested by Morrison and colleagues, 5D3 recognizes the 80-kDa membrane protein that serves as an LPS receptor, and engagement of the 80-kDa protein with 5D3 generates a transmembrane signal. Transmembrane signaling induces bmMOs to kill tumor cells and induces tolerance to the toxic effects of LPS and TNF in mice. In this regard, 5D3 is an agonist with properties that are similar to LPS. A concern is the presence of contaminating LPS in the preparation of 5D3; this issue has been addressed systematically by Morrison and colleagues and these investigators believe they have ruled out the potentially confounding effects of LPS contamination in preparations of 5D3.

Perhaps the strongest argument in favor of the 80-kDa protein being an LPS receptor and 5D3 acting as an LPS mimetic derives from results noted above using C3H/HeJ cells. However, even these data must be interpreted cautiously. It has been well documented that engagement of monocyte surface proteins with mAbs, with simultaneous engagement of Fc receptors, leads to monocyte stimulation. This phenomenon has been described in detail by MacIntyre *et al.*

(1989). To provide an unambiguous interpretation of the effects of 5D3, controls with hamster IgM mAbs recognizing an MO surface protein not involved with LPS recognition are required. Because C3H/HeJ MOs have reduced numbers of Fc receptors (Vogel *et al.*, 1983), it is important to have controls that exclude contributions of Fc receptor engagement.

The results of Morrison and co-workers support the concept that an 80-kDa membrane protein identified by photochemical crosslinking on lymphoreticular and other cell types may serve as a receptor for LPS that regulates lipid A-dependent cellular responses. Further analysis of the function of this protein awaits a full description of its molecular properties. This will permit the development of additional immunologic and molecular biologic reagents to evaluate the function of the 80-kDa protein in cellular recognition of LPS.

More recent studies by Dziarski (1991a,b) provide an alternative interpretation concerning the function of the 80-kDa protein described by Morrison and colleagues. Dziarski has used photochemical crosslinking strategies with ASD–peptidoglycan (PG) and ASD–LPS to demonstrate that PG and LPS bind to the same site, which has been identified as a 70-kDa protein identified by SDS–PAGE. Compelling evidence supporting the conclusion that ASD–LPS and ASD–PG bind to the same membrane protein is derived from competitive binding experiments and one-dimensional peptide maps of the crosslinked product formed with ASD–LPS or ASD–PG. It is suggested that this membrane protein similarly recognizes the repeating backbone of PG and the diglucosamine of lipid A. The difference in apparent size, 80 versus 70 kDa, is simply explained by differences in the protein standards used in SDS–PAGE. It is clear that additional studies will be required to elucidate the role of this protein in transducing signals from lipid A or PG. Further, the unambiguous assignment of identity between the putative "PG receptor" and the "lipid A receptor" will only be made after complete evaluation of the primary structure of the crosslinked molecule.

A novel and possibly very useful long-term approach to identifying MO receptors for LPS has recently been described by Hara-Kuge *et al.* (1990). The murine macrophage-like cell line J774.1 was shown to display specific binding of LPS that was sensitive to proteinase K treatment. The J774.1 cells were mutagenized with ethyl methane sulfonate, selecting for cells that are resistant to the cytotoxic effects of LPS. One such clone, termed LR-9, was shown not to bind O111:B4 LPS and not to display biologic responses to LPS. The biologic responses measured were LPS-induced priming for 12-*O*-

tetradecanoylphorbol-13-acetate (TPA)-induced superoxide anion production and LPS-induced arachidonate release; studies of LPS-induced cytokine production were not reported. The data reported by Hara-Kuge *et al.* (1990) are consistent with the contention that the phenotype of the LR-9 cells is that of a cell lacking a membrane receptor for LPS. To characterize further the interactions of LPS, the parental and mutant line cells were exposed to ^{125}I-labeled ASD–O111:B4 LPS at 4°C and then subjected to ultraviolet light (UV)-induced crosslinking. Analysis of crude cell membranes from crosslinked parental and LR-9 cells by SDS–PAGE and autoradiography revealed LPS crosslinking to two proteins, 65 and 55 kDa, in the J774.1 cells and the complete absence of such moieties in the LR-9 cells. Interestingly, there was no labeling of an 80-kDa protein noted in the autoradiograms. Recent evidence has been presented that the LPS-unresponsive mutants lack the membrane protein CD14 (M. Nishijima, personal communication). Additional discussion of the role of CD14 in LPS recognition will be considered in a subsequent section of this article.

Crosslinking of ^{125}I-labeled ASD–Re595 LPS has also been used by Kirkland, Tobias, and co-workers (Kirkland *et al.*, 1990) to identify LPS-binding proteins in a murine pre-B cell line, 70Z/3. This study failed to demonstrate crosslinking to an 80-kDa protein, but instead labeled membrane-localized proteins of 18 and 25 kDa. Crosslinking of these proteins was saturable with respect to LPS and was blocked by a variety of LPS and lipid A sources, including RSLA. No crosslinking was observed at 4°C. An estimate of at least 6000 binding sites per cell was calculated from the crosslinking data. The experimental approach utilized by Kirkland and colleagues appeared to be identical to that explored by Morrison and co-workers; reasons for the inability to detect the 80-kDa protein are not apparent. Potential differences between these studies include the use of different LPS preparations (smooth versus rough forms of LPS), the concentrations of ASD–LPS used to label cells, and the methods used to solubilize membrane proteins. No additional data about the structure of the cross-linked 18- or 25-kDa proteins are available.

Lipid IVa can be radiolabeled to high specific activity with ^{32}P (Hampton *et al.*, 1988), and the use of ^{32}P-labeled lipid IVa has been recently described to characterize lipid A-binding sites in a murine macrophage cell line, RAW264.7 (Hampton *et al.*, 1988). Initial studies were performed to characterize lipid IVa binding to RAW264.7 cells. An estimate of approximately 3×10^6 molecules of lipid IVa bound per cell was made, assuming the lipid IVa monomer is the

bound species. If higher molecular weight oligomers of LPS are bound, the calculated number for binding sites would be reduced. Similar binding data were obtained for another murine macrophage cell line (J774); in contrast, two fibroblast cell lines (CHO-K1 and L929) did not reveal saturable binding. Binding is blocked by unlabeled lipid IVa and Re595 LPS whereas various pure phospholipids are essentially without effect. Pretreatment of RAW cells with either trypsin or proteinase K prevents specific binding of lipid IVa.

To probe the identity of the putative receptors for lipid A, a ligand blotting strategy was utilized. To do this, cellular proteins are immobilized on nitrocellulose and examined for LPS binding using either radioactive LPS or anti-LPS antibodies to detect bound LPS. Lysates of whole cells or solubilized nuclear or plasma membrane proteins were run in SDS–PAGE, transferred to nitrocellulose, and analyzed in a ligand blotting assay with ^{32}P-labeled lipid IVa. These studies revealed binding of lipid IVa to 95-kDa protein that was present in cell membrane fractions. At present no additional information is available about the functional or biochemical properties of this protein.

A ligand blotting approach has also been used to study LPS-binding proteins in erythrocyte membranes. LPS has been shown to bind to murine red blood cells (RBCs) via Pronase-sensitive LPS-binding protein in the RBC membrane (Kirikae *et al.*, 1988). Membrane proteins from the RBCs were subjected to SDS–PAGE and transferred to nitrocellulose. Using a ligand blotting assay, wherein bound Re595 LPS is detected with immunopurified anti-Re595 antibody, a 96-kDa LPS-binding protein was detected. Erythrocytes obtained from the LPS-hyporesponsive C3H/HeJ mice revealed the presence of an identical 96-kDa LPS-binding protein. The relationship of this protein to the 95-kDa protein detected in RAW264.7 cells is unknown at present.

A series of reports by Wright and colleagues (Wright and Jong, 1986; Wright *et al.*, 1989a) suggested that heterodimeric surface receptors containing CD11a, CD11b, or CD11c and CD18 recognize LPS-bearing particles or bacteria. This interaction was demonstrable only with lipid A, lipid IVA, or with LPS from organisms with the rough-form phenotype. Wright and co-workers showed that unopsonized bacteria and LPS-coated erythrocytes bind to MOs and that this binding is blocked by a variety of monoclonal antibodies, including the anti-β chain antibody, IB4. Subsequent studies demonstrated that CR3 (CD11b/CD18) has distinct binding sites that separately recognize arginine/glycine/aspartate-containing peptides and lipid

A. It was suggested that these heterodimeric receptors may have a binding site recognizing sugar phosphates that are structurally similar to the sugar phosphates present in lipid A. Evidence for the participation of CD18 in recognizing lipid A-containing particles was also derived from the use of neutrophils deficient in CD18; these cells did not bind erythrocytes coated with the rough-form LPS or lipid IVa or rough-form *E. coli*. Thus, a variety of experimental approaches support the concept that heterodimeric receptors containing CD18 recognize lipid A. However, these studies failed to address the role of CD18-dependent recognition of lipid A in initiating cellular responses to LPS.

Adherence of smooth-form organisms via the CD11/CD18 pathway was negligible despite the presence of rough and semi-rough LPS in the outer membrane of smooth-form organisms. Furthermore only unopsonized particles were used in these studies and it seems highly unlikely that MOs or neutrophils would encounter unopsonized bacteria under physiologic conditions. No data are reported showing that smooth-form LPS isolates compete for adherence of either rough-form bacteria or LPS-coated particles, despite the knowledge that MOs respond equally well to both rough- and smooth-form LPS. Thus, data from adherence studies alone are not sufficient to invoke a general role for CD18 in MO recognition of lipid A leading to cellular responses. Studies have been reported using MOs and neutrophils from CD18-deficient patients (Wright *et al.*, 1990a). These experiments failed to show any decrease in LPS-induced cytokine production or priming by LPS for enhanced superoxide anion production. Therefore, CD18 and its associated proteins are not involved in cellular recognition of LPS that leads to transmembrane signaling.

Recognition of lipid IVa by the scavenger [acetylated low-density lipoprotein (LDL)] receptor has also been described (Hampton *et al.*, 1991). Binding to this receptor facilitates the entry and subsequent dephosphorylation of lipid IVa at the 1-position. However, it is clear that this receptor-mediated pathway has nothing to do with transmembrane signaling leading to cytokine induction because acetylated LDL completely blocks lipid IVa binding without altering LPS-induced cytokine production. The biologic function of lipid IVA with the scavenger receptor is uncertain, especially because uptake of smooth-form LPS via the scavenger receptor has not been described. The scavenger receptor or the CD11/CD18 receptors may represent pathways that facilitate uptake and intracellular degradation of LPS, but considerably more work will be required to establish the physiological importance of these pathways.

In summary, at least five different proteins have been identified as lipid A-binding proteins, but in no single case has a role for these proteins in mediating transmembrane signaling been ascertained. Until the structure of these proteins is elucidated their importance cannot be established. In contrast, recent studies described below clearly show a crucial role for CD14 as an LPS receptor.

VI. The Role of the LBP/CD14 Pathway in LPS-Induced Macrophage Stimulation

The discovery of lipopolysaccharide-binding protein and the characterization of its effects on LPS have led to a new awareness of the molecular details of how LPS is recognized by cells. Specifically, a series of experiments performed during the past several years by Ulevitch, Tobias and co-workers, and Wright *et al.* have unequivocally shown that LBP potentiates cellular responses to LPS by facilitating the binding of complexes of LPS and LBP to the glycosylphosphatidylinositol (GPI)-anchored plasma membrane protein, CD14. Thus CD14 serves as an LPS receptor, and the data summarized below provide strong evidence for the participation of LBP and CD14 in cellular recognition of LPS.

The discovery of LBP occurred as a result of experiments that compared binding of LPS from the rough strain of *S. minnesota* Re595 (Re595 LPS) to high-density lipoproteins (HDLs) in normal and acute-phase serum (Tobias and Ulevitch, 1983; Tobias *et al.*, 1982, 1985). Isopycnic equilibrium density gradient centrifugation in CsCl of mixtures of ^{3}H-labeled Re595 LPS and serum revealed that the rate of binding of LPS to HDL was markedly slowed in acute-phase serum because of the formation of a stable complex between LPS and protein(s) in acute-phase serum. This phenomenon was noted in acute-phase serum of humans and of laboratory animals such as rabbits, rats, and mice. Fractionation of acute-phase rabbit serum revealed that a 60-kDa serum glycoprotein was responsible for complexing with Re595 LPS in acute-phase serum; this protein was named LPS-binding protein (Tobias *et al.*, 1986). Subsequent efforts resulted in the isolation of human LBP (Schumann *et al.*, 1990) and it is likely in the near future that LBP will be isolated from other animal species.

LBP is synthesized in hepatocytes as a 55-kDa polypeptide; it is glycosylated and released into blood as a 60-kDa glycoprotein (Ramadori *et al.*, 1990) Levels of LBP in normal rabbit serum have been estimated to be <0.5 μg/ml and between 1 and 10 μg/ml in normal

human serum (D. Leturcq and P. Tobias, unpublished data). After an acute-phase response in rabbits, LBP concentrations rise up to 50 μg/ml; LBP concentrations in excess of 300 μg/ml have been detected in acute-phase human serum (D. Leturcq and P. Tobias, personal communication).

Tobias and co-workers developed a solid-phase competitive binding assay that facilitated analysis of the binding of a variety of different LPS isolates to purified LBP (Tobias *et al.*, 1989). It was shown that LBP binds similarly to LPS isolated from the rough (R) or smooth (S) forms of gram-negative bacteria. These data showed that the presence of core and/or O-antigen polysaccharide did not significantly influence binding to lipid A. High-affinity binding also was seen with synthetic lipid A and a limited series of partial lipid A structures, including lipid IVa. In contrast, LBP does not bind to other charged polymers such as RNA, DNA, or heparin and even binds poorly to high-molecular-weight dextran sulfate, lipoteichoic acid, or lipid X. Studies with Re595 LPS provided an estimate for a K_d in the nanomolar range and a binding stoichiometry of 1:1.

Tobias *et al.* (1988) first reported that LBP was related to another LPS/lipid A-binding protein, namely, bactericidal/permeability-increasing protein (BPI). BPI is a 50-kDa protein localized in a granule fraction of neutrophils; it is an antibacterial protein specific for gram-negative bacteria (Weiss *et al.*, 1978, 1984; Ooi *et al.*, 1987). Cloning of the cDNAs for LBP (Schumann *et al.*, 1990) or BPI (Gray *et al.*, 1989) and elucidation of the complete primary amino acid sequences confirmed the marked structural homology between these two LPS/lipid A-binding proteins. Protein sequence database searches also revealed that LBP and BPI are related to the plasma protein, cholesteryl ester transfer protein (CETP) (Drayna *et al.*, 1987).

The important question of the biological properties of complexes of LPS and LBP formed with LPS preparations containing varying lipid A : carbohydrate ratios has been recently addressed by Mathison and co-workers in studies with elicited rabbit peritoneal exudate MOs (PEMs) (Mathison *et al.*, 1992). Measurements of TNF induction with a series of different S-form isolates of LPS, with *S. minnesota* LPS isolated from wild-type and inner/outer core mutants (Re-Ra LPS), and with synthetic lipid A showed that the presence of LBP lowered the threshold stimulatory concentration of LPS and markedly enhanced the rate of TNF production with each of these different forms of LPS. These studies also showed that the LBP effects were observed with synthetic lipid A, but not with partial lipid

A structures such as lipid IVa. Heat-denatured LBP that no longer binds to LPS, serum albumin, or BPI did not substitute for LBP. In contrast to effects on LPS, LBP does not alter the characteristics of the TNF response induced by phorbol 12-myristate 13-acetate (PMA) or suspensions of heat-killed *Staphylococcus aureus* (HKSA). Thus the effects of LBP on MO stimulation are limited to LPS/lipid A.

Comparative studies measuring LPS-induced cytokine production in the presence and absence of LBP have been performed with several different MO sources that all express CD14 (R. J. Ulevitch, unpublished data): elicited rabbit peritoneal exudate MOs, murine bone marrow-derived MOs, human and rabbit alveolar MOs, and freshly isolated human blood monocytes or macrophage-like cell lines. Although the majority of studies have measured TNF production, the effects of LBP on LPS-stimulated release of cytokines such as IL-1, IL-6, or IL-8 are similar. Addition of LBP has several pronounced effects on cytokine production: the threshold stimulatory concentration of LPS is reduced by as much as 1000-fold and the rate of cytokine release is markedly accelerated. The latter effect results from more rapid and greater induction of cytokine mRNA. Another consequence of stimulation of MO with LPS–LBP complexes is an increase in the stability of mRNA when TNF mRNA half-lives are measured in LPS or LPS/LBP-stimulated cells treated with actinomycin D (Mathison *et al.*, 1992; Martin *et al.*, 1992). This effect most likely contributes to enhanced cytokine production induced with complexes of LPS and LBP.

Another function of LBP is to opsonize LPS-bearing particles such as intact gram-negative bacteria or erythrocytes coated with LPS (E-LPS) (Wright *et al.*, 1989b). This observation forms the basis of a quantitative rosetting assay that provided the initial recognition of the role of CD14 in recognizing LPS–LBP complexes. Addition of LBP to E-LPS promoted adherence of these particles to MOs, but did not result in phagocytosis or initiation of an oxidative burst. However, E-LPS opsonized with LBP significantly enhanced phagocytosis in the presence of suboptimal amounts of antierythrocyte IgG.

Initial attempts to characterize the membrane receptor for E-LPS–LBP revealed that it was mobile in the plane of the membrane, based on the finding that it could be downmodulated by coating surfaces with LPS–LBP complexes. From additional downmodulation experiments the known opsonic receptors, such as CR1, CR3, or FcR (I–III), were excluded as mediating the attachment of E-LPS–LBP, and monocytes from CD18-deficient patients bind E-LPS–LBP identically to cells from normal controls (Wright *et al.*, 1989b).

Subsequent experimentation revealed that the glycan phosphatidylinositol-anchored MO plasma membrane protein, CD14, mediates the attachment of E-LPS–LBP to MOs. In experiments utilizing a "mobile receptor downmodulation" strategy, Wright *et al.* demonstrated that coating surfaces with anti-CD14 monoclonal antibodies but not with monoclonal antibodies to other MO surface proteins prior to adherence of MOs to the surfaces markedly reduces binding of E-LPS–LBP (Wright *et al.*, 1990b). Pretreatment of MOs with phosphatidylinositol-specific phospholipase C (PI-PLC) under conditions in which >90% of the GPI-anchored CD14 is released also markedly inhibited adherence of E-LPS–LBP. Pretreatment of MOs with certain anti-CD14 monoclonal antibodies, such as 3C10, directly blocked rosetting of E-LPS–LBP.

Additional data supporting an important role for CD14 in mediating LPS-induced cytokine release is derived from studies with whole blood *ex vivo*. It is likely that the whole blood assay reflects events that occur *in vivo* after exposure to LPS. Exposure of whole blood, *ex vivo*, to LPS results in rapid release of cytokines such as TNF; concentrations of LSP <1 ng/ml are potent agonists in the whole blood assay (Desch *et al.*, 1989). Pretreatment of whole human blood preparations with anti-CD14 monoclonal antibodies such as 3C10 (Wright *et al.*, 1990b) results in a marked inhibition of LPS-induced TNF release. In contrast, addition of the monoclonal anti-CD18, IB4, had no effect on LPS-induced TNF release in the whole blood assay. Ulevitch, Tobias, and colleagues (Schumann *et al.*, 1990) also showed that immunodepletion of LBP from normal rabbit blood with goat antirabbit LBP IgG markedly inhibited LPS-induced TNF release; several other control antibodies, including goat antirabbit IgG or antirabbit fibronectin, were without effect. Moreover immunodepletion of LBP did not block TNF release induced by HKSA. Thus the LBP/CD14 pathway seems to play a central role in controlling cellular responses to LPS.

Lee and colleagues have provided rigorous proof for the importance of CD14 in controlling LPS responses. These studies utilized the LPS-responsive murine pre-B cell line, 70Z/3 (Lee *et al.*, 1992). The 70Z/3 cells respond to LPS by synthesizing κ light chains with consequent expression of membrane IgM (Weeks and Sibley, 1988). Using polymerase chain reaction (PCR) with primers specific for murine CD14, Lee *et al.* established that 70Z/3 cells do not contain detectable CD14 mRNA. The 70Z/3 cells were transfected with human or rabbit CD14 (hCD14 or rCD14) cDNA incorporated into a pRc/RSV vector; evidence for stable transfectants expressing CD14

was obtained using flow cytometry staining with anti-CD14 monoclonal antibodies. Pretreatment of 70Z/3–hCD14 cells with the enzyme PI-PLC resulted in a loss of surface staining by anti-CD14 monoclonal antibodies, indicating that the hCD14 is GPI anchored. Quantitation of CD14 with ^{125}I-labeled Fab fragments of an anti-Cd14 monoclonal antibody revealed that 70Z/3–hCD14 cells express 10,000–20,000 hCD14/cell (P. S. Tobias, personal communication).

When LPS is complexed with LBP the 70Z/3–hCD14 cells bind more LPS than do parental or 70Z/3 cells transfected with vector only (70Z/3–RSV). This increased binding is prevented by pretreatment of cells with PI-PLC or anti-CD14 monoclonal antibodies. When LPS dose–response curves for 70Z/3–hCD14 or 70Z/3–RSV cells are compared it was observed that expression of CD14 lowers the amount of LPS required to stimulate surface IgM expression by up to 10,000-fold. These studies were performed with R- or S-form LPS isolates as well as with synthetic lipid A. Stimulation of 70Z/3–hCD14 with LPS is accompanied by NF-κB activation. In contrast, the response of 70Z/3–hCD14, 70Z/3-RSV, or the parental cells to IFN-γ is identical.

The data of Lee *et al.* (1992) show that CD14 has a crucial role in recognition of LPS and initiation of cellular responses and provide support for the concept that stimulation by LPS may occur via CD14-dependent and -independent pathways. These studies raise several important questions: Is binding of LPS or LPS–LBP to CD14 sufficient to stimulate 70Z/3–CD14 cells? What is the relationship between CD14-dependent and -independent pathways? At present there are no data that address these issues. However, it is tempting to speculate that the LPS receptor on cells that normally express CD14, i.e., MOs or polymorphonuclear leukocytes, is a complex containing CD14 and an as yet unidentified additional membrane protein(s). This implies that binding of LPS or LPS–LBP complexes to CD14 alone is not sufficient to initiate cellular responses.

Two plausible models for a heterodimeric LPS receptor can be derived from what is known about the receptors for the cytokines IL-2 or IL-6. The IL-2 receptor (IL-2R) is a heterodimer (Hatakeyama *et al.*, 1989; Mills *et al.*, 1990) consisting of two ligand-binding subunits: the IL-2R α subunit, which binds IL-2 with low affinity but does not transduce signals or mediate ligand internalization, and the IL-2R β subunit, which binds IL-2 with low affinity but does transduce signals and mediate ligand internalization. When both the α and β subunits are present, high-affinity binding is observed (Hatakeyama *et al.*, 1989) and the threshold stimulatory dose of IL-2 is

substantially reduced. The receptor for IL-6 is a heterodimer consisting of an 80-kDa ligand-binding subunit and the gp130 subunit that does not bind ligand but does mediate transmembrane signaling (Hibi *et al.*, 1990; Akira *et al.*, 1990). Engagement of the 80-kDa subunit by IL-6 results in association of the ligand/receptor complex with gp130 and the initiation of transmembrane signaling.

VII. Summary

Research performed during the past 5 years has provided a considerable amount of evidence to support the contention that the initial interaction of LPS (lipid A) with cells is mediated by distinct plasma membrane proteins. Some of these interactions may be solely involved in removal and eventual degradation of LPS whereas others may play a critical role in transmembrane signaling. Interactions that appear to be limited to a removal function have been assigned to the lipoprotein scavenger receptor or CD18 where R-form LPS, lipid A, or partial lipid A structures such as lipid IVa appear to be the preferred ligands; S-form LPS appears not to interact with these membrane proteins. Whether these interactions reflect events that occur *in vivo* remains to be definitively established. Moreover, the scavenger receptor and CD18 do not have a role in mediating LPS-induced transmembrane signaling.

Photochemical crosslinking studies performed by Morrison and colleagues and by Dziarski (1991a,b) have revealed an LPS-binding membrane protein with an apparent molecular weight 70,000–80,000. This protein binds the lipid A of LPS as well as the carbohydrate backbone of peptidoglycan. Studies with monoclonal antibodies to this protein show that the presence of antibody blocks LPS binding, suggesting that engagement of this protein leads to transmembrane signaling. However, a definitive evaluation of the role of this protein in mediating LPS effects will require complete purification and/or gene cloning.

Perhaps the most important advance in our understanding of how LPS acts is derived from the studies of Ulevitch, Tobias, and colleagues wherein the LBP/CD14-dependent pathway of cell stimulation has been identified. This pathway has particular importance for LPS recognition and signaling by cells such as monocytes/macrophages or polymorphonuclear leukocytes that constitutively express CD14. The importance of the LBP/CD14-dependent pathway has been definitively demonstrated by experiments using immunologic, biochemical, and molecular biologic approaches. Available

data are consistent with a model for a heterodimeric LPS receptor that consists of CD14 and an as yet unidentified additional protein(s). Clearly a major goal for future research will be to elucidate fully the additional proteins involved in recognition of LPS.

Acknowledgment

This work was supported by National Institutes of Health Grants AI15136, GM28485, and GM37696. This is publication number 7381-IMM from the Department of Immunology of The Scripps Research Institute.

References

Akira, S., Hirano, T., Taga, T., and Kishimoto, T. (1990). *FASEB J.* **4,** 2860.

Beutler, B., and Cerami, A. (1986). *Nature (London)* **320,** 584.

Beutler, B., Milsark, I. W., and Cerami, A. (1985a). *Science* **229,** 869.

Beutler, B. A., Milsark, I. W., and Cerami, A. (1985b). *J. Immunol.* **135,** 3972.

Bright, S. W., Chen, T.-Y., Flebbe, L. M., Lei, M.-G., and Morrison, D. C. (1990). *J. Immunol.* **145,** 1.

Chen, T.-Y., Bright, S. W., Pace, J. L., Russell, S. W., and Morrison, D. C. (1990). *J. Immunol.* **145,** 8.

Desch, C. E., Kovach, N. L., Present, W., Broyles, C., and Harlan, J. M. (1989). *Lymphokine Res.* **8,** 141.

Drayna, D., Jarnagin, A., McLean, J., Henzel, W., Kohr, W., Fielding, C., and Lawn, R. (1987). *Nature (London)* **327,** 632.

Dziarski, R. (1991a). *J. Biol. Chem.* **266,** 4713.

Dziarski, R. (1991b). *J. Biol. Chem.* **266,** 4719.

Flebbe, L. M., Chapes, S. K., and Morrison, D. C. (1990). *J. Immunol.* **145,** 1505.

Gray, P. W., Flaggs, G., Leong, S. R., Gumina, R. J., Weiss, J., Ooi, C. E., and Elsbach, P. (1989). *J. Biol. Chem.* **264,** 9505.

Hampton, R. Y., Golenbock, D. T., and Raetz, C. R. H. (1988). *J. Biol. Chem.* **263,** 14802.

Hampton, R. Y., Golenbock, D. T., Penman, M., Krieger, M., and Raetz, C. R. H. (1991). *Nature (London)* **352,** 342.

Hara-Kuge, S., Amano, F., Nishijima, M., and Akamatsu, Y. (1990). *J. Biol. Chem.* **265,** 6606.

Hatakeyama, M., Tsudo, M., Minamoto, S., Kono, T., Doi, T., Miyata, T., Miyasaka, M., and Taniguchi, T. (1989). *Science* **244,** 551.

Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990). *FASEB J.* **4,** 2881.

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kishimoto, T. (1990). *Cell (Cambridge, Mass.)* **63,** 1149.

Hunt, J. S., Soares, M. J., Lei, M.-G., Smith, R. N., Wheaton, D., Atherton, R. A., and Morrison, D. C. (1989). *J. Immunol.* **143,** 1606.

Jann, K., and Jann, B. (1984). *In* "Handbook of Endotoxin. Chemistry of Endotoxin" (E. T. Rietschel, ed.), Vol. I, pp. 138–186. Elsevier, Amsterdam.

Jansson, P.-E., Lindberg, A. A., Lindberg, B., and Wollin, R. (1981). *Eur. J. Biochem.* **115,** 571.

Johnston, R. B., Jr. (1991). *N. Engl. J Med.* **38,** 747.

Kirikae, T., Inada, K., Hirata, M., Yoshida, M., Kondo, S., and Hisatsune, K. (1988). *Microbiol. Immunol.* **32,** 33.

Kirkland, T. N., Virca, G. D., Kuus-Reichel, T., Multer, F. K., Kim, S. Y., Ulevitch, R. J., and Tobias, P. S. (1990). *J. Biol. Chem.* **265,** 9520.

Kirkland, T. N., Qureshi, N., and Takayama, K. (1991). *Infect. Immun.* **59,** 131.

Kotani, S., Takada, H., Tsujimoto, M., Ogawa, T., Mori, Y., Sakuta, M., Kawasaki, A., Nage, M., Kusumoto, S., Shiba, T., and Kasai, M. (1983). *Infect. Immun.* **41,** 758.

Kovach, N. L., Yee, E., Munford, R. S., Raetz, C. R. H., and Harlan, J. M. (1990). *J. Exp. Med.* **172,** 77.

Lee, J. D., Kato, K., Tobias, P. S., Kirkland, T. N., and Ulevitch, R. J. (1992). *J. Exp. Med.* **175,** 1697.

Lei, M.-G., and Morrison, D. C. (1988a). *J. Immunol.* **141,** 1006.

Lei, M.-G., and Morrison, D. C. (1988b). *J. Immunol.* **141,** 996.

Lei, M.-G., Stimpson, S. A., and Morrison, D. C. (1991). *J. Immunol.* **147,** 1925.

Loppnow, H., Brade, H., Durrbaum, I., Dinarello, C. A., Kusumoto, S., Rietschel, E. T. H., and Flad, H.-D. (1989). *J. Immunol.* **142,** 3229.

MacIntyre, E. A., Roberts, P. J., Jones, M., VanDerSchoot, C. E., Favalaro, E. J., Tidman, N., and Linch, D. C. (1989). *J. Immunol.* **142,** 2377.

Martin, T. R., Mathison, J. C., Tobias, P. S., Maunder, R. J., and Ulevitch, R. J. (1992). *J. Clin. Invest.* **90,** 2209.

Mathison, J. C., Wolfson, E., and Ulevitch, R. J. (1988). *J. Clin. Invest.* **81,** 1925.

Mathison, J. C., Virca, G. D., Wolfson, E., Tobias, P. S., Glaser, K., and Ulevitch, R. J. (1990). *J. Clin. Invest.* **85,** 1108.

Mathison, J. C., Tobias, P. S., Wolfson, E., and Ulevitch, R. J. (1992). *J. Immunol.* **149,** 200.

Mayer, H. (1984). *In* "The Cell Membrane" (E. Haber, ed.), pp. 71–83. Plenum, New York.

Michie, H. R., Manogue, K. R., Spriggs, D. R., Revhaug, A., O'Dwyer, S., Dinarello, C. A., Cerami, A., Wolff, S. M., and Wilmore, D. W. (1988). *N. Engl. J. Med.* **318,** 1482.

Mills, G. B., May, C., McGill, M., Fung, M., Baker, M., Sutherland, R., and Greene, W. C. (1990). *J. Biol. Chem.* **265,** 3561.

Munford, R. S., and Hall, C. L. (1989). *J. Biol. Chem.* **264,** 15613.

Nathan, C. F. (1987). *J. Clin Invest.* **79,** 319.

Ooi, C. E., Weiss, J., Elsbach, P., Frangione, B., and Mannion, B. (1987). *J. Biol. Chem.* **262,** 14891.

Pabst, M. J., Hedegaard, H. B., and Johnston, R. B., Jr. (1982). *J. Immunol.* **128,** 123.

Quereshi, N., Takayama, K., and Kurtz, R. (1991). *Infect. Immun.* **59,** 441.

Raetz, C. R. H. (1990). *Annu. Rev. Biochem.* **59,** 129.

Ramadori, G., Meyer zum Buschenfelde, K.-H., Tobias, P. S., Mathison, J. C., and Ulevitch, R. J. (1990). *Pathobiology* **58,** 89.

Rietschel, E. T., Galanos, C., Luderitz, O., and Westphal, O. (1982). *In* "Immunopharmacology and the Regulation of Leukocyte Function" (D. R. Webb, ed.), pp. 183–229. Dekker, New York.

Rietschel, E. T., Brade, H., Brade, L., Kaca, W., Kawahara, K., Lindner, B., Luderitz, T., Tomita, T., Schade, U., Seydel, U., and Zahringer, U. (1985). *In* "Bacterial Endotoxins: Structure, Biomedical Significance, and Detection With the Limulus Amebocyte Lysate Test," pp. 31–50. Alan R. Liss, New York.

Roeder, D. J., Lei, M.-G., and Morrison, D. C. (1989). *Infect. Immun.* **57,** 1054.

Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch, R. J. (1990). *Science* **249,** 1429.

Strittmatter, W., Weekesser, J., Salimath, P. V., and Galanos, C. (1983). *J. Bacteriol.* **155,** 153.
Takada, H., and Kotani, S. (1989). *CRC Crit. Rev. Microbiol.* **16,** 477.
Takayama, K., Qureshi, N., Beutler, B., and Kirkland, T. N. (1989). *Infect. Immun.* **57,** 1336.
Thomas, L. (1974). "The Lives of a Cell: Notes of a Biology Watcher." Viking Press, New York.
Tobias, P. S., and Ulevitch, R. J. (1983). *J. Immunol.* **131,** 1913.
Tobias, P. S., McAdam, K. P. W. H., and Ulevitch, R. J. (1982). *J. Immunol.* **128,** 1420.
Tobias, P. S., McAdam, K. P. W. H., Soldau, K., and Ulevitch, R. J. (1985). *Infect. Immun.* **50,** 73.
Tobias, P., Soldau, K., and Ulevitch, R. (1986). *J. Exp. Med.* **164,** 777.
Tobias, P. S., Mathison, J. C., and Ulevitch, R. J. (1988). *J. Biol. Chem.* **263,** 13479.
Tobias, P. S., Soldau, K., and Ulevitch, R. J. (1989). *J. Biol. Chem.* **264,** 10867.
Tracey, K. J. (1991). *Circ. Shock* **35,** 123.
Tracey, K. J., Fong, Y., Hesse, D. G., Manogue, K. R., Lee, A. T., Kuo, G. C., Lowry, S. F., and Cerami, A. (1987). *Nature (London)* **330,** 662.
Ulevitch, R. J., Wolfson, N., Virca, G. D., Kim, S., Kline, L., and Mathison, J. C. (1989). *Prog. Clin. Biol. Res.* **229,** 193.
Vas, S. I., Roy, R. S., and Robson, H. G. (1973). *Can. J. Microbiol.* **19,** 767.
Vogel, S. N., Finbloom, D. S., English, K. E., Rosenstreich, D. L., and Langreth, S. G. (1983). *J. Immunol.* **130,** 1210.
Weeks, R. S., and Sibley, C. H. (1988). *J. Immunol.* **140,** 1312.
Weiss, J., Elsbach, P., Olsson, I., and Odeberg, H. (1978). *J. Biol. Chem.* **253,** 2664.
Weiss, J., Muello, K., Victor, M., and Elsbach, P. (1984). *J. Immunol.* **132,** 3109.
Wollenweber, H.-W., and Morrison, D. C. (1985). *J. Biol. Chem.* **260,** 15068.
Wright, S. D., and Jong, M. T. C. (1986). *J. Exp. Med.* **164,** 1876.
Wright, S. D., Levin, S. M., Jong, M. T. C., Chad, Z., and Kabbash, L. G. (1989a). *J. Exp. Med.* **169,** 175.
Wright, S. D., Tobias, P. S., Ulevitch, R. J., and Ramos, R. A. (1989b). *J. Exp. Med.* **170,** 1231.
Wright, S. D., Detmers, P., Aida, Y., Adamowski, R., Anderson, D. C., Chad, Z., Kabbash, L. G., and Pabst, M. J. (1990a). *J. Immunol.* **144,** 2566.
Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990b). *Science* **249,** 1431.

This article was accepted for publication on 23 September 1992.

ADVANCES IN IMMUNOLOGY, VOL. 53

Cell Adhesion Molecules as Targets of Autoantibodies in Pemphigus and Pemphigoid, Bullous Diseases Due to Defective Epidermal Cell Adhesion

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I. Introduction

The skin, as an organ, hardly ever fails, even in extreme old age. However, if the crucial barrier function of the skin, subserved by the epidermis, does fail, the result is almost invariably fatal due to infection and/or fluid loss. This barrier function of the skin is maintained by an epidermis in which keratinocytes adhere to each other as well as to the basement membrane at the dermal–epidermal junction.

The theme of this review will be that, in various autoimmune blistering diseases of skin, autoantibodies are directed against epidermal cell adhesion molecules or molecules found in cell adhesion junctions. The blisters and dysfunction of the epidermal barrier in these diseases result from loss of adhesion of cells either to the basement membrane or to other cells.

This theme will be developed for three diseases, bullous pemphigoid (BP), pemphigus foliaceus (PF), and pemphigus vulgaris (PV). Studies of the autoantibodies from patients with these diseases have not only shed light on potential pathophysiologic mechanisms, but, in a more general sense, have enabled us to start to understand how the epidermis maintains its intact state.

II. Clinical, Histologic, and Immunopathologic Findings

BP, PF, and PV are all bullous diseases of skin. Each has a distinctive clinical appearance (*1–4*) (Table I), and the blisters in each result from loss of adhesion at a specific level of the epidermis (2) (Fig. 1). BP and pemphigus also have characteristic immunofluorescence findings (Table I; Fig. 2); however, the immunofluorescence findings in PF and PV are very similar (5–9) (Table I).

TABLE I
CLINICAL AND IMMUNOPATHOLOGIC FINDINGS IN BULLOUS PEMPHIGOID, PEMPHIGUS FOLIACEUS, AND PEMPHIGUS VULGARIS[a]

Disease	Skin Findings	Mucous Membrane Findings	Direct IF (skin)	Indirect IF (serum)
BP	Tense blisters, urticarial lesions	10–35% have oral erosions	IgG and C3 at the epidermal basement membrane	IgG binds normal stratified squamous epithelial basement membrane
PF	Scaly, crusted erythematous macules and erosions	None	IgG on the keratinocyte cell surface	IgG binds normal stratified squamous epithelial cell surface
PV	Flaccid bullae, large erosions	Almost all have oral or other mucous membrane erosions. Oral erosions often presenting sign	IgG on the keratinocyte cell surface	IgG binds normal stratified squamous epithelial cell surface

[a] Abbreviations: BP, bullous pemphigoid; PF, pemphigus foliaceus; PV, pemphigus vulgaris; IF, immunofluorescence.

A. BULLOUS PEMPHIGOID

BP is predominantly a disease of the elderly, although people of all ages may be affected. The characteristic lesion in BP is a tense blister arising on either normal skin or on an erythematous base. The blisters characteristically occur on the lower abdomen, thighs, and flexural surfaces of the extremities, but may become more generalized. On the other hand, lesions of BP may be localized, most commonly to the lower legs. Because the lesions in BP result in loss of the barrier function of the epidermis, the disease may be fatal, especially if widespread in elderly patients.

Histology indicates that the blister in BP forms at the epidermal basement membrane (Fig. 1). In cell biologic terms, the pathology results from loss of adhesion of the epidermal basal cells to the un-

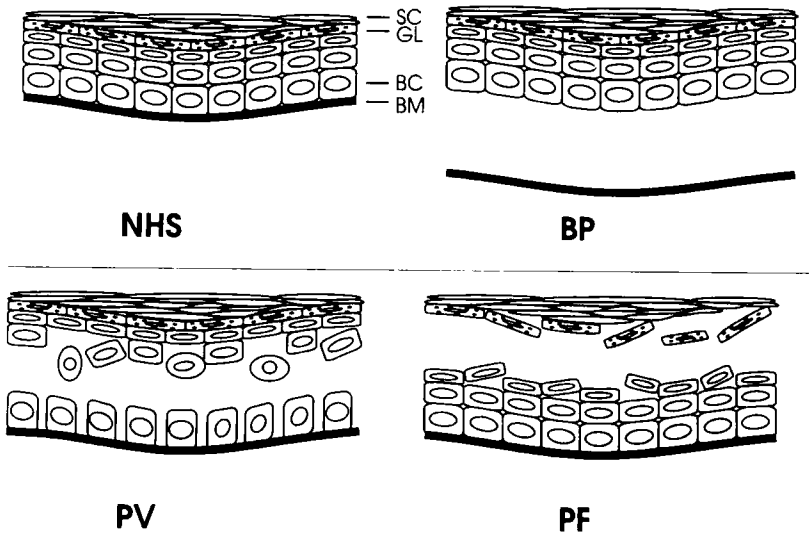

FIG. 1. Site of blister formation in pemphigoid and pemphigus. NHS, Normal human skin; BP, bullous pemphigoid; PV, pemphigus vulgaris; PF, pemphigus foliaceus; SC, stratum corneum; GL, granular layer; BC, basal cells; BM, basement membrane.

derlying basement membrane. Electron microscopic studies demonstrate that early blisters occur just below the basal cell membrane, in the lamina lucida of the basement membrane zone, with the lamina densa remaining on the floor of the blister cavity (*10*). The earliest lesion shows tearing of the anchoring filaments within the hemidesmosome below the basal cell membrane, with subsequent disappearance of the entire hemidesmosome (*11,12*) (a schematic diagram of these structures is shown in Fig. 3). It is of interest that the hemidesmosome, an organelle thought to be important in basal cell–basement membrane attachment, is destroyed in lesions of BP.

Lesions of BP also show an inflammatory cell infiltrate to varying degrees. This infiltrate characteristically contains eosinophils, which localize to the upper dermis, along the basement membrane zone, and in the blister cavity. In so-called cell-poor lesions, this infiltrate is minimal; however, it is always seen to some degree in all lesions. Finally, light microscopic and electron microscopic studies have shown degranulation of mast cells near the epidermal basement membrane in early BP lesions (*13,14*).

Direct immunofluorescence of perilesional skin from BP patients shows deposits of IgG and C3 at the epidermal basement membrane

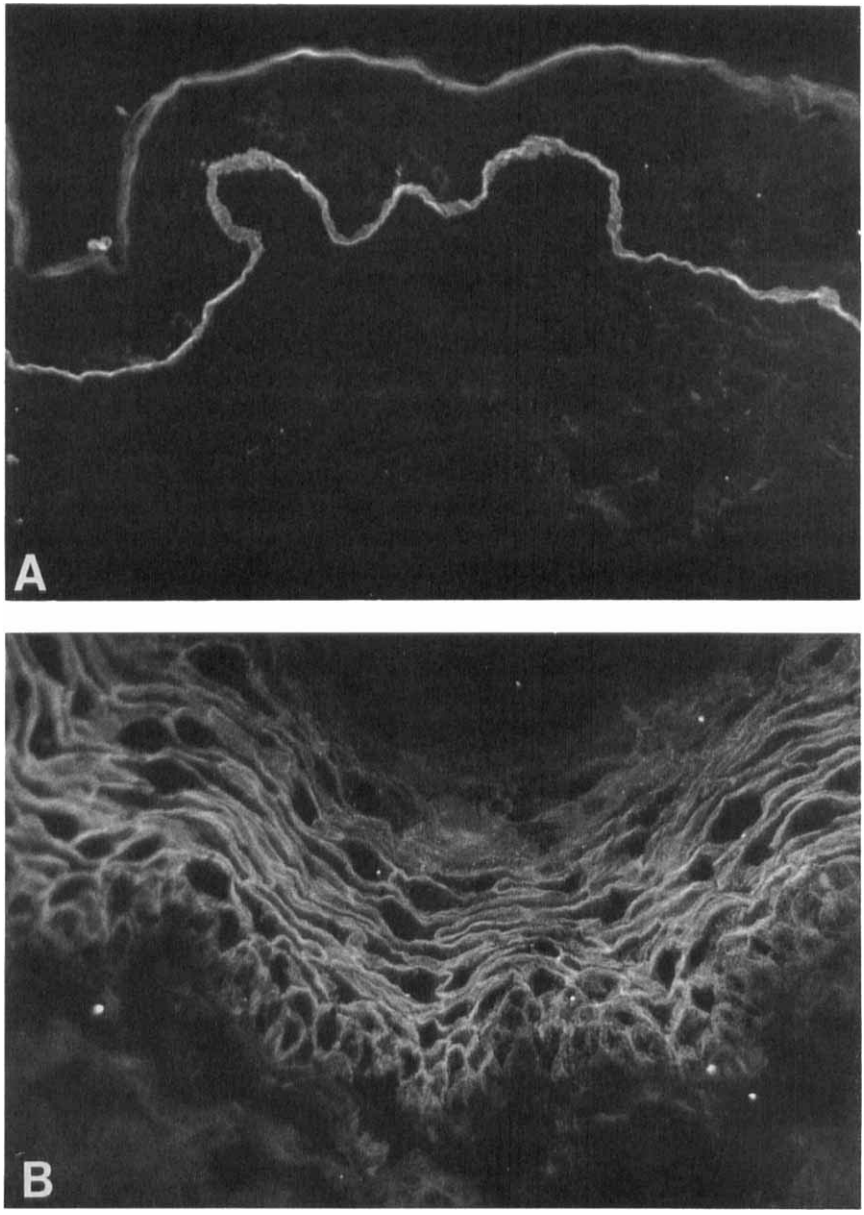

FIG. 2. Indirect immunofluorescence with pemphigoid and pemphigus sera. (A) BP serum on pig skin shows staining of epidermal basement membrane zone. (B) PF serum on guinea pig esophagus shows cell surface staining of stratified squamous epithelium. (C) PV staining of cultured human keratinocytes shows cell surface fluorescence that in more oblique views is seen as dots, suggesting junctional staining (×160).

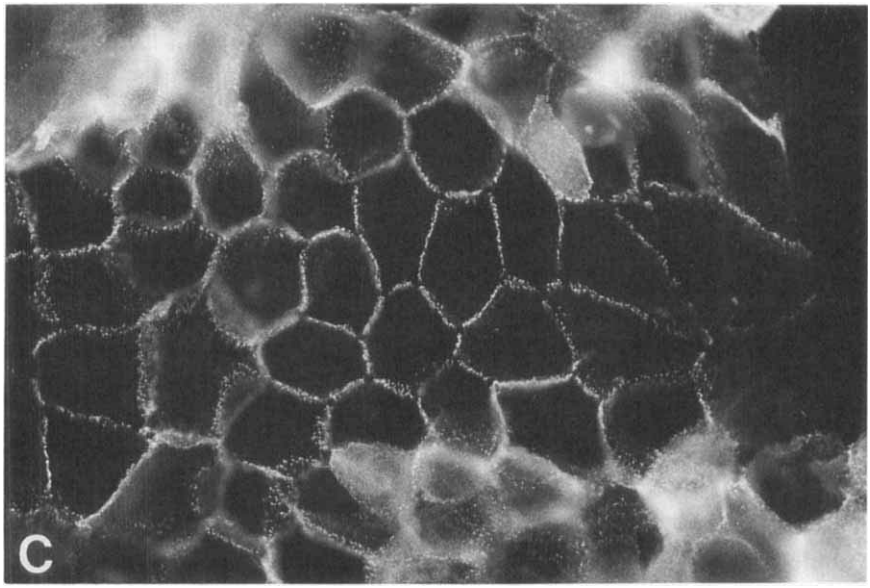

FIG. 2. (*Continued*)

zone. In addition, most patients have circulating antibodies that bind the basement membrane zones of normal stratified squamous epithelia and of certain complex epithelia (e.g., trachea, urinary bladder, gall bladder), but not simple epithelia or nonepithelial tissues (*8,15*) (Table I; Fig. 2A). Thus, BP autoantibodies define a tissue-specific antigen.

In summary, then, BP is a subepidermal blistering disease in which autoantibodies localize to the site of pathology (the epidermal basement membrane) and define a normal antigen of stratified squamous and complex epithelial basement membranes.

B. PEMPHIGUS

There are two major types of pemphigus, PV and PF (Table I). PV is characterized by erosions of mucous membranes and flaccid blisters of the skin. Almost all PV patients have involvement of the mucous membranes, most often the oral mucosa. Frequently patients present with oral erosions and, if untreated, these may persist for months before skin lesions eventually develop. Before the advent of

corticosteroid therapy for PV the disease was almost invariably fatal because of lesions on large areas of skin and mucous membrane erosions, with resultant loss of epidermal barrier function.

PF patients develop small flaccid blisters that rapidly evolve into scaly crusted lesions, characteristically localized to the face and upper trunk, but the lesions may become generalized. As opposed to PV patients, PF patients do not develop mucous membrane lesions or large skin erosions. PF in an elderly patient with widespread involvement may be fatal, but, in general, it has a much better prognosis than does PV.

Histology of the lesions in PV and PF indicates that an intraepidermal blister occurs because of loss of epidermal cell-to-cell adhesion. However, in PV this defect in cell adhesion occurs deep with the epidermis, just above the basal cell layer, whereas in PF the loss of cell adhesion is in the granular layer, the upper part of the living epidermis (Fig. 1). Early blisters of pemphigus often show minimal or no inflammatory infiltrate.

Electron microscopic studies of PV blisters have been somewhat contradictory. Early PV blisters demonstrate separation of the plasma membrane of keratinocytes between desmosomes, with eventual separation of the desmosomes in later lesions (*12,16*). However, electron microscopic studies of uninivolved skin from PV patients and of skin organ culture exposed to PV antibodies suggest abnormalities in desmosomes early in blister formation (*17,18*) (see Fig. 3 for a schematic diagram of the desmosome). Electron microscopic examination of early PF lesions shows abnormalities of desmosomes, with separation of the keratin filaments from the desmosome attachment plaque. Eventually, cells separate, with loss of desmosomes (*19*).

These types of ultrastructural studies are difficult to interpret regarding the time course of pathologic findings, but they do show that when keratinocytes come apart in pemphigus lesions, the desmosome, an organelle thought to serve the function of cell-to-cell attachment, is destroyed.

Autoantibodies against the keratinocyte cell surface are characteristic of both types of pemphigus (*20*). By direct immunofluorescence of perilesional skin from PV and PF patients, IgG can be detected on the cell surface of keratinocytes throughout the epidermis. The pattern of fluorescence is the same in both types of pemphigus. As seen by indirect immunofluorescence, PV and PF patients have circulating IgG antibodies that bind the cell surface of stratified squamous epithelial cells, but not other epithelial cells (*8,21*) (Table I; Figs. 2B and 2C).

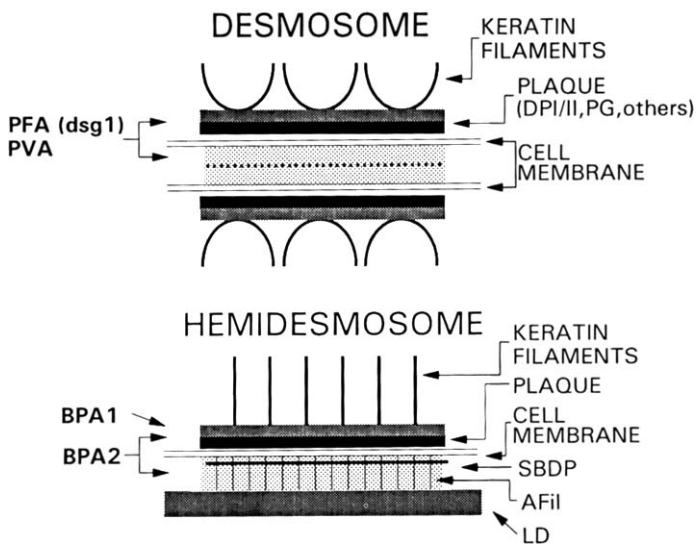

FIG. 3. Diagram of the ultrastructure of desmosomes and hemidesmosomes. PFA, PF antigen; PVA, PV antigen; BPA1, the 230-kDa BP antigen; BPA2, the 180-kDa BP antigen; DPI/II: desmoplakin I/II; PG, plakoglobin; SBDP, subbasal dense plate; AFil, anchoring filaments; LD, lamina densa.

In summary, then, PV and PF are diseases in which blisters and erosions form due to the loss of the normal cell-to-cell adhesion of keratinocytes, and in which autoantibodies are directed against antigens found on the cell surface of keratinocytes.

III. Immunochemical Characterization of Pemphigoid and Pemphigus Antigens

A. Pemphigoid Antigens Are Hemidesmosomal Proteins

Immunoelectron microscopic studies have been used to localize, at an ultrastructural level, the site of binding of BP antibodies. The original studies were immunoperoxidase localization of *in vivo*-bound IgG or C3 in the skin of BP patients. These studies showed a diffuse localization of peroxidase reaction products to the lamina lucida, the electron-lucent area between the lamina densa and the basal cell membrane (*22–24*). However, when the localization of BP antigen was investigated with circulating antibodies from patient sera, an unexpected finding was that most of the IgG bound inside the cell. For example, immunofluorescence studies of isolated epi-

dermal basal cells and cultured keratinocytes revealed that permeabilization of the cell membrane was necessary for BP IgG to bind (25–27). Similarly, very little BP IgG was able to bind the basement membrane zone of viable epidermis, as detected by immunofluorescence, but permeabilization of the membrane or exposure of intracellular sites by cryosectioning allowed much greater binding with markedly increased fluorescence (25,28,29). Futhermore, immunoperoxidase and immunogold electron microscopic studies demonstrated that major labeling with BP sera occurred inside the basal cell and, more specifically, was found on the hemidesmosome plaque (Fig. 3) (15,25–27,30–32). The hemidesmosome is a specialized structure organized at sites of attachment of the basal cell to the lamina densa and is, therefore, thought to be important in anchoring basal epithelial cells to the underlying basement membrane (33,34).

More recent immunoelectron microscopic studies with BP sera have confirmed that BP IgG mainly binds the plaque of the hemidesmosome inside the cell but that there is some binding in the lamina lucida, external to the cell membrane and beneath the hemidesmosome plaque (28,35).

In summary, these immunofluorescence and ultrastructural studies with whole BP sera suggest that BP antigen is localized mainly to the plaque of the hemidesmosome, but also has some epitopes in the extracellular hemidesmosome.

To further characterize the molecule or molecules that BP sera recognize, investigators have used immunoprecipitation and immunoblotting techniques. Preliminary immunofluorescence studies demonstrated that keratinocytes in culture display, and presumably synthesize, BP antigen (36,37). Thus, extracts of cultured keratinocytes, whose newly synthesized proteins were labeled with radioactive amino acids, were used for immunoprecipitation assays with BP sera, disease control sera, and normal human sera. These studies demonstrated that human and rodent keratinocytes in culture synthesize BP antigen, which is [as estimated from sodium dodecyl sulfate (SDS) and polyacrylamide gel electrophoresis (PAGE)] a protein of approximately 230 kDa (reported in various publications as 220–240 kDa) (38,39). These observations have subsequently been confirmed by several laboratories (27,40,41). Greater than 90% of sera from BP patients precipitates this protein, whereas no disease control (such as pemphigus and epidermolysis bullosa acquisita) or normal sera precipitate it. Studies with mouse keratinocytes grown in low-calcium medium (in which they have a basal cell phenotype) as compared to high-calcium medium (in which they differentiate) demon-

strated that this BP antigen was synthesized predominantly by the basal cells, as is expected for an antigen that is only found in this cell population *in vivo* (*42*). This 230-kDa BP antigen has also been identified by immunoblotting of extracts of normal human epidermis, bovine cornea, and bovine tongue with BP sera (*15,43–50*). Two-dimensional gel electrophoresis of immunoprecipitates of keratinocyte extracts and immunoblots of epidermal extracts indicated that the 230-kDa BP antigen migrates as a single spot with a basic p*I* of about 8 (*41*). Further evidence for the identity of this molecule comes from immunofluorescence studies in which antibodies affinity purified on immunoblots of this 230-kDa antigen bind the basement membrane zone of epidermis in a pattern indistinguishable from whole BP sera (*41,51*).

The 230-kDa BP antigen has been localized to the plaque of keratinocyte hemidesmosomes. Consistent with a localization in hemidesmosomes, at least some of the 230-kDa BP antigen is found organized in the nonionic detergent-insoluble particulate fraction of cultured keratinocytes and epidermis (*51*). It is a major polypeptide in hemidesmosome-enriched fractions of bovine corneal epithelial cells (*46*). A monoclonal antibody raised against this protein binds stratified squamous epithelial basement membrane in the same pattern as is seen using BP antibodies (*46*). Furthermore, antibodies from BP sera that are affinity purified on the 230-kDa BP antigen bind the human epidermal hemidesmosome by immunoperoxidase electron microscopy (52) and the bovine tongue hemidesmosome plaque as determined by immunogold electronmicroscopy (*49*). Finally, a human monoclonal antibody, isolated from a patient with BP, binds the 230-kDa antigen and also binds the hemidesmosome plaque by immunoelectron microscopy (53). As discussed below, ultrastructural localization of the 230-kDa BP antigen to the hemidesmosome plaque has also been confirmed with rabbit antibodies raised against portions of the cloned 230-kDa BP antigen.

Perhaps surprisingly, the 230-kDa BP antigen is not the only molecule specifically recognized by BP antibodies. That there might be more than one BP antigen was first suggested by immunofluorescence studies in which patient sera tested against a panel of different human skin substrates gave various patterns of reactivity (*54*). In other words some sera, but not others, might bind a particular skin specimen, whereas all sera bind other skin specimens. However, this type of study only suggests that different epitopes are recognized by different sera, but does not determine if these epitopes are on different molecules. Subsequently, immunoblotting studies with epider-

mal extracts demonstrated that different BP sera bind two major polypeptides (*44,55*). Although the majority of BP patient sera recognized the 230-kDa antigen, about 50% recognized a 180-kDa polypeptide (by SDS–PAGE, reported as 165–180 kDa in various publications). Some patient sera recognized both the 230- and the 180-kDa antigens, whereas others recognized only one of these antigens. Many groups have subsequently confirmed this heterogeneity as determined by immunoblotting (*45,47,49,50,56*). The 180-kDa antigen has also been detected by immunoprecipitation of cultured keratinocyte extracts, but not consistently (*41*). This variability in detection of the 180-kDa antigen may be because it is particularly prone to proteolytic degradation during keratinocyte extraction procedures or because of differences in extraction procedures used in different studies (*48,57*). Although one study suggested an immunological cross-reactivity of the 230- and 180-kDa molecules (*52*), subsequent cloning studies have indicated that these are unrelated polypeptides that are distinct gene products (see below).

Analogous to the findings with the 230-kDa BP antigen, the 180-kDa BP antigen has been localized to hemidesmosomes of stratified squamous epithelia. A 180-kDa glycoprotein is enriched in the hemidesmosomal fraction of bovine corneal epithelial cells (*46*). Monoclonal antibodies directed against this 180-kDa glycoprotein showed an immunofluorescence staining pattern and tissue distribution similar to that of BP sera and to that of a monoclonal antibody against the 230-kDa BP antigen (*46*). Antibodies affinity purified from BP sera on the 180-kDa antigen were localized by immuoperoxidase electron microscopy to human hemidesmosomes (*52*) and by immunogold electron microscopy to the hemidesmosome plaque of bovine tongue epithelia (*49*). Finally, antibodies raised against a fragment of the cloned 180-kDa BP antigen bind to the epidermal basal cell hemidesmosome as determined by immunoelectron microscopy (see below) (*58*).

In summary, then, two distinct BP antigens have been characterized immunochemically. Both are localized in the hemidesmosome (mainly on the plaque). Interestingly, there is no correlation between the antigens recognized by individual patients and their clinical presentation (*59–61*).

B. Pemphigus Foliaceus Antigen Is Desmoglein, a Desmosomal Glycoprotein

The first studies to try to characterize the PF antigen were immunoblot analyses of SDS extracts of normal human epidermis. These

studies demonstrated that about one-third of PF sera identified a polypeptide of about 160-kDa (155–160 kDa in various publications) (*62–66*). Antibodies from PF sera that were affinity purified on this 160-kDa polypeptide bound the epidermis by immunofluorescence in a pattern indistinguishable from that of PF sera (*62*). The other two-thirds of PF sera did not specifically bind any polypeptides on these immunoblots, presumably because they could no longer bind PF antigen epitopes that were denatured during the SDS–PAGE procedure.

This 160-kDa polypeptide was subsequently shown, by the evidence outlined below, to be desmoglein (previously called desmoglein I), a desmosomal transmembrane glycoprotein (Fig. 3). Analogous to hemidesmosomes that are thought to anchor basal cells to basement membrane, desmosomes are organelles found in areas of cell-to-cell contact and are thought to be important in maintaining cell-to-cell adhesion (Fig. 3) (*33,34,67–69*). Desmoglein is a transmembrane glycoprotein that extends into the core (or center) of desmosomes and is presumed to be directly involved in cell adhesion (*32,70*). Desmoglein is found in the desmosomes of all tissues (*71*), and has also been shown to bind calcium, which is known to be required to induce desmosome formation in keratinocytes (*72–74*).

What is the evidence that PF autoantibodies bind desmoglein? First, monoclonal antibodies against desmoglein and PF antibodies bind human epidermis in identical patterns, as seen by immunofluorescence (*75*). Second, on immunoblots of extracts of normal human epidermis, both polyclonal and monoclonal antibodies against desmoglein and PF IgG bind to comigrating polypeptides (which are ~160 kDa and have a p*I* of ~5.5) electrophoresed in one (SDS–PAGE) and two (isoelectric focusing followed by SDS–PAGE) dimensions (*75,76*). Third, PF sera bind desmoglein in bovine desmosomal extracts (*64,75*). Fourth, IgG affinity purified from PF sera on the 160-kDa PF antigen from human epidermal extracts binds to desmoglein extracted from bovine muzzle desmosomes (*75*). Finally, PF antigen, obtained by sonication of trypsin-resistant human epidermis, can absorb out the antidesmoglein antibodies from PF sera (*77*).

However, as stated above, only about one-third of PF sera can be shown to bind the 160-kDa desmoglein by immunoblotting. What about the other two-thirds of sera? With the technique of immunoprecipitation of nonionic detergent extracts of epidermis, which, as opposed to immunoblotting, does not denature proteins, it was shown that all PF sera bind to a complex of polypeptides that includes the 160-kDa desmoglein as well as an 85-, 260-, and minor 110-kDa mol-

ecule, all in a stoichiometric ratio (78). Antibodies to desmoglein precipitate this same complex. Diagonal gel electrophoresis (unreduced in the horizontal direction, reduced in the vertical second direction) indicates that the desmoglein is linked by disulfide and noncovalent bonds to the 85-kDa peptide in this complex (79). Much, if not all, of this disulfide bonding might take place after extraction of these molecules from the epidermis when they are exposed to a more oxidizing environment. In fact, extraction in the presence of iodoacetamide, which alkylates free sulfhydryl groups and thereby prevents disulfide bond formation, demonstrates much less disulfide linkage and more noncovalent association of these molecules as determined by diagonal gel electrophoresis (unpublished observation, 1988). Sequential immunoprecipitation experiments, in which this complex was denatured, reduced, and alkylated, then reprecipitated, confirm immunoblotting results that demonstrate that the PF antigen epitopes are on desmoglein, not on the 85-kDa polypeptide of this complex (*80*). The noncovalent interaction of desmoglein and this 85-kDa polypeptide must be a strong and specific one, because the complex is stable in nonionic detergent and because the molecules always show the same relative stoichiometry.

Immunofluorescence studies have suggested that PF antibody binding to human epidermis might be dependent on the presence of calcium (*81*). Consistent with this hypothesis, most PF sera could precipitate the PF complex only in the presence of calcium (78). In fact, the PF sera that were negative by immunoblotting were the same sera that required calcium to precipitate this complex, and those that were immunoblot positive (i.e., those that could bind denatured antigen) were able to precipitate this complex even when the calcium was chelated. These results suggested that most PF sera bind to a calcium-sensitive conformational epitope on desmoglein. Further studies of immunoprecitation of a fragment of PF antigen released from keratinocytes by trypsin have demonstrated that, in fact, all PF sera have antibodies that bind a calcium-sensitive epitope and that this epitope is extracellular (82).

If PF antigen is desmoglein and it is linked in the epidermis to an 85-kDa molecule in a stoichiometric fashion, then is the 85-kDa molecule also desmosome associated? In fact, the sequential immunoprecipitation studies discussed above demonstrated that the 85-kDa molecule, once dissociated from the PF antigen complex, could be immunoprecipitated by antibodies to plakoglobin, a plaque protein found not only in desmosomes (Fig. 3) but also in another type of intercellular junction called adherens junctions (*80,83*). These stud-

ies also demonstrated that the 260-kDa polypeptide probably consists of both the desmoglein and plakoglobin linked by covalent bonds that are not reducible (*80*). Again, it is not known if these covalent bonds form after extraction from the epidermis.

If PF antigen is desmoglein, it should be localized in desmosomes. Early immunoelectron microscopic studies, in which investigators used the immunoperoxidase technique, localized IgG along the entire membrane of keratinocytes *in vivo* both in PF patients and in neonatal mice that had been injected with PF antibodies (*84–86*). However, more recent immunoperoxidase studies of IgG localization in patients' skin indicates that the major binding is to the extracellular aspects of desmosomes (*87*). In addition, indirect immunoelectron microscopic studies with immunogold labeling indicate that PF IgG binds to the extracellular portion of desmosomes in cultured keratinocytes and in normal human skin (*87,88*).

The finding that PF antigen is desmoglein resulted in a paradox, because PF antigen, as detected by immunofluorescence, was known to be limited in distribution to stratified squamous epithelia, as discussed above. Yet, desmoglein was found in the desmosomes of all tissues. The answer to this paradox started to become apparent when it was noted that the desmoglein in different tissues was not identical. For example, it displayed slightly different mobilities on SDS–PAGE (*89*). In addition, as with PF antibodies, some monoclonal antibodies also only recognized epitopes of desmoglein expressed in stratified squamous epithelia (*21,89,90*). The final solution to this paradox has been provided by cDNA cloning of desmoglein, which reveals that there is a gene family encoding these molecules, enabling expression of different gene products in different tissues (see below).

In summary, then, PF autoantibodies recognize stratified squamous epithelial-specific, and calcium-sensitive conformational, epitopes on desmoglein, a desmosomal transmembrane glycoprotein. Recent cDNA cloning of desmoglein, as described below, provides a framework in which to understand these findings. PF antibodies also have been used to show that desmoglein in keratinocytes is found in a stoichiometric complex with plakoglobin, a desmosome plaque protein.

C. Pemphigus Vulgaris Antigen Is a 130-kDa Glycoprotein Complexed with Plakoglobin

The initial studies done to characterize PV antigen at a molecular level were immunoprecipitation studies of nonionic detergent extracts of mouse and human cultured keratinocytes that, by immuno-

fluorescence, were known to express PV antigen (*36,37,91*). These studies indicated that PV sera precipitated a glycosylated 130-kDa glycoprotein complexed, in a stoichiometric ratio, with an 85-kDa polypeptide (also reported as 80 kDa) (*62,92*). Under nonreducing conditions these two chains were disulfide crosslinked as a 210-kDa molecule. This PV antigen complex was synthesized by keratinocytes but not fibroblasts (*62,92*). PF sera did not precipitate any polypeptides from these keratinocyte extracts, probably because keratinocytes in culture synthesize little PF antigen and/or it is not extractable with nonionic detergent.

Further studies of immunoprecipitation of human epidermal extracts showed that PV sera precipitated the same 130- and 85-kDa complex (*79*). In these complexes the 210-kDa molecule was also apparent even on reduced gels, suggesting that it consisted of covalently linked nonreducible chains. No PF sera precipitated this PV antigen complex; however, interestingly, two-thirds of PV sera, in addition to precipitating the PV antigen complex, also precipitated the PF antigen complex, as defined above.

As with PF antigen, immunofluorescence of PV sera on normal human skin and monkey esophagus showed less binding (i.e., decreased titer) when calcium was chelated (*81,93*). Immunoprecipitation analysis of epidermal extracts also showed that decreased amounts of PV antigen complex were precipitated in the absence of calcium, although this effect was not as dramatic as with PF sera (*79*).

Two-dimensional (SDS–PAGE and isolelectric focusing) and diagonal (nonreduced and reduced) gel electrophoresis demonstrated interesting parallels between the PV and PF antigen complexes (*79*). First, both complexes contained a glycosylated chain linked by noncovalent, disulfide, and nonreducible covalent bonds to an 85-kDa polypeptide. (Some or all of these covalent bonds might form simply from the oxidizing environment outside the cell, as discussed above, or during iodination of the extracted proteins.) Second, the 85-kDa polypeptides from both the PF and the PV antigen complexes showed identical migration in two-dimensional gels. Furthermore, as in the PF antigen complex, the 85-kDa molecule in the PV antigen complex was also identified by sequential immunoprecipitation as plakoglobin (*80*). Finally, the 130-kDa glycopeptide in the PV antigen complex had the same isoelectric point as the 160-kDa PF antigen (desmoglein). These studies, as well as the calcium sensitivity of their immunoreactivity, suggested a biochemical similarity between the 130-kDa PV antigen glycoprotein and desmoglein, a fact later confirmed by cDNA sequencing (see below).

The actual antigenic site for PV antibodies was shown by sequential immunoprecipitation to be on the 130-kDa glycopeptide (*80*). In addition, immunoblotting studies of SDS extracts of normal human epidermis showed binding to a 130-kDa polypeptide (*64,65*).

Is the 130-kDa PV antigen found in desmosomes? The data on this are conflicting. Immunoblot studies of bovine desmosomes do demonstrate that an approximately 130-kDa (reported as 135–140 kDa) antigen is detected (*64,65,94,95*). However, this does not address the issue of whether PV antigen is concentrated in desmosomes or simply distributed all along the keratinocyte cell membrane. Immunofluorescence studies of cultured mouse keratinocytes suggest that PV sera might bind desmosomes, although light microscopic studies such as these are not definitive (*95*) (see also Fig. 2C for immunofluorescence of the PV antigen in cultured human keratinocytes). Electron microscopic studies are also conflicting. As seen by direct immunoelectron microscopy of PV patients' skin (using the immunoperoxidase technique), PV IgG is distributed diffusely along the keratinocyte cell membrane (*96*). Indirect immunoferritin electron microscopy on trypsinized guinea pig keratinocytes, immunoperoxidase electron microscopy of the skin of neonatal mice injected with PV IgG, and immunogold ultrastructural studies of a rabbit antibody raised against the presumed PV antigen from bovine tongue desmosomes all showed localization diffusely along the cell membrane (*97–99*). On the other hand, direct immunoelectron microscopy of PV patients' epidermis with immunoferritin localization and indirect immunogold ultrastructural localization of PV antigen on cultured keratinocytes have shown a predominantly desmosomal location of PV antigen (*88,100*).

In summary, then, these immunochemical studies demonstrate that PV antigen is a 130-kDa glycoprotein complexed in the keratinocyte with plakoglobin. The PV antigen is biochemically similar to the PF antigen (desmoglein) and also seems to have some calcium-dependent epitopes. As with PF antigen, recent cDNA cloning of PV antigen puts these observations in perspective (see below).

IV. cDNA Cloning of Autoantigens

Recently, cDNAs reflecting coding sequences for the 230- and 180-kDa BP antigens, desmoglein (PF antigen), and pemphigus vulgaris antigen have been cloned. The deduced amino acid sequences for these antigens provide explanations for many of the observed immunochemical findings described above. In addition, the cloning of the

230-kDa BP antigen has defined a new gene family of adhesion plaque proteins, and the cloning of the pemphigus antigens has definitively assigned them to a gene subfamily of adhesion molecules.

A. The 230-kDa Pemphigoid Antigen Is Homologous to Desmoplakin I, a Desmosome Plaque Protein; the 180-kDa Pemphigoid Antigen Is Unique

Initial cloning of cDNA with coding sequences for BP antigen was performed with sera from BP patients by immunoperoxidase staining of a λgt11 human keratinocyte cDNA expression library (*101*). Antibodies from BP serum affinity purified on the expressed protein of this clone stained the epidermal basement membrane by indirect immunofluorescence and immunoprecipitated the 230-kDa BP antigen. These results demonstrated that a partial cDNA clone for the 230-kDa BP antigen was obtained. The full-length mRNA encoding the 230-kDa BP antigen was shown by Northern analysis to be 9 kb. Another group used a human monoclonal antibody directed against the 230-kDa BP antigen to screen a λgt11 mouse keratinocyte cDNA expression library (*102*). This allowed isolation of a partial cDNA for the mouse 230-kDa BP antigen. Comparison of the human and mouse amino acid sequences showed 77% identity in corresponding regions, which demonstrates evolutionary conservation of this antigen and suggests that an important physiologic function has been maintained. The partial human cDNA clone was then used for extension cloning to isolate overlapping cDNAs encoding the 230-kDa BP antigen (*103–105*).

What did this cDNA cloning tell us about the antigen? Consistent with the immunofluorescence protein localization studies discussed above, Northern analysis revealed that the 9-kb mRNA for the 230-kDa BP antigen was found in keratinocytes but not in fibroblasts or nonstratified squamous epithelial cells (*103*). Rabbit antibodies raised against a fusion protein within the carboxy-terminal region of the antigen stained the epidermal basement membrane zone by immunofluorescence and were localized by immunoelectron microscopy to the plaque of the hemidesmosome, confirming the studies, discussed above, that were performed with patients' antibodies (*106,107*). Antibodies have also been raised against synthetic peptides, 17–19 amino acids long, representing hydrophilic regions in the carboxy-terminal region of this antigen, and these antibodies bind the epidermal basement membrane as seen by immunofluorescence (*106*) and localize to the hemidesmosome plaque (unpublished observation, 1990). Southern analysis of genomic DNA indicates that

the 230-kDa BP antigen is encoded by a single-copy human gene (*103,108*); related genes are detectable in mammals but not in the chicken, frog, or fish (*108*). The antigen has been localized to the short arm of human chromosome 6 (6p11–6p12) (*109,110*).

Most interestingly from a cell biology perspective, cDNA cloning of the 230-kDa BP antigen revealed its sequence and structural homology with desmoplakin I/II, a desmosome plaque protein (*111,112*) (Figs. 3 and 4). Analysis of the amino acid sequence of human desmoplakin I/II, as determined from cloned cDNA representing most of the molecule but lacking the far amino-terminal domain, predicted a central coiled-coil α-helical rod domain formed by two molecules lined up in parallel without stagger (*113*). This prediction is consistent with rotary shadowing images of desmoplakin I/II (*114*). The rod domain of desmoplakin also shows a regular periodicity of the acidic and basic residues, occurring about every 10.4 amino acids. The basic and acidic residue periodicities are 180° out of phase, suggesting a mechanism by which desmoplakin could self-aggregate by ionic interactions. In addition, at the carboxy terminus there are three domains (called A, B, and C), of 176 amino acids each, that show marked amino acid homology with each other and consist of 38-residue internal repeats with homology to each other. An interesting feature of these domains is that they display acidic and basic

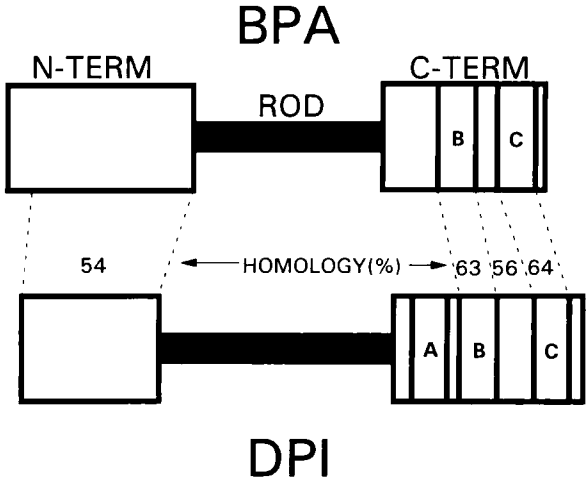

FIG. 4. Structural and amino acid homologies (percentage identical and chemically similar) of BP antigen (BPA) and desmoplakin I (DPI).

amino acids in a regular periodicity, about every 9.5 residues, the same as that of the 1B rod domain of keratins, suggesting a means of ionic interactions between keratins and desmoplakins.

At the time of the original cloning of desmoplakin I/II, only part of the carboxy terminus of BP antigen had been cloned. It was noted that in this part of the BP antigen there was a 174-residue domain that had 40% identity to the B domain of desmoplakin (*113*). In addition, in comparison to the B domain of desmoplakin, this B domain of BP antigen contained a similar internal 38-residue repeat and had approximately the same periodicity of acidic and basic residues (*32,113*). Extension cloning of BP antigen revealed further, and striking, homologies with desmoplakin I/II in both sequence and structure (Fig. 4) (*104,105*). BP was discovered not only to have a desmoplakin I/II-like B domain, but also a desmoplakin-like C domain (*104,105*). In addition, the 230-kDa BP antigen and desmoplakin I/II displayed regions of homology in the amino acids linking the B and C domains and in those amino terminal to the B domains (*104*). In addition, BP antigen, like desmoplakin I/II, was predicted to have a central coiled-coil rod domain (*104,105*), and, remarkably, with the same acidic and basic periodicity (*104*). Finally, when the amino-terminal domains of desmoplakin I/II (*115*) and BP antigen (*105*) were cloned, these too had remarkable sequence homology (*116*). Interestingly, the chromosomal localization of desmoplakin I/II, like that of the 230-kDa BP antigen, was determined to be on the short arm of human chromosome 6 (*117*). It is therefore possible that a gene duplication on this chromosome gave rise to both genes. These studies, then, suggested that BP antigen, a hemidesmosomal plaque protein, and desmoplakin I/II, a desmosomal plaque protein, belonged to a gene family of adhesion plaque proteins, perhaps fulfilling similar functions, including the binding of keratin filaments to the adhesion structure. Lending support to this hypothesis, a recent study in which COS cells and fibroblasts were transfected with cDNA encoding the carboxy-terminal domains of desmoplakin I/II does demonstrate binding of this region to the intermediate filaments (*118*).

Another member of this gene family is a recently described intermediate filament-binding large protein called plectin (*119*). Cloning and amino acid sequence analysis of this 466-kDa polypeptide indicated structural and amino acid homology with the 230-kDa BP antigen and with desmoplakin I/II (*120*). As in these latter two molecules, plectin was predicted to have a coiled-coil rod central domain (which was confirmed by rotary shadowing) and, remarkably, had the same acidic and basic perioidicity, 180° out of phase, of approxi-

mately 10.4 residues throughout much of this domain (*116,120*). The carboxy terminus of plectin contains six highly homologous domains, containing internal repeats of either 19 or 38 residues, that are similar to the B and C domains of BP antigen and desmoplakin I/II (*116,120*). Finally, the amino terminus of plectin shares sequence homology with BP antigen and desmoplakin I/II (*116*).

In summary, then, cloning of the 230-kDa BP antigen indicated rather striking structural and sequence homology to desmoplakin I/II, another adhesion junction plaque protein that may have a function similar to that of binding keratin filaments, and to that of plectin, which may also function by binding intermediate filaments. These molecules form a new gene family, and presumably are important in cell–cell adhesion and/or maintaining the structural integrity of the cells through interactions with their intermediate filaments.

Cloning of the cDNA encoding most of the 180-kDa BP antigen has recently been reported (*58,121,122*). A BP serum with antibodies against both the 230- and 180-kDa BP antigens was used for immunoscreening of a λgt11 library representing keratinocyte mRNA sequences (*58*). Antibodies from BP serum, affinity purified on one of the selected clones, bound the 180-kDa BP antigen as detected by immunoblotting. In addition, a rabbit antiserum made against the fusion protein of this clone bound the 180-kDa BP antigen and the epidermal hemidesmosome. The mRNA encoding the 180-kDa BP antigen was determined, by Northern blotting with this partial cDNA clone, to be about 6 kb, clearly distinct from the 9-kb message for the 230-kDa BP antigen. This clone was also used to localize the 180-kDa BP gene to the long arm of chromosome 10, which provides further and definitive evidence that the 180- and 230-kDa BP antigens are distinct gene products (*123*).

Additional, overlapping cDNA clones with coding sequences for the 180-kDa BP antigen were obtained by screening cDNA libraries with hybridization so that almost the full-length coding sequence was obtained (*122*). Analysis of the deduced amino acid sequence for the 180-kDa BP antigen indicated a unique and interesting structure (*121,122*). A hydrophobic region of 23 amino acids is predicted to be a transmembrane domain that would span the membrane once. On the carboxy-terminal side of this hydrophobic region, presumably extracellular, is a polypeptide of about 100 kDa made up of 15 collagenous domains of varying sizes. These collagenous domains are interrupted by noncollagenous sequences. On the amino-terminal side of the putative transmembrane region is a very basic peptide containing four internal 24- to 26-residue homologous repeating

units. Studies using collagenous digestion confirm the collagenous nature of part of this molecule.

Therefore, it is speculated that the 180-kDa BP antigen, as a transmembrane molecule, might interact with the extracellular matrix through its collagenous domains and stabilize basal keratinocyte attachment to the basement membrane.

B. Pemphigus Foliaceus Antigen Is in the Cadherin Gene Superfamily

As discussed above, PF antigen was identified as desmoglein by immunochemical techniques. Subsequently, cDNAs with desmoglein coding sequences were isolated from libraries constructed with mRNA from bovine and human stratified squamous epithelia and human keratinocytes (*124–127*). Analysis of these cDNA clones indicated that desmoglein belonged to the cadherin gene superfamily of cell adhesion molecules (Fig. 5).

Cadherins are calcium-dependent cell adhesion molecules that mediate homophilic adhesion (i.e., a molecule on one cell binds to the same molecule on another cell) (*128,129*). The originally described cadherins (or "typical" cadherins) were E-cadherin (also called uvomorulin; L-CAM in the chicken), N-cadherin, and P-cadherin. These cadherins have the property that they are protected by calcium from proteolytic (trypsin) degradation. Cloning of these typical cadherins indicated that they had certain sequence and structural similarities (Fig. 5). Each has a transmembrane domain that spans the cell membrane once, with the amino terminus outside the cell. These transmembrane molecules are synthesized in the cell as inactive precursor proteins that have to be cleaved at a domain of highly basic amino acids to yield an active adhesion molecule (*130*). The extracellular region of cadherins can be divided into five domains of about equal size (approximately 100 amino acids). The first four of these domains have sequence homology with each other (*131,132*). An amino acid sequence in the first (furthest amino-terminal) extracellular domain, histidine–alanine–valine (HAV), is conserved in sequence and location in all the originally described cadherins, and this site is thought to be important in adhesion (*133*). These cadherins have six putative calcium-binding motifs in their extracellular domains, with sequences such as aspartic acid–any amino acid–aspartic acid (DXD), aspartic acid–any amino acid–asparagine–aspartic acid–asparagine (DXNDN), or similar motifs (*129,134,135*). It was shown by site-directed mutagenesis that substituting one amino acid in one of these domains prevented calcium

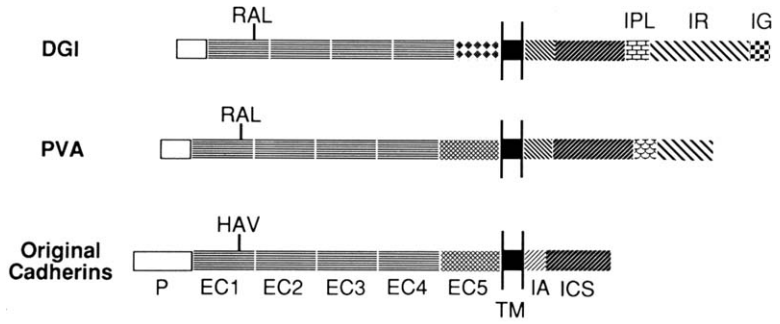

FIG. 5. Comparison of desmoglein (DGI), PV antigen (PVA), and the originally described cadherins (e.g., E-cadherin, N-cadherin, and P-cadherin). Boxes with the same pattern have amino acid sequences that are homologous to each other. RAL and HAV are conserved amino acid sequences that are thought to be important in adhesion function (see text). P, Segment of precursor polypeptide that is cleaved to form the mature protein; EC, extracellar domain; TM, transmembrane segment; IA, intracellular anchor domain; ICS, intracellular cadherin-type segment; IPL, intracellular proline-rich linker; IR, intracellular repeating domain (contains NVXVTE motifs; see text); IG, intracellular glycine/serine-rich domain. (Adapted from Refs. *124* and *142*.)

binding and abolished the adhesive function of E-cadherin (*135*). It was speculated that calcium influences the adhesive function of cadherins through its effect on the conformation of the molecule. Finally, these typical cadherins also have very well-conserved cytoplasmic domains. The cytoplasmic domains of these typical cadherins bind to cytoplasmic proteins called α-, β-, and γ-catenins, molecules that are presumably important in linking the cadherins to the actin cytoskeleton (*129,136*). Of these three molecules, β-catenin seems to be the most tightly linked to the cadherins (*137,138*). Recent cDNA cloning of β-catenin indicates that it is a homolog of plakoglobin (*139*).

Like the typical cadherins, desmoglein was found to be a transmembrane protein whose cleavage at a basic domain would result in a mature protein (Fig. 5). Desmoglein has an extracellular domain structure similar to typical cadherins, and has a conservatively substituted arginine–alanine–leucine (RAL) in the same location as the HAV sequence in typical cadherins. Desmoglein has putative calcium-binding motifs in the extracellular domains in locations equivalent to those in typical cadherins. The cytoplasmic portion of desmoglein has a domain that is homologous to that of typical cadherins, but a marked difference is that the cytoplasmic domain of desmoglein is much longer, with another approximately 300 amino

acids at the carboxy terminus. This extra cytoplasmic region contains a repeating amino acid sequence of asparagine–valine–any amino acid–valine–threonine–glutamic acid (NVXVTE). Another marked difference in cytoplasmic domains is that there are cysteines in desmoglein but not in typical cadherins.

More recent cloning studies have indicated that desmoglein is actually a product of two distinct genes (termed *DSG1* and *DSG2*), both localized to chromosome 18 (*140–142*). Preliminary data (*in situ* hybridization and Northern analysis) indicate that the first type of desmoglein (dsg1), discussed above, is probably expressed by stratified squamous epithelial cells, whereas the second type (dsg2) is expressed by basal-type cells or simple epithelial cells (*141,142*).

These data on the cloning of desmoglein explain many of the observations made about PF antigen. The fact that PF antigen was identified as desmoglein, yet could only be detected in stratified squamous epithelia, could be explained if PF antigen corresponds to dsg1. The finding of a calcium-sensitive epitope on the extracellular domain of PF antigen is consistent with the calcium-binding properties of cadherins and the finding of putative calcium-binding domains in desmoglein. The immunoreactivity of PF antigen, as determined by immunoflourescence, has been shown to be protected from proteolytic destruction by calcium (*81*), consistent with a property of cadherins. The ability of PF antigen to bind plakoglobin by disulfide bonds is explained by the presence of cysteines in its cytoplasmic domain. Perhaps most importantly, the finding that PF, a disease in which epidermal cells come apart, is a disease in which autoantibodies are directed against a member of the cadherin family of adhesion molecules implies a functional importance of cadherins in maintaining the integrity of the epidermis and in the pathophysiology of disease.

C. Pemphigus Vulgaris Antigen Is a Cadherin Closely Related to Pemphigus Foliaceus Antigen

PV antibodies, affinity purified on the 130-kDa PV antigen, were used to clone, from a human keratinocyte expression library, cDNA with coding sequences for PV antigen (*143*). The clone was verified as coding for PV antigen because (1) antibodies from PV serum, affinity purified on the fusion protein of this clone, bound the cell surface of monkey esophageal epithelium as detected by indirect immunofluorescence, in a pattern indistinguishable from that produced by PV sera, and bound the 130-kDa antigen as detected by immunoblotting; (2) PV sera, but not control sera, bound a fusion protein made

with this cDNA; and (3) rabbit antibodies raised against this fusion protein also showed PV-like immunofluorescence and bound the 130-kDa PV antigen as detected by immunoblotting. Northern blot analysis indicated that PV antigen was encoded by an approximately 6-kb mRNA that was restricted in distribution to stratified squamous epithelia. Southern analysis indicated that PV antigen was encoded by a single human gene (*143*).

Analysis of the deduced amino acid sequence of PV antigen indicated that it, too, belonged to the cadherin superfamily, but was more closely related to the desmoglein subfamily than to typical cadherins (Figs. 5 and 6) (*143*). Like typical cadherins and desmoglein, it has a transmembrane domain predicted to span the membrane once and has a basic domain predicted to be the cleavage site to produce the mature protein. The extracellular domain structure is typical of cadherins. PV antigen has the conserved RAL sequence of desmoglein, as well as six putative calcium-binding motifs and two potential N-glycosylation sites corresponding to those in desmoglein. The three most amino-terminal domains of PV antigen show greater homology to desmoglein than to typical cadherins, whereas the fourth domain shows about equal homology to both. The greatest homology between PV antigen and desmoglein (dsg1) was between the most external (amino-terminal) domains—about 70% identity and 80% similarity compared to 30% identity and 50% similarity with typical cadherins. The homology also extends into the cytoplasmic domains in which, again, desmoglein shows more similarity to PV antigen than do typical cadherins. PV antigen has a longer cytoplasmic tail (360 amino acids) than do typical cadherins (160 amino acids), although the tail is not as long as that of the desmogleins (480 amino acids). Five cysteines are in similar locations in the cytoplasmic regions of PV antigen and desmoglein. Finally, PV antigen has two repeats corresponding to the NVXVTE repeats of desmoglein. Interestingly, the gene for PV antigen is on chromosome 18, like that of the other desmogleins (*144*). Thus, PV antigen can be classified as being in the desmoglein subfamily of cadherins.

As discussed above with respect to PF antigen, the cloning of PV antigen provides explanations for many of its characteristics. For example, the calcium sensitivity of some of the epitopes on PV antigen as well as the observation, as determined by immunofluorescence, that calcium protects its immunoreactivity from destruction by trypsin (*81*) are consistent with the properties of cadherins. As with PF antigen, the disulfide binding with plakoglobin could use cysteines in the cytoplasmic domain, which is presumably in close contact with

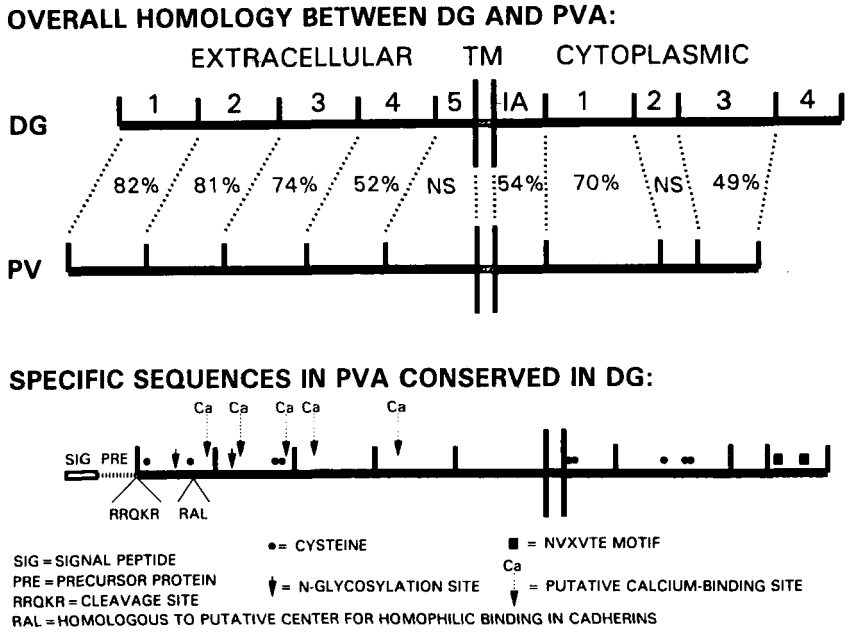

FIG. 6. Amino acid sequence homologies (percentage of identical and chemically similar amino acids) and conserved sites between desmoglein (DG) and PV antigen (PVA). TM, Transmembrane; NS, not significant.

plakoglobin. But most importantly, PV is another autoantibody-mediated disease of cell adhesion, which suggests an important function for cadherin-like molecules in maintaining the integrity of the epidermis.

This critical function of PV antigen and the pathophysiologic action of antibodies directed against this antigen have recently been demonstrated (*145*). PV sera reactivity with fusion proteins representing different domains of PV antigen demonstrated that dominant epitopes were present in the most amino-terminal extracellular domains, regions in typical cadherins known to be critical for homophilic adhesion (*146*). Antibodies from PV sera affinity purified on this region induced loss of cell-to-cell adhesion in the epidermis when injected into neonatal mice. Antibodies from PV sera affinity purified against more carboxy-terminal extracellular domains did not induce this pathology. These studies, therefore, demonstrated that critical adhesion functions could be perturbed by antibodies against PV antigen and, by implication, suggest the critical function of cadherins in maintaining epithelial integrity.

V. Conclusions and Future Directions

When the blistering diseases pemphigus and pemphigoid were orginally classified according to their clinical presentation and histology, it became clear that the pathology was due to a loss of either epidermal cell-to-cell or cell-to-basement membrane adhesion. Later, immunofluorescence was used to show that these were autoimmune diseases with antibodies directed against the sites of pathology. More recently, these autoantibodies have been used to characterize immunochemically and to clone molecularly their corresponding autoantigens. Most interestingly, in light of the pathology of these diseases, the targets of these autoantibodies have all been discovered to be in gene families of known adhesion molecules or to be associated with adhesion junctions or both. In two of these diseases, BP and PV, previously unidentified molecules have been defined. Molecular cloning of the 230-kDa BP antigen, the first known molecular component of hemidesmosomes, not only defined a new molecule but also established a new gene family of adhesion junction plaque proteins and, perhaps, intermediate filament-binding proteins, including desmoplakin I/II and plectin. Molecular cloning of the 180-kDa BP antigen defined a unique type of transmembrane molecule. Finally, the PV antigen was shown to be a new member of the desmoglein subfamily of cadherins.

Analyses of the deduced amino acid sequences of these cloned autoantigens resulted in predictions of certain structures and functions. Future research will be directed at trying to confirm these predictions experimentally. For the 230-kDa BP antigen the following questions might be among those that will be explored: Does rotary shadowing show the predicted rod structure? Does the predicted coiled-coil rod domain lead to aggregation into a plaque? Does the carboxy terminus bind keratin filaments? Regarding the 180-kDa BP antigen, do the collagenous domains bind the extracellular matrix and, if so, which molecules are bound? What is the function of the various regions of the cytoplasmic domain? Regarding the PF and PV antigens, are these molecules protected by calcium against trypsin degradation, as are typical cadherins? Is the PV antigen localized to desmosomes and/or adherens junctions? How do the cytoplasmic domains of these antigens function? Can these molecules be shown experimentally to mediate homophilic adhesion and, if so, which domains are important in this function?

In addition, important questions relating to the pathophysiology of disease remain. Specifically, do the autoantibodies in these patients directly mediate loss of cell adhesion? Which epitopes are critical in

antibody-mediated pathology? For BP, how can the 230-kDa BP antigen, which is inside the cell, be a target for autoantibodies? Is the 180-kDa transmembrane antigen the critical target and, if so, why do only about 50% of patients have antibodies against it?

Finally, another area that may be fruitful for investigation is whether these autoantigens are genetic targets in certain hereditary diseases (such as junctional epidermolysis bullosa, Hailey–Hailey disease, and Darier's disease) that show loss of epidermal basal cell-to-substrate or cell-to-cell adhesion.

I would predict that the symbiotic relationship, discussed throughout this review, between the study of these autoimmune diseases and the cell biologic study of adhesion structures and molecules will continue to shed light on both of these areas in the future.

ACKNOWLEDGMENTS

Dr. Masayuki Amagai helped design the schematic diagrams and reviewed this paper. Dr. Sarolta Karpati performed the immunofluorescence with pemphigus sera on keratinocytes.

REFERENCES

1. Lever, W. F. (1953). Pemphigus. *Medicine* **32,** 1–123.
2. Lever, W. F. (1965). "Pemphigus and Pemphigoid." Charles C. Thomas, Springfield, Illinois.
3. Stanley, J. R. (1992). Bullous pemphigoid, cicatricial pemphigoid, and chronic bullous disease of childhood. *In* "Dermatology in General Medicine" (T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg, and K. F. Austen, eds.), 4th Ed. McGraw-Hill, New York. (In press.)
4. Stanley, J. R. (1992). Pemphigus. *In* "Dermatology in General Medicine" (T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg, and K. F. Austen, eds.), 4th Ed. McGraw-Hill, New York. (In press.)
5. Jordon, R. E., Beutner, E. H., Witebsky, E., Blumental, G., Hale, W. L., and Lever, W. F. (1967). Basement zone antibodies in bullous pemphigoid. *JAMA* **200,** 751–756.
6. Beutner, E. H., and Jordon, R. E. (1964). Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescent staining. *Proc. Soc. Exp. Biol. Med.* **117,** 505–510.
7. Beutner, E. H., Lever, W. F., Witebsky, E., Jordon, R., and Chertock, B. (1965). Autoantibodies in pemphigus vulgaris. *JAMA* **192,** 682–688.
8. Beutner, E. H., Jordon, R. E., and Chorzelski, T. P. (1968). The immunopathology of pemphigus and bullous pemphigoid. *J. Invest. Dermatol.* **51,** 63–80.
9. Beutner, E. H., Chorzelski, T. P., and Jablonska, S. (1987). Clinical significance of immunofluorescence tests of sera and skin in bullous diseases: A cooperative study. *In* "Immunopathology of the Skin" (E. H. Beutner, T. P. Chorzelski, and V. Kumar, eds.), 3rd Ed., pp. 177–205. John Wiley & Sons, New York.
10. Lever, W. F. (1979). Pemphigus and pemphigoid. *J. Am. Acad. Dermatol.* **1,** 2–31.
11. Kobayasi, T. (1967). The dermo-epidermal junction in bullous pemphigoid. *Dermatologica* **134,** 157–165.

12. Lever, W. F., and Hashimoto,K. (1969). The etiology and treatment of pemphigus and pemphigoid. *J. Invest. Dermatol.* **53,** 373–389.
13. Wintroub, B. U., Mihm, M. C., Goetzi, E. J., Soter, N. A., and Austen, K. F. (1978). Morphologic and functional evidence for release of mast-cell products in bullous pemphigoid. *N. Engl. J. Med.* **298,** 417–421.
14. Dvorak, A. M., Mihm, Jr., M. C., Osage, J. E., Kwan, T. H., Austen, K. F., and Wintroub, B. U. (1982). Bullous pemphigoid, an ultrastructural study of the inflammatory response: Eosinophil, basophil and mast cell granule changes in multiple biopsies from one patient. *J. Invest. Dermatol.* **78,** 91–101.
15. Owaribe, K., Kartenbeck, J., Stumpp, S., Magin, T. M., Krieg, T., Diaz, L. A., and Franke, W. W. (1990). The hemidesmosomal plaque. I. Characterization of a major constituent protein as a differentiation marker for certain forms of epithelia. *Differentiation* **45,** 207–220.
16. Hashimoto, K. and Lever, W. F. (1967). An electron microscopic study on pemphigus vulgaris of the mouth and the skin with special reference to the intercellular cement. *J. Invest. Dermatol.* **48,** 540–552.
17. Barnett, M. L. (1978). Effect of pemphigus antibodies on desmosomal structure *in vitro*. *J. Invest. Dermatol.* **70,** 141–142.
18. Grando, S. A., Terman, A. K., Stupina, A. S., Glukhenky, B. T., and Romanenko, A. B. (1991). Ultrastructural study of clinically uninvolved skin of patients with pemphigus vulgaris. *Clin. Exp. Dermatol.* **16,** 359–363.
19. Wilgram, G. F., Caulfield, J. B., and Madgic, E. B. (1964). An electron microscopic study of acantholysis and dyskeratosis in pemphigus foliaceus. *J. Invest. Dermatol.* **43,** 287–299.
20. Krasny, S. A., Beutner, E. H., and Chorzelski, T. P. (1987). Specificity and sensitivity of indirect and direct immunofluorescent findings in the diagnosis of pemphigus. In "Immunopathology of the Skin" (E. H. Beutner, T. P. Chorzelski, and V. Kumar, eds.), 3rd Ed., pp. 207–247. Wiley, New York.
21. Rubinstein, N., and Stanley, J. R. (1987). Pemphigus foliaceus antibodies and a monoclonal antibody to desmoglein I demonstrate stratified squamous epithelial-specific epitopes of desmosomes. *Am. J. Dermatopathol.* **9,** 510–514.
22. Schaumburg Lever, G., Rule, A., Schmidt Ullrich, R., and Lever, W. F. (1975). Ultrastructural localization of *in vivo*-bound immunoglobulins in bullous pemphigoid—A preliminary report. *J. Invest. Dermatol.* **64,** 47–49.
23. Holubar, K., Wolff, K., Konrad, K., and Beutner, E. H. (1975). Ultrastructural localization of immunoglobulins in bullous pemphigoid skin. Employment of a new peroxidase-antiperoxidase multistep method. *J. Invest. Dermatol.* **64,** 220–227.
24. Schmidt Ullrich, B., Rule, A., Schaumburg Lever, G., and Leblanc, C. (1975). Ultrastructural localization of *in vivo*-bound complement in bullous pemphigoid. *J. Invest. Dermatol.* **65,** 217–219.
25. Mutasim, D. F., Takahashi, Y., Labib, R. S., Anhalt, G. J., Patel, H. P., and Diaz, L. A. (1985). A pool of bullous pemphigoid antigen(s) is intracellular and associated with the basal cell cytoskeleton-hemidesmosome complex. *J. Invest. Dermatol.* **84,** 47–53.
26. Regnier, M., Vaigot, P., Michel, S., and Prunieras, M. (1985). Localization of bullous pemphigoid antigen (BPA) in isolated human keratinocytes. *J. Invest. Dermatol.* **85,** 187–190.
27. Westgate, G. E., Weaver, A. C., and Couchman, J. R. (1985). Bullous pemphigoid antigen localization suggests an intracellular association with hemidesmosomes. *J. Invest. Dermatol.* **84,** 218–224.

28. Mutasim, D. F., Morrison, L. H., Takahashi, Y., Labib, R. S., Skouge, J., Diaz, L. A., and Anhalt, G. J. (1989). Definition of bullous pemphigoid antibody binding to intracellular and extracellular antigen associated with hemidesmosomes. *J. Invest. Dermatol.* **92,** 225–230.
29. Ikeda, S., Yaguchi, H., and Ogawa, H. (1990). Expression of extracellular and intracellular bullous pemphigoid antigens at the dermal-epibolic junction in organ culture of human skin. *J. Invest. Dermatol.* **94,** 336–340.
30. Yamasaki, Y., and Nishikawa, T. (1983). Ultrastructural localization of *in vitro* binding sites of circulating anti-basement membrane zone antibodies in bullous pemphigoid. *Acta Derm. Venereol. (Stockholm)* **63,** 501–506.
31. Jones, J. C. R., Yokoo, K. M., and Goldman, R. D. (1986). Is the hemidesmosome a half desmosome? An immunological comparison of mammalian desmosomes and hemidesmosomes. *Cell Motil. Cytoskel.* **6,** 560–569.
32. Schwarz, M. A., Owaribe, K., Kartenbeck, J., and Franke, W. W. (1990). Desmosomes and hemidesmosomes: Constitutive molecular components. *Annu. Rev. Cell Biol.* **6,** 461–491.
33. Odland, G. F. (1958). The fine structure of the interrelationship of cells in the human epidermis. *J. Biophysic. Biochem. Cytol.* **4,** 529–538.
34. Kelly, D. E. (1966). Fine structure of desmosomes, hemidesmosomes, and an adepidermal globular layer in developing newt epidermis. *J. Cell Biol.* **28,** 51–72.
35. Shimizu, H., McDonald, J. N., Kennedy, A. R., and Eady, R. A. J. (1989). Demonstration of intra- and extracellular localization of bullous pemphigoid antigen using cryofixation and freeze substitution for postembedding immunoelectron microscopy. *Arch. Dermatol. Res.* **281,** 443–448.
36. Diaz, L. A., and Marcelo, C. L. (1978). Pemphigoid and pemphigus antigens in cultured epidermal cells. *Br. J. Dermatol.* **98,** 631–637.
37. Stanley, J. R., Hawley Nelson, P., Poirier, M., Katz, S. I., and Yuspa, S. H. (1980). Detection of pemphigoid antigen, pemphigus antigen, and keratin filaments by indirect immunofluorescence in cultured human epidermal cells. *J. Invest. Dermatol.* **75,** 183–186.
38. Stanley, J. R., Hawley Nelson, P., Yuspa, S. H., Shevach, E. M., and Katz, S. I. (1981). Characterization of bullous pemphigoid antigen: A unique basement membrane protein of stratified squamous epithelia. *Cell (Cambridge, Mass.)* **24,** 897–903.
39. Stanley, J. R., Hawley Nelson, P., Yaar, M., Martin, G. R., and Katz, S. I. (1982). Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. *J. Invest. Dermatol.* **78,** 456–459.
40. Okada, N., Kitano, Y., Miyagawa, S., Sakamoto, K., and Steinberg, M. L. (1986). Expression of pemphigoid antigen by SV40-transformed human keratinocytes. *J. Invest. Dermatol.* **86,** 399–401.
41. Mueller, S., Klaus-Kovtun, V., and Stanley, J. R. (1989). A 230-kD basic protein is the major bullous pemphigoid antigen. *J. Invest. Dermatol.* **92,** 33–38.
42. Stanley, J. R., and Yuspa, S. H. (1983). Specific epidermal protein markers are modulated during calcium-induced terminal differentiation. *J. Cell Biol.* **96,** 1809–1814.
43. Stanley, J. R., Woodley, D. T., and Katz, S. I. (1984). Identification and partial characterization of pemphigoid antigen extracted from normal human skin. *J. Invest. Dermatol.* **82,** 108–111.
44. Labib, R. S., Anhalt, G. J., Patel, H. P., Mutasim, D. F., and Diaz, L. A. (1986). Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. *J. Immunol.* **136,** 1231–1235.

45. Bernard, P., Didierjean, L., Denis, F., Saurat, J. H., and Bonnetblanc, J. M. (1989). Heterogeneous bullous pemphigoid antibodies: Detection and characterization by immunoblotting when absent by indirect immunofluorescence. *J. Invest. Dermatol.* **92,** 171–174.
46. Owaribe, K., Nishizawa, Y., and Franke, W. W. (1991). Isolation and characterization of hemidesmosomes from bovine corneal epithelial cells. *Exp. Cell Res.* **192,** 622–630.
47. Meyer, L. J., Taylor, T. B., Kadunce, D. P., and Zone, J. J. (1990). Two groups of bullous pemphigoid antigens are identified by affinity-purified antibodies. *J. Invest. Dermatol.* **94,** 611–616.
48. Meyer, L. J., Taylor, T. B., Kadunce, D. P., Thuong-Nguyen, V., and Zone, J. J. (1991). Bullous pemphigoid antigens: Extraction and degradation of antigens during epidermal preparation. *J. Invest. Dermatol.* **96,** 991–993.
49. Klatte, D. H., Kurpakus, M. A., Grelling, K. A., and Jones, J. C. R. (1989). Immunochemical characterization of three components of the hemidesmosome and their expression in cultured epithelial cells. *J. Cell Biol.* **109,** 3377–3390.
50. Riddelle, K. S., Green, K. J., and Jones, J. C. R. (1991). Formation of hemidesmosome *in vitro* by a transformed rat bladder cell line. *J. Cell Biol.* **112,** 159–168.
51. Thacher, S. M., and Hefti, P. L. (1991). Characterization of 230-kD bullous pemphigoid antigen associated with the detergent-insoluble fraction of cultured keratinocytes. *J. Invest. Dermatol.* **96,** 139–143.
52. Robledo, M. A., Kim, S. C., Korman, N. J., Stanley, J. R., Labib, R. S., Futamura, S., and Anhalt, G. J. (1990). Studies of the relationship of the 230-kD and 180-kD bullous pemphigoid antigens. *J. Invest. Dermatol.* **94,** 793–797.
53. Shimizu, H., Hashimoto, T., Nishikawa, T., and Eady, R. A. J. (1991). Human monoclonal anti-basement membrane zone antibodies derived from virally transformed lymphocytes of a patient with bullous pemphigoid recognize epitopes associated with hemidesmosomes. *Br. J. Dermatol.* **124,** 217–220.
54. Zhu, X. J., and Bystryn, J. C. (1983). Heterogeneity of pemphigoid antigens. *J. Invest. Dermatol.* **80,** 16–20.
55. Morrison, L. H., Labib, R. S., Zone, J. J., Diaz, L. A., and Anhalt, G. J. (1988). Herpes gestationis autoantibodies recognize a 180-kD human epidermal antigen. *J. Clin. Invest.* **81,** 2023–2026.
56. Cook, A. L., Hanahoe, T. H., Mallett, R. B., and Pye, R. J. (1990). Recognition of two distinct major antigens by bullous pemphigoid sera. *Br. J. Dermatol.* **122,** 435–444.
57. Ebihara, T., Hashimoto, T., Nishikawa, T., Kudoh, J., Gamou, S., Shimizu, N., and Owaribe, K. (1992). Detection of the 170KD bullous pemphigoid antigen by immunoprecipitation technique. *J. Invest. Dermatol.* **98,** 586 (abstr.).
58. Diaz, L., Ratrie, H., Saunders, W. S., Futamura, S., Squiquera, H. L., Anhalt, G. J., and Giudice, G. J. (1990). Isolation of a human epidermal cDNA corresponding to the 180-kD autoantigen recognized by bullous pemphigoid and herpes gestationis sera. *J. Clin. Invest.* **86,** 1088–1094.
59. Venning, V. A., Whitehead, P. H., Leigh, I. M., Allen, J., and Wojnarowska, F. (1991). The clinical expression of bullous pemphigoid is not determined by the specificity of the target antigen. *Br. J. Dermatol.* **125,** 561–565.
60. Domloge-Hultsch, N., Utecht, L., James, W., and Yancey, K. B. (1990). Autoantibodies from patients with localized and generalized bullous pemphigoid immunoprecipitate the same 230-kd keratinocyte antigen. *Arch. Dermatol.* **126,** 1337–1341.
61. Soh, H., Hosokawa, H., Miyauchi, H., Izumi, H., and Asada, Y. (1991). Localized

pemphigoid shares the same target antigen as bullous pemphigoid. *Br. J. Dermatol.* **125,** 73–75.

62. Stanley, J. R., Koulu, L., and Thivolet, C. (1984). Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *J. Clin. Invest.* **74,** 313–320.
63. Stanley, J. R., Klaus Kovtun, V., and Sampaio, S. A. (1986). Antigenic specificity of fogo selvagem autoantibodies is similar to North American pemphigus foliaceus and distinct from pemphigus vulgaris autoantibodies. *J. Invest. Dermatol.* **87,** 197–201.
64. Hashimoto, T., Ogawa, M. M., Konohana, A., and Nishikawa, T. (1990). Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. *J. Invest. Dermatol.* **94,** 327–331.
65. Hashimoto, T., Konohana, A., and Nishikawa, T. (1991). Immunoblot assay as an aid to the diagnoses of unclassified cases of pemphigus. *Arch. Dermatol.* **127,** 843–847.
66. Ogawa, M. M., Hashimoto, T., Konohana, A., Castro, R. M., and Nishikawa, T. (1990). Immunoblot analyses of Brazilian pemphigus foliaceus antigen using different antigen sources. *Arch. Dermatol. Res.* **282,** 84–88.
67. Chambers, R., and de Renyi, G. S. (1925). The structure of the cells in tissues as revealed by microdissection. I. The physical relationships of cells in epithelia. *Am. J. Anat.* **35,** 385.
68. Staehelin, L. A. (1974). Structure and function of intercellular junctions. *Int. Rev. Cytol.* **39,** 191–283.
69. Cowin, P., Franke, W. W., Grund, C., Kapprell, H. P., and Kartenbeck, J. (1985). The desmsosome-intermediate filament complex. *In* "The Cell in Contact. Adhesions and Junctions as Morphogenetic Determinants" (G. M. Edelman and J. P. Thiery, eds.), pp. 427-460. Wiley, New York.
70. Gorbsky, G., and Steinberg, M. S. (1981). Isolation of the intercellular glycoproteins of desmosomes. *J. Cell Biol.* **90,** 243–248.
71. Cowin, P., and Garrod, D. R. (1983). Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity. *Nature (London)* **302,** 148–150.
72. Steinberg, M. S., Shida, H., Giudice, G. J., Shida, M., Patel, N. H., and Blaschuk, O. W. (1987). On the molecular organization, diversity and functions of desmosomal proteins. *Ciba. Found. Symp.* **125,** 3–25.
73. Hennings, H., and Holbrook, K. A. (1983). Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. *Exp. Cell Res.* **143,** 127–142.
74. Watt, F. M., Mattey, D. L., and Garrod, D. R. (1984). Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. *J. Cell Biol.* **99,** 2211–2215.
75. Koulu, L., Kusumi, A., Steinberg, M. S., Klaus Kovtun, V., and Stanley, J. R. (1984). Human autoantibodies against a desmosomal core protein in pemphigus foliaceus. *J. Exp. Med.* **160,** 1509–1518.
76. Stanley, J. R., Koulu, L., Klaus Kovtun, V., and Steinberg, M. S. (1986). A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J. Immunol.* **136,** 1227–1230.
77. Calvanico, N. J., Martins, C. R., and Diaz, L. A. (1991). Characterization of pemphigus foliaceus antigen from human epidermis. *J. Invest. Dermatol.* **96,** 815–821.
78. Eyre, R. W., and Stanley, J. R. (1987). Human autoantibodies against a desmo-

somal protein complex with a calcium-sensitive epitope are characteristic of pemphigus foliaceus patients. *J. Exp. Med.* **165,** 1719–1724.

79. Eyre, R. W., and Stanley, J. R. (1988). Identification of pemphigus vulgaris antigen extracted from normal human epidermis and comparison with pemphigus foliaceus antigen. *J. Clin. Invest.* **81,** 807–812.
80. Korman, N. J., Eyre, R. W., Klaus-Kovtun, V., and Stanley, J. R. (1989). Demonstration of an adhering-junction molecule (plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. *N. Engl. J. Med.* **321,** 631–635.
81. Matis, W. L., Anhalt, G. J., Diaz, L. A., Rivitti, E. A., Martins, C. R., and Berger, R. S. (1987). Calcium enhances the sensitivity of immunofluorescence for pemphigus antibodies. *J. Invest. Dermatol.* **89,** 302–304.
82. Labib, R. S., Rock, B., Robledo, M. A., and Anhalt, G. J. (1991). The calcium-sensitive epitope of pemphigus foliaceus antigen is present on a murine tryptic fragment and constitutes a major antigenic region for human autoantibodies. *J. Invest. Dermatol.* **96,** 144–147.
83. Cowin, P., Kapprell, H. P., Franke, W. W., Tamkun, J., and Hynes, R. O. (1986). Plakoglobin: A protein common to different kinds of intercellular adhering junctions. *Cell (Cambridge, Mass.)* **46,** 1063–1073.
84. Konrad, K., Stingl, G., and Holubar, K. (1977). Ultrastructural localization of *in vivo* bound immunoglobulin in epidermis of Brazilian pemphigus foliaceus (fogo selvagem). *Br. J. Dermatol.* **96,** 449–451.
85. Sotto, M. N., Shimizu, S. H., Costa, J. M., and Brito, T. De (1980). South American pemphigus foliaceus: Electron microscopy and immunoelectron localization of bound immunoglobulin in the skin and oral mucosa. *Br. J. Dermatol.* **102,** 521–527.
86. Futamura, S., Martins, C., Rivitti, E. A., Labib, R. S., Diaz, L. A., and Anhalt, G. J. (1989). Ultrastructural studies of acantholysis induced *in vivo* by passive transfer of IgG from endemic pemphigus foliaceus (Fogo Selvagem). *J. Invest. Dermatol.* **93,** 480–485.
87. Rappersberger, K., Roos, N., and Stanley, J. R. (1992). Immunomorphological and biochemical identification of the pemphigus foliaceus autoantigen within desmosomes. *J. Invest. Dermatol.* **99** (in press).
88. Akiyama, M., Hashimoto, T., Sugiura, M., and Nishikawa, T. (1991). Ultrastructural localization of pemphigus vulgaris and pemphigus foliaceus antigens in cultured human squamous carcinoma cells. *Br. J. Dermatol.* **125,** 233–237.
89. Giudice, G. J., Cohen, S. M., Patel, N. H., and Steinberg, M. S. (1984). Immunological comparison of desmosomal components from several bovine tissues. *J. Cell. Biochem.* **26,** 35–45.
90. Jones, J. C. R., Vikstrom, K. L., and Goldman, R. D. (1987). Evidence for heterogeneity in the 160/165,000 M_r glycoprotein components of desmosomes. *J. Cell Sci.* **88,** 513–520.
91. Farb, R. M., Dykes, R., and Lazarus, G. S. (1978). Anti-epidermal-cell-surface pemphigus antibody detaches viable epidermal cells from culture plates by activation of proteinase. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 459–463.
92. Stanley, J. R., Yaar, M., Hawley Nelson, P., and Katz, S. I. (1982). Pemphigus antibodies identify a cell surface glycoprotein synthesized by human and mouse keratinocytes. *J. Clin. Invest.* **70,** 281–288.
93. Ogawa, H., Taneda, A., and Morioka, S. (1978). Characterization of pemphigus antigen. *J. Invest. Dermatol.* **70,** 194–196.
94. Jones, J. C. R., Arnn, J., Staehelin, L. A., and Goldman, R. D. (1984). Human

autoantibodies against desmosomes: Possible causative factors in pemphigus. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2781–2785.

95. Jones, J. C. R., Yokoo, K. M., and Goldman, R. D. (1986). Further analysis of pemphigus autoantibodies and their use in studies on the heterogeneity, structure, and function of desmosomes. *J. Cell Biol.* **102,** 1109–1117.
96. Wolff, K., and Schreiner, E. (1971). Ultrastructural localization of pemphigus auto-antibodies within the epidermis. *Nature (London)* **229,** 59–60.
97. Takigawa, M., Imamura, S., and Ofuji, S. (1978). Surface distribution of pemphigus antibody-binding substance(s) on isolated guinea pig epidermal cells. An immunoferritin electron microscopic study. *J. Invest. Dermatol.* **71,** 182–185.
98. Takahashi, Y., Patel, H. P., Labib, R. S., Diaz, L. A., and Anhalt, G. J. (1985). Experimentally induced pemphigus vulgaris in neonatal BALB/c mice: A time-course study of clinical, immunologic, ultrastructural, and cytochemical changes. *J. Invest. Dermatol.* **84,** 41–46.
99. Jones, J. C. R., Yokoo, K. M., and Goldman, R. D. (1986). A cell surface desmosome-associated component: Identification of a tissue-specific cell adhesion molecule. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7282–7286.
100. Iwatsuki, K., Takigawa, M., Imaizumi, S., and Yamada, M. (1989). *In vivo* binding site of pemphigus vulgaris antibodies and their fate during acantholysis. *J. Am. Acad. Dermatol.* **20,** 578–582.
101. Stanley, J. R., Tanaka, T., Mueller, S., Klaus-Kovtun, V., and Roop, D. (1988). Isolation of cDNA for bullous pemphigoid antigen by use of patients' autoantibodies. *J. Clin. Invest.* **82,** 1864–1870.
102. Amagai, M., Hashimoto, T., Tajima, S., Inokuchi, Y., Shimizu, N., Saito, M., Miki, K., and Nishikawa, T. (1990). Partial cDNA cloning of the 230-kD mouse bullous pemphigoid antigen by use of a human monoclonal anti-basement membrane zone antibody. *J. Invest. Dermatol.* **95,** 252–259.
103. Sawamura, D., Li, K. H., Nomura, K., Sugita, Y., Christiano, A. M., and Uitto, J. (1991). Bullous pemphigoid antigen: cDNA cloning, cellular expression, and evidence for polymorphism of the human gene. *J. Invest. Dermatol.* **96,** 908–915.
104. Tanaka, T., Parry, D. A. D., Klaus-Kovtun, V., Steinert, P. M., and Stanley, J. R. (1991). Comparison of molecularly cloned bullous pemphigoid antigen to desmoplakin I confirms that they define a new family of cell adhesion junction plaque proteins. *J. Biol. Chem.* **266,** 12555–12559.
105. Sawamura, D., Li, K., Chu, M. L., and Uitto, J. (1991). Human bullous pemphigoid antigen (BPAG1). Amino acid sequences deduced from cloned cDNAs predict biologically important peptide segments and protein domains. *J. Biol. Chem.* **266,** 17784–17790.
106. Tanaka, T., Korman, N. J., Shimizu, H., Eady, R. A. J., Klaus-Kovtun, V., Cehrs, K., and Stanley, J. R. (1990). Production of rabbit antibodies against carboxy-terminal epitopes encoded by bullous pemphigoid cDNA. *J. Invest. Dermatol.* **94,** 617–623.
107. Thacher, S. M., Malone, K. L., Dave, K., and Zhao, S. (1991). Localization of the 230-kilodalton bullous pemphigoid antigen in cultured keratinocytes: Formation of a prehemidesmosome. *Exp. Cell Res.* **194,** 238–247.
108. Amagai, M., Elgart, G. W., Klaus-Kovtun, V., and Stanley, J. R. (1991). Southern analysis of the 230-kD bullous pemphigoid antigen gene in normal humans, animals, and patients with junctional epidermolysis bullosa. *J. Invest. Dermatol.* **97,** 249–253.
109. Sawamura, D., Nomura, K., Sugita, Y., Mattei, M. G., Chu, M. L., Knowlton, R.,

and Uitto, J. (1990). Bullous pemphigoid antigen (BPAG1): cDNA cloning and mapping of the gene to the short arm of human chromosome 6. *Genomics* **8**, 722–726.

110. Minoshima, S., Amagai, M., Kudoh, J., Fukuyama, R., Hashimoto, T., Nishikawa, T., and Shimizu, N. (1991). Localization of the human gene for 230-kDa bullous pemphigoid autoantigen (BPAG1) to chromosome 6pter–q15. *Cytogenet. Cell Genet.* **57**, 30–32.

111. Franke, W. W., Schmid, E., Grund, C., Muller, H., Engelbrecht, I., Moll, R., Stadler, J., and Jarasch, E. D. (1981). Antibodies to high molecular weight polypeptides of desmosomes: Specific localization of a class of junctional proteins in cells and tissues. *Differentiation* **20**, 217–241.

112. Mueller, H., and Franke, W. W. (1983). Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of the desmosomal plaque. *J. Mol. Biol.* **163**, 647–671.

113. Green, K. J., Parry, D. A. D., Steinert, P. S., Virata, L. A., Wagner, R. M., Angst, B. D., and Nilles, L. A. (1990). Structure of the human desmoplakins. Implications for function in the desmosomal plaque. *J. Biol. Chem.* **265**, 2603–2612.

114. O'Keefe, E. J., Erickson, H. P., and Bennett, V. (1989). Desmoplakin I and desmoplakin II. Purification and characterization. *J. Biol. Chem.* **264**, 8310–8318.

115. Virata, M. L. A., Wagner, R. M., Parry, D. A. D., and Green, K. J. (1992). Molecular structure of the human desmoplakin I and II amino terminus. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 544–548.

116. Green, K. J., Virata, M. L. A., Elgart, G. W., Stanley, J. R., and Parry, D. A. D. (1992). Comparative structural analysis of desmoplakin, bullous pemphigoid antigen and plectin: Members of a new gene family involved in organization of intermediate filments. *Int. J. Biol. Macrobiol.* **14**, 145–153.

117. Arnemann, J., Spurr, N. K., Wheeler, G. N., Parker, A. E., and Buxton, R. S. (1991). Chromosomal assignment of the human genes coding for the major proteins of the desmosome junction, desmoglein DGI (DSG), desmocollins DGII/III (DSC), desmoplakins DPI/II (DSP), and plakoglobin DPIII (JUP). *Genomics* **10**, 640–645.

118. Stappenbeck, T. S., and Green, K. J. (1992). The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J. Cell Biol.* **116**, 1197–1209.

119. Wiche, G. (1989). Plectin: General overview and appraisal of its potential role as a subunit protein of the cytomatrix. *Crit. Rev. Biochem. Mol. Biol.* **24**, 41–67.

120. Wiche, G., Becker, B., Luber, K., Weitzer, G., Castanon, M. J., Hauptmann, R., Stratowa, C., and Stewart, M. (1991). Cloning and sequencing of rat plectin indicates a 466-kD polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. *J. Cell Biol.* **114**, 83–99.

121. Giudice, G. J., Squiquera, H. L., Elias, P. M., and Diaz, L. A. (1991). Identification of two collagen domains within the bullous pemphigoid autoantigen, BP180. *J. Clin. Invest.* **87**, 734–738.

122. Giudice, G. J., Emery, D. J., and Diaz, L. A. (1992). Cloning and primary structural analysis of the bullous pemphigoid autoantigen, BP180. *J. Invest. Dermatol.* **99**, 243–250.

123. Li, K., Sawamura, D., Giudice, G. J., Diaz, L. A., Mattei, M. G., Chu, M. L., and Uitto, J. (1991). Genomic organization of collagenous domains and chromosomal assignment of human 180-kDa bullous pemphigoid antigen-2, a novel collagen of stratified squamous epithelium. *J. Biol. Chem.* **266**, 24064–24069.

124. Koch, P. J., Walsh, M. J., Schmelz, M., Goldschmidt, M. D., Zimbelmann, R., and Franke, W. W. (1990). Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion molecules. *Eur. J. Cell Biol.* **53**, 1–12.
125. Goodwin, L., Hill, J. E., Raynor, K., Raszi, L., Manabe, M., and Cowin, P. (1990). Desmoglein shows extensive homology to the cadherin family of cell adhesion molecules. *Biochem. Biophys. Res. Commun.* **173**, 1224–1230.
126. Nilles, L. A., Parry, D. A. D., Powers, E. E., Angst, B. D., Wagner, R. M., and Green, K. J. (1991). Structural analysis and expression of human desmoglein: A cadherin-like component of the desmosome. *J. Cell Sci.* **99**, 809–821.
127. Wheeler, G. N., Parker, A. E., Thomas, C. L., Ataliotis, P., Poynter, D., Arnemann, J., Rutman, A. J., Pidsley, S. C., Watt, F. M., Rees, D. A., Buxton, R. S., and Magee, A. I. (1991). Desmosomal glycoprotein DGI, a component of intercellular desmosome junctions, is related to the cadherin family of cell adhesion molecules. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4796–4800.
128. Takeichi,M. (1990). Cadherins: A molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* **59**, 237–252.
129. Takeichi,M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451–1455.
130. Ozawa, M., and Kemler, R. (1990). Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. *J. Cell Biol.* **111**, 1645–1650.
131. Hatta, K., Nose, A., Nagafuchi, A., and Takeichi, M. (1988). Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: Its identity in the cadherin gene family. *J. Cell Biol.* **106**, 873–881.
132. Shimoyama, Y., Yoshida, T., Terada, M., Shimosato, Y., Abe, O., and Hirohashi, S. (1989). Molecular cloning of a human Ca^{2+}-dependent cell-cell adhesion molecule homologous to mouse placental cadherin: Its low expression in human placental tissues. *J. Cell. Biol.* **109**, 1787–1794.
133. Blaschuk, O. W., Sullivan, R., David, S., and Pouliot, Y. (1990). Identification of a cadherin cell adhesion recognition sequence. *Dev. Biol.* **139**, 227–229.
134. Ringwald, M., Schuh, R., Vestweber, D., Eistetter, H., Lottspeich, F., Engel, J., Dolz, R., Jahnig, F., Epplen, J., Mayer, S., Muller, C., and Kemler, R. (1987). The structure of cell adhesion molecule uvomorulin. Insights into the molecular mechanism of Ca^{2+}-dependent cell adhesion. *EMBO J.* **6**, 3647–3653.
135. Ozawa, M., Engel, J., and Kemler, R. (1990). Single amino acid substitutions in one Ca^{2+} binding site of uvomorulin abolish the adhesive function. *Cell (Cambridge, Mass.)* **63**, 1033–1038.
136. Ozawa, M., Ringwald, M., and Kemler, R. (1990). Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4246–4250.
137. McCrea, P. D., and Gumbiner, B. M. (1991). Purification of a 92-kDa cytoplasmic protein tightly associated with the cell-cell adhesion molecule E-cadherin (uvomorulin). Characterization and extractability of the protein complex from the cell cytostructure. *J. Biol. Chem.* **266**, 4514–4520.
138. Ozawa, M., and Kemler, R. (1992). Molecular organization of the uvomorulin-catenin complex. *J. Cell Biol.* **116**, 989–996.
139. McCrea, P. D., Turck, C. W., and Gumbiner, B. (1991). A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science* **254**, 1359–1361.
140. Koch, P. J., Goldschmidt, M. D., Walsh, M. J., Zimbelmann, R., and Franke,

W. W. (1991). Complete amino acid sequence of the epidermal desmoglein precursor polypeptide and identification of a second type of desmoglein gene. *Eur. J. Cell Biol.* **55,** 200–208.

141. Koch, P. J., Goldschmidt, M. D., Zimbelmann, R., Troyanovsky, R., and Franke, W. W. (1992). Complexity and expression patterns of the desmosomal cadherins. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 353–357.

142. Buxton, R. S., and Magee, A. I. (1992). Structure and interactions of desmosomal and other cadherins. *Semin. Cell Biol.* **3,** 157–167.

143. Amagai, M., Klaus-Kovtun, V., and Stanley, J. R. (1991). Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell (Cambridge, Mass.)* **67,** 869–877.

144. Arnemann, J., Spurr, N. K., and Buxton, R. S. (1992). The human gene (DSG3) coding for the pemphigus vulgaris antigen is, like the genes coding for the other two known desmogleins, assigned to chromosome 18. *Hum. Genet.* **89,** 347–350.

145. Amagai, M., Karpati, S., Prussick, R., Klaus-Kovtun, V., and Stanley, J. R. (1992). Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic. *J. Clin. Invest.* **90,** 919–926.

146. Nose, A., Tsuji, K., and Takeichi, M. (1990). Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell. (Cambridge, Mass.)* **61,** 147–155.

This article was accepted for publication on 23 September 1992.

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